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EDITORIAL

The contributions presented in this issue of Acta Biologica Hungarica are dedicated to Professor János Salánki, Ordinary Member of the Hungarian Academy of Sciences, and among others, Editor-in-Chief of this periodical, to celebrate his 70th birthday, and to pay tribute to his achievements in scientific research and roles in different communities of the scientific world during his long career.

János Salánki studied medicine at the Medical University of Debrecen, but he has been interested very early in medical-biological research, instead of becoming a medical doctor. Before graduation, he has joined the staff of the Institute of Physiology and had his first scientific publications. From then on, his engagement with biological research has remained unbroken, and his activities have been devoted to comparative neurobiology and enviromental studies. Debrecen–Moscow–Debrecen–Tihany, these are the major stations of his way, by the end of which he has been anchored, up to the present days, at the Balaton Limnological (at that time Biological) Research Institute of the Hungarian Academy of Sciences. As a young director at that time, he has established a flourishing research field in the institute, dealing with the neurobiology of invertebrates, mainly of molluscs, and introduced intracellular microelectrophysiology as a routine method. He always preferred and generated studies, using different methods when approaching problems, underlying regulatory processes of different behaviours. By time, he started to combine some aspects, mainly cellular electrophysiology, of invertebrate neurobiology with problems of enviromental pollution, putting emphasis onto the effect of heavy metals. He has succeeded to draw attention to the significance of slow accumulation of environmental pollutants in influencing and deteriorating the physiological state of aquatic organisms living in Lake Balaton and its surroundings.

He has always been an active person and personality of different scientific communities, serving in this way, too, the good reputation of his fields of research and the scientific reputation of our country as well. His many-sided international connection and recognition have peaked by his election to President of the International Union of Biological Sciences, and the foundation and first presidency of the International Society for Invertebrate Neurobiology.

The Editorial Board of Acta Biologica Hungarica joins the contributors of this special issue, congratulating and wishing all the best to Professor János Salánki, in the hope that in good health he will remain as active as before in the scientific life.

KÁROLY ELEKES
Editor

Akadémiai Kiadó, Budapest

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MY WAY IN SCIENCE

János SALÁNKI

As far as I can recall, I realised around 1951, as a student of the Medical University of Debrecen, that I am much more interested in dealing with theoretical problems of medicine and its basic science, biology, than becoming a general practitioner in a town or a specialist in a hospital to cure people. Starting from the fourth year of my studies in medicine, I spent most of my time as an externist at the Department of Biology and Pathophysiology headed by Professor L. Kesztyűs, and participated in teaching lower classes as well as in the research of the Department, instead of listening lectures on clinical subjects and attending demonstrations as well as practicals held with patients as part of the physician's education.

At that time Lóránd Kesztyűs, a 34 years old enthusiastic, theoretically oriented professor was interested in immunology and so, together with two other students I joined his research team and made experiments on rabbits to study the question, whether the nervous system takes part in the development of specific response of the organism to exogenous proteins by transmitting signals from the antigen sensing receptors to the system responsible for sensitisation or antibody production. Parallel with my university duties and exams, I continued research on this problem for two more years, and when I graduated as a medical doctor in 1954 I had three publications in this topic, the first of them with the question in its title we were studying "Können sich unter sekundenlanger Einwirkung von Antigenreizen spezifische Abwehrstoffe bilden?" [13]. Today it is known, that the participation of neural processes in immune response is not so direct as we presumed, nevertheless, it has turned out that there exists a link between neural and immune systems at least in the

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form of a functional interrelationship between primary neurotransmitters, second messengers, and the cytokines designated as signal molecules involved in the neural, humoral and immune regulation.

As a result of my earlier work and affection towards research, after graduation I applied for and achieved to become a member of the Department of Pathophysiology. Moreover, in 1955 I got a chance to continue my studies as an aspirant (PhD student) in comparative physiology at the Moscow State University (the 'Lomonosov University'), pushing me even farther from "real" medicine.

Due to historical circumstances, that time physiology was represented in Hungary only at Medical Universities as a human-oriented subject, in contrary to western European countries, Russia and United States, where also comparative animal physiology belonged to recognised disciplines at science faculties. By the fifties, in new discoveries of biology a number of invertebrate animals obtained a great importance, like *Drosophila* in genetics, sea urchin eggs in cell biology, *Loligo* axon in neurophysiology, etc., and the idea to develop experimental biology and so comparative physiology in Hungary became more and more emphasised by the Academy of Sciences. With the support of the Ministry of Education, a program was initiated, and I got a proposal to continue my studies and research in comparative physiology, which I accepted. As it turned out later, this episode was the first step in establishing a comparative physiology group dealing with invertebrate neurobiology at the Biological Research Institute of the Hungarian Academy of Sciences, at the shore of Lake Balaton, in Tihany. The setting up of this group is firmly connected to my further scientific carrier, as it will be seen later.

In the '50s the Department of Comparative Animal Physiology of the Moscow State University was headed by Professor H. S. Koshtoyants, a worldwide known physiologist, having both medical and biological education, working in the twenties in Jordan's laboratory in Utrecht. As main lecturer of several summer courses held at the Tihany Biological Research Institute organised by the Biological Section of the Academy for young scientists interested in comparative physiology, and being also Honorary Member of the Hungarian Academy of Sciences, he was familiar with Hungarian medical and biological teaching and research. I first met him in 1955 at one of these courses, and in the same year became his PhD student. In compliance with his initiative instead of continuing my research in immunology I started to work on the physiology of invertebrate animals.

The problem I was proposed to deal with concerned a basic and fascinating question of the fifties: are behavioural motor patterns genetically determined in the brain or they can be modified by external factors. For example, the theory of "inherent rhythms" suggested that among aquatic animals the periodicity of activity and rest of the mussels, described as a behavioural phenomenon decades earlier, has constant phases characteristic to the animal (like diurnal rhythms) fixed in the central nervous system (biological clock). The other idea was that for such a motor pattern only the structural and functional specificities are determined genetically, and time relations can change, depending on the need of accommodation to circumstances. To answer such general questions, model animals can be used, and my model was the freshwa-

ter bivalve, *Anodonta cygnaea.* Studying the effect of chemical stress on the freshwater mussel I could demonstrate that the duration of both activity and rest supposed earlier to be centrally determined can change significantly in response to variation in the chemism of the environment, supporting the second theory [16]. I succeeded in proving that potassium, cadmium and mercury, if present in excess in the water surrounding the animal, have a concentration-dependent effect on the time relations of the rhythmic and periodic activity of the adductor muscles responsible for closing and opening the valves, and influencing by this way the water filtering function, the most characteristic behavioural pattern in this animal [23]. The results summed up in my candidate's dissertation suggested that the central mechanisms responsible for the regulation of the periodicity are under the control of environmental components, affecting the functioning of the brain either indirectly, through the peripheral sensory system, or directly, influencing the metabolism and excitability of the central neuronal elements.

This fundamental observation inspired me to deal with two, basically different questions in my further studies on mussels. The first was to clarify the mechanism underlying the regulation of periodicity, and the second one, to use the phenomenon in testing and monitoring water quality. In extending my research to these directions lucky circumstances played a decisive role. After obtaining the candidate's degree by the end of 1959 in Moscow, I started to work at the Department of Physiology of the Medical University in Debrecen. I continued my experiments with the regulation of periodic activity and taking part at the same time in teaching human physiology, when I received an invitation from Professor Brunó Straub, head of the Biological Section of the Academy to apply for a vacancy announced for the directorship of the Biological Research Institute at Tihany. I did it and my application was accepted, so I became Director of the Institute in September 1962. Being 33 years old the challenge was great, but beside undertaking the thankless duties of scientific administration of an Institute with 60 members, I had the chance to continue, and also to broaden my research with the participation of a number of young fellows. The first group of colleagues with whom I started research at Tihany included Katalin S.-Rózsa (my wife, who won PhD in comparative physiology also in Moscow), two MDs, Elemér Lábos physiologist and Imre Zs.-Nagy morphologist as well as László Hiripi chemist and István Varanka biologist, all moving with me from eastern part of Hungary to Transdanubia.

My idea was to organise a complex laboratory using physiological, morphological, and biochemical technics and approaches in conducting research with neural regulation of invertebrates, so to deal not with comparative physiology only, but – as we called – with invertebrate neurobiology. Within several years this goal could be achieved. More and more publications have been published by this group, and in 1967, taking the opportunity of the 40th anniversary of the Institute's opening, we organised the first international symposium on invertebrate neurobiology. At this symposium we presented our results before a wide international scientific audience and also started to expand international scientific co-operation to East and West, what was far not as simple and easy in the sixties as it is today. It was very stimulating

when our efforts and achievements were recognized by awarding the team with an Academic Prize.

Using a complex, multi-methodical approach in studying the underlying mechanisms of rhythmicity in mussels the long duration, continuous recording of the adductor's activity and the neurochemical-pharmacological approach proved to be the most profitable, complemented to some degree with light and electron microscopy. There was no doubt, that we are facing central nervous processes, and our question was, which substances are involved in the regulation of the rhythmic and periodic activity?

In the early sixties, less than ten bioactive compounds were recognised as neurotransmitters and transmitter precursors, so it was not too complicated to examine their action on the nervous system of mussels meanwhile the activity of the animal was recorded. We found, that only 5-hydroxytriptamine (5-HT, serotonin), first isolated from molluscs, and its precursors, tryptophane and 5-OH-tryptophane were able to eliminate the several hours long tonic contraction of the adductors when applied to the central nervous system, whereas the effect of adrenaline and noradrenaline was week in this respect [24]. Serotonin released tonic contraction also when injected into the adductor muscle directly [35]. The suggestion that 5-HT can be the relaxing agent was supported by fluorimetric measurements and density gradient centrifugation showing high 5-HT content in the ganglia, mainly in the synaptic fraction [11]. Another monoamine we detected and measured in high concentration in the mussel's ganglia was dopamine. In long-term experiments, after pharmacological blocking of dopamine breakdown, tonic contraction became prevailing, suggesting that this monoamine can be responsible for the "catch" [10]. It has been supported when high dopamine and low serotonin concentrations were detected in the ganglia of mussels living at winter in Lake Balaton and performing long (20–30 hours) rest periods alternating with short (3–5 hours) activities [42, 43]. As a result of these studies it could be concluded that a serotonin–dopamine antagonism plays a decisive role in the underlying mechanism of the periodic activity. In agreement with this conclusion the double innervation of the adductors, one responsible for the catch and the other for the relaxation has been revealed by electrophysiological experiments [34]. Acetylcholine (ACh), a third neurotransmitter we studied, is not involved in inducing or eliminating long-lasting tonic contraction, which are the main events in the pattern of the periodic activity, but it evokes rapid, phasic contractions of the adductors when they are out of catch [35].

To have an insight into the ontogenesis of rhythm regulation, the effect of a number of bioactive agents was studied in the larvae of the mussel, called glochidia, performing also a rhythmic activity pattern due to the embryonic adductor muscle, having yet no definite nervous system. We found that the rhythmic activity can be enhanced for a short time by cholinomimetics, catecholamines and indole-alkyl-amines. Among indole-alkyl-amines the effect of 5-hydroxytriptamine, being the activating agent in adult mussels, was moderate, however, tryptamine proved to be a highly effective stimulatory substance in this early stage of ontogenesis [17, 18], referring to the embryonic and postnatal evolution of transmitter substances and

transformation of transmitter receptors during development. Albeit this was an exciting question, we did not continue our research in this direction, which could have brought interesting new discoveries for developmental neurobiology.

Being convinced that activation and/or inhibition of certain neurones by transmitters is a key process in the regulation of behaviour, we looked for a suitable technique to study the mechanisms of excitation at the cellular level, instead of investigating the whole ganglion or nerve activities. The methodical approach could be microelectrophysiology. The intracellular technique with glass microelectrodes has been developed in the fifties, and was applied successfully on nerve cells in a number of physiological laboratories, but not in Hungary. In 1965, I was given the chance to spend six months in Gerald Kerkut's laboratory in England, and trained for working with microelectrodes. I wanted to study mussel's neurones, but due to the very small size of the cells and the soft tissue they are embedded in, no impalement was possible with microelectrodes at that technical level. Since my trial failed with the mussel, I turned to another mollusc, the gastropod *Helix,* having very nice, *in vivo* visible, giant neurones. The question I wanted to study remained the same: what is the response of nerve cells to various neurotransmitters, do they respond similarly or differently to biologically active substances and is the same cell sensitive only to one or to more neurotransmitter? Today the answers are in textbooks, but that time our knowledge was very poor in this respect.

I started to work in Southampton on the isolated ganglion of *Helix aspersa,* but after coming back to Tihany and setting up the technique, I continued experiments with *Helix pomatia* and the pond snail, *Lymnaea stagnalis,* outstanding with its reddish, well accessible giant neurones.

Let me stop here at the phrase "setting up the technique". Those days were the heroic times of microelectrophysiology. It was not the case of the contemporary biologist wishing to work with microelectrodes: "look the catalogues of companies advertising and selling all necessary equipment, select and buy them and with the help of a trained colleague you can start experimenting, what will be more or less successful after a couple of weeks". The first problem was how to fabricate the micropipettes. I saw in Koshtoyants laboratory Natasha Kokina, working with the unicellular *Opalina,* pulling microelectrodes with hands over a small Bunsen-flame. In other laboratories, researchers constructed their own microelectrode puller, vertical or horizontal, with electric heating. We had to do the same, and the puller constructed at that time was in use for years, even when we already had our Kopf-puller. To fill micropipettes was also a tiresome process while using various tricks. The second task was to build a D.C. amplifier with high input impedance, since we had no hard currency to buy it from abroad. For recording electric signals, light sensitive paper or film was used, and for data analysis, rulers and compasses served as basic instruments. It was our colleague Mihály Véró, an electric engineer, who constructed, adapted and built equipments, put together set-ups, including the first voltage clamp amplifier and analizator for collecting and computing bioelectric signals.

Studying the chemical sensitivity of snail neurones, first I checked their reaction to ACh, which was known from the paper of Tauc and Gerschenfeld [62] to have

either depolarising (D) or hyperpolarising (H) effect on a number of *Aplysia* nerve cells. Similar results were obtained in *Helix* and *Lymnaea,* however, when I tested the effect of glutamate on the neurones which responded to ACh, it turned out, that only D-cells reacted to this amino acid, until that time only presumed interneuronal transmitter, but H-cells were insensitive to it [26]. This was the first publication revealing glutamate sensitive neurones in molluscs, and was among the early papers showing that the membrane of the same neurone can have receptors to more than one neurotransmitter. Using chemical sensitivity and the type of firing pattern as characterising criteria, with Dmitri Sakharov from Moscow we constructed the first neuronal map of *Helix* ganglia [22], which was employed and complemented later in Tihany and by other laboratories. A similar map was created later on *Lymnaea,* when together with István Kiss we described the heterogenic chemical sensitivity of central neurones and the membrane potential dependent ACh response in this snail [14]. These were the years when, due to the great advantage of invertebrate animals compared to vertebrates in carrying out single cell recordings, identifiable large size neurones of gastropods attracted many laboratories, and research on biophysical, physiological and pharmacological membrane properties, as well as on brain–organ complex preparations of gastropods began to flourish throughout the world.

In the early seventies, with Katalin S.-Rózsa, who worked out a semi-intact preparation from the snail *Helix pomatia,* consisting of the brain and the cardio-renal system with the connecting nerve, we first achieved to record both excitatory and inhibitory responses in central neurones during heart stimulation [53]. We could also identify single neurones capable of increasing or arresting heart beat when depolarised by current injection [52]. For identification and localisation of the neurones and pathways we used both intracellular and retrograde $CoCl_2$ staining technique [54], being a new method at that time introduced by Pitman et al. [21] on insects. On another type of *Helix* preparation the central representation of chemosensory input from the lip was studied with my Vietnamese PhD student Truong Van Bay, and the involvement of a rather large number of neurones was shown in the central chemo-discriminating process [36]. Working with Jahan-Parwar in Worchester (USA) on *Aplysia,* we could also prove the cellular representation of the input from the statocyst and tentacle nerves in identified neurones of the cerebral ganglia [33]. As a continuation of these studies, in collaboration with L. Hernádi and Gy. Kemenes, both the sensory and the motor neurones of the cerebral ganglia connected to the lip were examined and mapped on *Helix* [8, 12], serving as a starting point of further studies of the feeding behaviour and learning of snails by Gy. Kemenes.

The most famous Gastropoda species for giant neurones, the sea hear, *Aplysia,* is not available in Hungary. However, we could work on it at the Adriatic see, in Kotor (Yugoslavia) in two consecutive summers, and could show, that the central neurones collecting sensory input from and sending motor output to the peripheral organs, similarly to *Helix,* are distributed scattered in nearly all units of the brain, representing diffusely organised networks including multifunctional neurones [60, 61]. The specificities, connections and functioning of central neurones were investigated in detail

by Katalin S.-Rózsa, resulting later in her theory on overlapping, variable, dynamic organisation and re-organisation of neuronal networks.

The functional identification of molluscan neurones and the clarification of their individual membrane properties contributed to a great degree to the understanding of basic principles of neuronal regulation. The great heterogeneity found not only in the chemical sensitivity but also in the ionic processes underlying excitation and inhibition led to particular studies on selected, identified neurones possessing of special properties. For example, the peculiar activity pattern of the "oscillatory" or "bursting" neurone discovered by Arvanitaki and Chalazonitis in the early sixties in the *Aplysia* visceral ganglion (cell R15) attracted the attention of many laboratories. Shortly after the first Invertebrate Neurobiology Symposium in Tihany where they presented the latest results [1] and mentioned that a similar neurone exists also in terrestrial snails, together with D. Sakharov we have found and localised the homologue of this bursting cell in the parietal ganglion of *Helix pomatia* [22]. The cell, marked on the map as RPa1 neurone, became for a time our favourite object. With the participation of S.-Rózsa and Véró, and of a younger group of colleagues, including K. Elekes, D. Budai, I. Vadász and Ágnes Vehovszky, basic morphological and functional properties of this neurone were discovered. First we made a mistake, believing that RPa1 neurone has no output to the periphery but takes part in intraganglionic regulation [45], but this was corrected when we demonstrated its axon in the intestinal nerve by parallel recording of extracellular activity with spiking of the neurone and by retrograde staining [27]. In a series of experiments we showed that the spike generation is Na-dependent but TTX insensitive [51], meanwhile the interburst hyperpolarization is metabolic dependent, disappearing at low temperature [46], in Ca-free medium and after intracellular injection of metabolic inhibitors or Ca-chelator [40, 63]. We could not prove any synaptic output of the neurone to peripheral organs, however, we detected by electrophysiological recordings and ultrastructural analysis that it received large number of excitatory and inhibitory input from the CNS and the periphery [4, 39, 65]. The generation of bursting activity in RPa1 neurone resembles in many respect to that described for the *Aplysia* R15 and similar cells found in nearly all gastropods, however, the real function of this type of activity has not been clarified until now. The suggestion that it effectuates the release of peptides at nerve endings still requires experimental confirmation.

The idea of peptides as putative central neurotransmitters in vertebrates emerged in the seventies. Our laboratory joined this trend with invertebrates to clarify whether high molecular-weight substances are functioning in the neural regulation already at lower levels of phylogeny or not, since it was a question at that time. As a first evidence, the presence of opiate receptors in the brain of *Anodonta* and *Helix* was shown by a study of Hiripi, S.-Rózsa and Stefano [9], and later, working both in Tihany and in the United States with D. Carpenter (Albany) and B. Stefano (Old Westbury), the specific membrane effects of substance P, FMRFamide and enkephalins on identified *Helix* and *Aplysia* neurones were demonstrated by electrophysiological experiments [48, 58, 59]. It was followed by a complex study with opioids in *Aplysia* brain [3],

contributing to the recognition of peptides in interneuronal communication throughout the animal kingdom.

Similarly to many marine laboratories, like in Naples, Plymouth, Arcachon and others, the Tihany Institute had since its foundation two main sections, a hydrobiological, called Balaton Department and an Experimental Zoology Department. It was the zoological part which became engaged from 1962 with invertebrate neurobiology. I myself, although being the Head of the Institute, was not involved directly in the research of the other Department, but took part in co-ordinating and managing its activities. Nevertheless, working also on filtration behaviour of mussels and the effect of water pollution on it, being the second branch of my studies started still in Moscow, my research was close to hydrobiology and ecotoxicology. This trend has strengthened from the second part of the seventies, due to the growing interest towards environmental problems in biology, and especially from 1982, following the decisions of our superising authority, the Hungarian Academy of Sciences, declaring Balaton research as the main task of the Institute.

The resolution adopted by the Academy was a part of a governmental program responding to worrying problems occurring in the human environment, threatening also Lake Balaton. In 1965 and 1975, two massive fishkills occurred in the lake, and due to the increase of nutrient supply a growing eutrophycation became also a real danger. To clarify the reasons of the water quality deterioration and to work out proposals for restoring and maintaining favourable conditions of the lake, a state-supported program has been initiated and implemented. The issue of environmental protection was widely discussed that time on international level, and the efforts of Hungarian officials and scientists were in accordance with it. The International Biological Program started in the seventies, its continuation called the Man and Biosphere Program and the scientific program on Biological Monitoring of the State of the Environment launched in 1982 by the International Union of Biological Sciences characterise the initiatives in this respect. The Tihany Institute participated in all of these programs. My personal research interest and earlier studies determined my involvement in the Biomonitoring program, in the elaboration of which, being at that time member of the Executive Committee of IUBS, together with Rita Colwell (USA) and John Vallentyne (Canada) I took an active role.

As it has become evident in the past decades, toxic heavy metals, pesticides and other pollutants are often responsible for the deterioration and killing organisms in aquatic ecosystems. On the other hand, many organisms are able to give a characteristic response without serious damage to the changes in the quality of the environment, and so can be used to monitor it. Based on these principles, we were studying the behaviour of mussels under the effect of environmental pollutants, employing their filtration activity in monitoring water quality. My studies, mentioned earlier, showed the characteristic, well measurable response of the freshwater mussel to some heavy metals, being at the same time very tolerant to these substances. Working at the famous "Aquarium" in Naples in 1965, I have repeated my experiments with twelve marine bivalve species, to clarify whether the periodicity of activity found in *Anodonta* is also a typical behavioural pattern of them, and if so, does it change under

the influence of toxic substances effective on freshwater mussel. It turned out, that ten of the studied species behaved and responded exactly in the same way as *Anodonta* did. The two exceptions were *Pecten* and *Cardium,* which have no "catch" muscle and are not able to keep their shells closed for long period of time [25]. Ten years later working at Friday Harbour Laboratory I checked the behaviour and reaction of the American *Oyster,* and found it also similar to *Anodonta.* As a conclusion I recommended to use bivalves for functional monitoring of water quality in relation to toxic substances [28, 31], under both laboratory and natural conditions [66]. Another method we have developed on mussels with Turpajev and Nechaeva (from Moscow) for rapid laboratory testing of the harmful effect of pollutants was the recording and measuring of water flow through the siphon [44]. By employing these methods, the toxic effect of various heavy metals, insecticides and herbicides occurring in or used around Lake Balaton could be traced [15, 29, 37, 38], and utilised in protection of the water quality.

Mussels are animals capable of accumulating and storing abiotic chemicals in their body, so they can be used as passive biomonitors of the quality of the environment, as it has been shown earlier in marine ecosystems. Other aquatic animals, including fish, are also suitable organism for this purpose. Following the strategy of measuring the concentration of toxic metals in various tissues of animals as markers of the contamination, together with Katalin V.-Balogh, Anna Farkas and I. Varanka in a several years program, we have studied the level of pollution in Lake Balaton. We came to the conclusion, that the animals of the lake are polluted with toxic metals to a low degree [5, 47, 64], although, because of the permanent environmental impact a regular control of the level of pollution is necessary. Further on, due to possible sublethal effect of toxicants, studies on the reaction of the animals of Lake Balaton to low concentrations of pollutants are indispensable.

Toxic substances released into the environment cause often neurological disorders in humans, being the main targets of the action the neuronal structures. In modifying the behaviour of aquatic animals by toxicants the basic mechanisms also at neuronal level can be supposed. As a first experimental evidence, the effects of cadmium and mercury ions on the chemosensitivity of neuronal somata of *Lymnaea stagnalis* have been described [55], proving that receptors of the neuronal membrane are involved in heavy metal action. Working in 1986 with ACh sensitive *Aplysia* neurones under voltage clamp in the laboratory of David Carpenter at Albany, I checked the effect of cadmium ions on the reaction to the transmitter at different holding potentials. Working on a neurone located in the caudo-dorsal part of the pleural ganglion responding to ACh with inhibition by activating both chloride and potassium channels, a rather unexpected result was obtained: the outward current consisting at –50 mV holding potential of a fast chloride and a slow potassium current was partly inhibited by cadmium ions, namely the potassium component was blocked, whereas the chloride component remained intact [30]. The fact that cadmium ions prevented only the activation of ACh dependent potassium channels suggested that its toxicity cannot be explained with a general membrane damage, but with a rather specific one. These observations were the starting point of a new trend of research in Tihany

and also of a fruitful collaboration between Tihany and the Research Laboratories of the N.Y. Health Department at Albany, dealing with the effect of toxic heavy metals on Gastropoda and vertebrate neurones.

In these studies, with the participation of Katalin S.-Rózsa, T. Kiss, J. Győri, O. Osipenko, S. Rubakhin and A. Szűcs, all working in Tihany, not only the modulatory effects of cadmium, copper, mercury, lead and silver on the excitability of the neuronal membrane and on the activation of currents were described, but in some instances also differences in modulation of synaptic events could be revealed [7, 19, 56, 57]. In experiments with ACh, 5HT, DA and Glu activated currents a great heterogeneity of effects was proved, showing, that toxic metals have very specific targets at the neuronal membrane, and probably at the intracellular metabolism as well [6, 41, 50].

Beside detecting acute effects of heavy metals on neuronal membranes and synaptic transmission, also their influence on synthesis and release of transmitters as well as, under chronic treatment, ultrastructural alterations of peripheral receptors were observed [2, 20, 32, 49], indicating, that heavy metal ions may have very profound and widespread, specific effects at different levels of neural regulation.

Working in a research Institute and having no teaching obligations I could spend much more time in the laboratory than my colleagues at universities. However, since the Institute is situated in a small village, even if Lake Balaton is a fashionable resort at summer, communication and collaboration with other laboratories were not easy. To reduce isolation, by exploiting the advantages of the surroundings, the Institute from its foundation served as a summer conference, research and holiday place for Hungarian biologists. This tradition was continued and extended by organising special training courses and also by inviting guest-scientists for joint research. As the overall political relaxation from the second part of the sixties allowed, I initiated also to organise international conferences at Tihany, which soon became a meeting point for eastern and western scientists who otherwise could not have got together. On the basis of our neurobiology research, not only series of conferences were held, but the International Society for Invertebrate Neurobiology has been founded in 1987, of which I served as first president. Conferences and training courses took place also in hidrobiology and environmental biology, and this way the international relations of the Institute has grown significantly preceding the time when the iron curtain ceased and the Berlin-wall fell down in 1989.

In addition to my activity in scientific administration inspired and stimulated by personal and local institutional motives in Tihany, I became involved in the co-ordination of research at a broader scale, too. In the sixties, the Academy of Sciences started in launching scientific programs, as part of the overall policy of planning of the government. In life sciences preference was given to molecular biology, but other fields were also considered. I was nominated to co-ordinate Academy-supported projects in neurobiology. Starting in 1972, report conferences were organised yearly in Tihany and later in sequence at university cities, already not only for project members, but also with the participation of all Hungarian neurobiologists. These meetings turned to be prestigious scientific events, giving an impetus first to the establishment

of a special section within the Physiological Society and in 1993 of the independent Hungarian Neuroscience Society, when – becoming the past president of the new Society – my co-ordinating activity came to end.

I have also been involved in the activity of a number of other Societies like the Biological and the Biophysics Society, as well as in scientific committees of the Academy. Among others between 1985–1997 I fulfilled the post of the President of the Veszprém Academic Committee, one of the five regional committees of the Hungarian Academy of Sciences. I am acting as the Editor of Acta Biologica Hungarica since 1977, and as member of the Editorial Board of several international Journals. I dealt also with popularisation of science, being nearly for a decade the president of the Biological Section of the relevant National Society, organising local and nation-wide conferences mainly for school teachers.

In spite of the isolation policy in Eastern European countries at the time of the cold war, the Academy of Sciences could maintain relations with international bodies, what turned to be more and more flexible from late sixties. As a result of that, Hungary became an active member of the International Council of Scientific Unions and its family Organisations, among them of the International Union of Biological Sciences. At the time when Professor Imre Törő was the Chairman of the Hungarian National Committee for IUBS I have been the Secretary and following his resignation due to his advanced age, I took over the chairmanship in 1980. As the representative of Hungary I became member of the Executive Committee of the Union at its Bangalore's General Assembly in 1976. Six years later I have been elected Vice-president, and in 1988 President of the IUBS. In this capacity I could influence the science policy of the Union, strengthen the involvement of less favoured countries in the scientific programs and help a good number of Hungarian biologists extend their international co-operation. With establishing in IUBS the Interdisciplinary Commission on Biological Monitoring, of which I am still the Chairman, workshops and a series of conferences were organised, Proceedings and a Manual of Methods have been published and international co-operation of laboratories was initiated.

As a response to growing interest and worry of the public concerning the quality of fresh water resources, an International Lake Environment Committee was created at Lake Biwa with the support of the Japanese Government in the eighties, to consider and attract attention to the problem of large lakes of the world. Upon the initiative of Hungarian authorities, Balaton as an endangered great shallow lake was included into the program. As Director of the Tihany Institute I was invited to the Scientific Committee of ILEC, and was authorised to organise the third International Conference on Conservation and Management of Lakes, which was held at Keszthely in 1988. The research carried out on Lake Balaton, and the efforts made in order to restore earlier favourable conditions of the water quality got an international recognition through this Conference and the Proceedings published afterwards. I remained for eight more years member of the Committee, taking part in the preparation of succeeding conferences and organising training courses for ILEC/UNESCO in Tihany. It has also enriched my knowledge and experiences, becoming very use-

ful at my governmental assignment to take part in planning and to co-ordinate Balaton research with the participation of 28 laboratories between 1995–98.

Due to my heavy engagement as the head of the Institute and other obligations, I was unable to participate in regular lecturing at Universities distant from Tihany, although in the late sixties I have had a special course in Budapest for several years. As a result of that I have received the title of Titular Professor of the Eötvös Lóránd University. In the early nineties, the Chemical University of Veszprém has been transformed to a general University, and I was invited to join the staff of the Department of Informatics in lecturing basic neurobiology. Since by the end of 1990 my appointment as Director of the Balaton Limnological Research Institute had expired, I took the responsibility of regular teaching at Veszprém, located only 30 km from Tihany, and continued working in the Institute with my team as a research professor.

Remembering and summing up in this short review the main trends and results of my scientific activity, I do not want to give any evaluation of the way I have passed. My education, the circumstances and at some crucial occasions my own choice determined and governed this path, influenced also by my colleagues I had been working with. The main research topics I was involved in were proposed by my masters or were chosen as recognition of trends or intuition of further development in science or were forced by existing circumstances and events requiring to react to current problems. Looking back to the years when I have started in science, I often remember the technical conditions and circumstances we were living in. During the past forty-six years an enormous technical development has happened in research. While working in Moscow and even in Naples with kymograph and smutty paper in recording the activity of fixed mussels, today for the same purpose we use on-line collection and evaluation of transducer produced data obtained from free-moving animals with the help of electronic sensors. For doing experiments in cellular neurobiology, instead of home made instruments we have in the laboratory sophisticated, programmable pullers, generators, amplifiers, and naturally computers for programming experiments, processing results and printing diagrams. The technical and conceptual development I have passed as well as the achievements I have obtained with my colleagues are reflected in about 240 research papers. Beside that, I have edited or co-edited more than 20 Volumes, mostly Proceedings of conferences I have organised. My results and achievements were honoured by electing me to the Academy as a correspondent member in 1976 and as an ordinary member in 1987. Nevertheless, there is always a rightful criticism when one looks back and recalls his own earlier concepts, approaches, experiments and results, even if they seemed to be novelties that time and were recorded among the contributions to the development of our knowledge in biology. The real satisfaction is if some of them will remain recognised by the scientific community and stimulate further research of the coming generations.

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AN ATTEMPT FOR THE APPLICATION OF THE BASIC LAW OF PSYCHOPHYSICS (FECHNER–STEVENS LAW) IN THE DOMAIN OF VISCERAL STIMULI IN HUMANS*

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The validity of the basic Law of Psychophysics proposed originally by Fechner and revised by Stevens had been tested in the domain of visceral perception. The experiments were carried out on six adult colonostomy patients. The distension of the sigmoid colon (the ϕ-value of the Fechner–Stevens equation) had been undertaken by the aid of a rubber balloon inserted into the intestinal stoma orifice and controlled by a computerized pneumatic system. The tracking of sensations of "gut-feelings" by the subjects (the ψ-value of the Fechner–Stevens equation) had been made possible by applying the subjects' manipulation of a sliding potentiometer of a Visual Analogue Scale (VAS-device). The results seem to prove the extension of the validity of the Fechner–Stevens power equation to the field of visceral perception.

Keywords: Fechner–Stevens Law – psychophysics – visceroception – gut stimuli

INTRODUCTION

The endeavour for quantification of sensory events taking place in the animal and human organism was a powerful claim since the advent of modern Experimental Physiology. This fundamental effort happened to be successful in the framework of the pioneering experimental data of Fechner [6], who first formulated the quantitative relations between the magnitude of the sensory experience (Ψ) and the intensity of the physical stimulus (ϕ). His main proposition, the basic *Law of Psychophysics* happened to be validated in the domain of every sensory modality (visual, auditive, tactile, etc.) studied in this Century. It is essential, however, to underline that instead of the logarithmic relations originally formulated by the above author, nowadays we apply the power function proposed by Stevens [9], namely: $\Psi = k \cdot \phi^{\alpha}$, in which the exponent α depends on the modality of the stimulus tested.

* Dedicated to Professor János Salánki for his 70th birthday.

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In the course of our several efforts to elucidate the role of the viscerosensory input in the processing of information arriving from internal organs [2, 3], the claim has been expressed by us many years ago [1] to apply the Fechner–Stevens Law in the domain of visceral sensory phenomena. Namely it was our strong belief that the visceral sensation and perception must and should be included among the Special Senses. A considerable amount of experimental data summarized recently by us [3] had reinforced this view, but the extension of the validity of the Fechner–Stevens Law to the field of viscerosensory events remained up to the present study merely an unfulfilled claim. In order to fulfil this plan, based on the presumption outlined above, the aim of the present study has been the elucidation of the relation of non-painful, weak visceral stimuli arising from the gut, to some dim feelings, in other words minimal abdominal sensations, experienced by our human subjects.

MATERIALS AND METHODS

Subjects

The experiments have been undertaken on six adult (three male and three female) subjects (mean age 62 years) who underwent the surgery of colonostomy. This intervention has the aim to create an artificial intestinal orifice (usually the transsected oral end of the sigmoid colon) and to suture it to the skin of the abdominal wall. The subjects, usually suffering from recto-sigmoid cancer, defecated, following this surgery, through the artificial stoma, which served as an "anus praeter naturalis". The patients were recruited from the volunteer members of the Budapest Club of Ileo-Colonostomy Patients (ILCO), a branch of the International ILCO Federation. The stoma patients are usually enthusiastic and devoted supporters of such investigations. All subjects were completely informed about the aim and the methods of the investigation and gave a written consent. The patients defecated 2–3 times daily by means of irrigation, consequently the insertion of a balloon-catheter into the stoma orifice during the experimental session did not raise any problem.

Technical equipment

A rubber balloon-catheter, manufactured in the Laboratory, has been inserted into the abdominal orifice of the colonic stoma 10–15 cm deep. The inflation of the balloon with air was undertaken with the help of a computer-driven pneumatic system which enabled the precise dosing of the air and the exact recording of the volume and the pressure circumstances in the system on the hard disc of a computer. The computer-operated pneumatic system was described in detail by us previously elsewhere [7].

The subjects were instructed to follow with careful attention the distention changes in that part of their gut (the sigmoid colon) in which the balloon was situated and to try to tune accordingly the scale from 0 to 100 of a special sliding poten-

tiometer, a Visual Analogue Scale (VAS) being within the subject's reach. The VAS was equipped with a sliding handle and also with a button enabling the subject to signall when the tuning had been completed. The manipulation of the handle of the VAS was visualized on the screen of a monitor placed 2 meters in front of the subject. By the way, for safety reasons, the VAS-device carried additionally a special "emergency" STOP-button, to enable the interruption of the experiment by the subject in case of some inconvenience or alleged danger (this event had never occurred during the whole investigation).

The experimental procedure

Every subject took part in three consecutive sessions on 3 different days. Thus the results of a total of 18 sessions have been statistically evaluated. Each session consisted of *three* successive stages and lasted about 2 hours.

During *Stage 1* the *minimal threshold* as well as the minimal limit of the feeling of a marked *discomfort* had to be detected by each subject following a rather smooth gut distention with uniform acceleration (1 ml/sec). The balloon was inflated and deflated three times during one session and the patient was instructed to push the signalling button on the VAS immediately experiencing successively one of the 2 situations: (i) Detecting a feeling of minimal, "dim" tension or gut movement in the site of the stoma. This value was regarded in the two next stages of the session to come as the minimal threshold, and was the starting point "0" in the course of our data analysis displayed on the horizontal x-axis of our three Figures (see Figs 1, 2, and 3). (ii) Detecting a sensation of *minimal, but marked discomfort* or eventual unpleasantness, accidentally pain. This second value was regarded in the two stages to come as the point "100" of the data analysis and are shown likewisely on x-axis of the same three Figures of this study. This Stage 1, regarded by us as a *"digital"* Stage, was in effect the visceral application of the *method of limits* originally described by Békésy [4] for audiometric purposes and served in the next Stages 2 and 3 (see below) as fix points of orientation concerning minimal detectable (0-value) and minimal discomfort (100-value) figures concerning the volumes of balloon distension. All subsequent balloon inflations had been carried out taking into consideration this "0 to 100-span" serving as the x-axis of the coordinate system diplayed on the three Figures.

Stage 2 consisted of the close, continuous and faithful following by the subject of the computer driven inflation and deflation of the balloon by manipulating the sliding handle of the VAS according to his/her increasing or decreasing "gut-feeling" of pressure and discomfort. Stage 2 was regarded as the *"analogue"* Stage of the sessions. The horizontal slipping of the handle has been made visible on the screen of the computer as outlined above. The subject was instructed to follow-up and "imitate" the computer-triggered either slow (1 ml/sec) or rapid (5 ml/sec) distension and the subsequent sagging of the balloon (more precisely of the intestinal wall) by moving manually the handle, to guide and control in this way visually on the screen his/her own manipulations. Both the increasing-decreasing volume and pressure val-

ues of the balloon as well as the follow-up values of the sliding handle were recorded in 100 steps (from zero to one hundred) on the hard disc of the computer which otherwise controlled the entire experimental session.

Stage 3 consisted of the rapid, stroke-like application of nine balloon-inflations and deflations several times during this last stage of the session. The intensity of these sudden impulses amounted to 11%, 22%, 33%, 44%, 55%, 66%, 77%, 88%, and 99% of the "0 to 100-span" quantified in Stage 1 as described above. This final stage of the session was regarded as the *"gut-impulse"* Stage, in which the 9 balloon-impulses were applied in a random order at least 5–5 times during the Stage asking the subject to tune the VAS-handle accordingly.

Analysis of the experimental data

Stage 1 In the course of the analysis of the data of these "digital" trials the grand averages (a) of all *minimal threshold* values (expressed in the volume of the balloon) just detectable by the given subject during balloon inflations and deflations have been calculated and, subsequently, all *discomfort* values of the same subject have been averaged. Consequently each of the six subjects had his/her own "0 to 100-span" as described above, and in the Stages to follow we worked taking into consideration these individual dual threshold values when compiling the three coordinate-figures of the results.

Stage 2. In the course of the analysis of the data of these "analogue" trials the grand averages of the values of the "gut-feelings" (the ψ values) of all six subjects have been computed and averaged. These ψ-values were the representations of the tuning, i.e. of the manipulation of the VAS-handle, which had accompanied the automatic, computer driven balloon-inflations and deflations. The air inflations of the balloon were *continuously,* i.e. unabruptly supplied and withdrawn from the pneumatic system by the computer starting from minimal threshold (0-value) up to the discomfort limit (100-value) forth and back. On the other hand, the *sampling of the ψ-numbers* was a *fractional* event: the ψ-series being abruptly divided into *100 fractions,* each fraction reflecting the momentary situation of the manipulation of the VAS-handle in those moments, when the balloon-inflation or deflation had changed up or down with *one percent* of the "0 to 100-span". By applying this means of computation a cluster of 100 points had been obtained representing a special "cloud of gut-feelings" during balloon inflation and deflation. This cluster of 100 points has been covered later with a *fitted curve of the Fechner–Stevens equation.* The above described Fechner–Stevens power relation had been additionally calculated and represented over the cluster of the 100 ψ-values. In this power equation the exponent α equalled 1.7 to 2.1.

Stage 3. In the course of the analysis of data of this "gut-impulse" Stage a grand average of all ψ-values detected with the help of the VAS-device following the nine different impulse-like distensions were calculated in such a way that each of the 9 volumes from 11% up to 99% of the "0 to 100-span" was graphically represented as

one single square on the coordinate. The results of this final Stage were also additionally computed according to the Fechner–Stevens equation and the appropriate curve was fitted to the row of the nine ψ-squares.

RESULTS

The results are summarized on the three Figures. As seen in Figure 1, which summarizes the results of the Stage 2 *slow* (1 ml/sec) "analogue" tracking trials , the cluster of the hundred ψ-points represents a marked ascending string which climbs from the ψ-intensity of about 10 up to the ψ-value of roughly 60. This climbing row shows a definite relation with the fitted curve of the Fechner–Stevens equation. The same ascending cloud of the 100 ψ-values can be observed in Figure 2, where the results of the Stage 2 *rapid* (5 ml/sec) "analogue" follow-up trials have been represented. Although the cluster of the 100 ψ-points seem here somewhat more dispersed than in the case of the slow (1 ml/sec) balloon inflation trials, the upward climbing of the spreaded ψ-cloud is apparent. And, what is even more convincing, its linear relation with the fitted curve of the Fechner–Stevens equation seems probable. This coher-

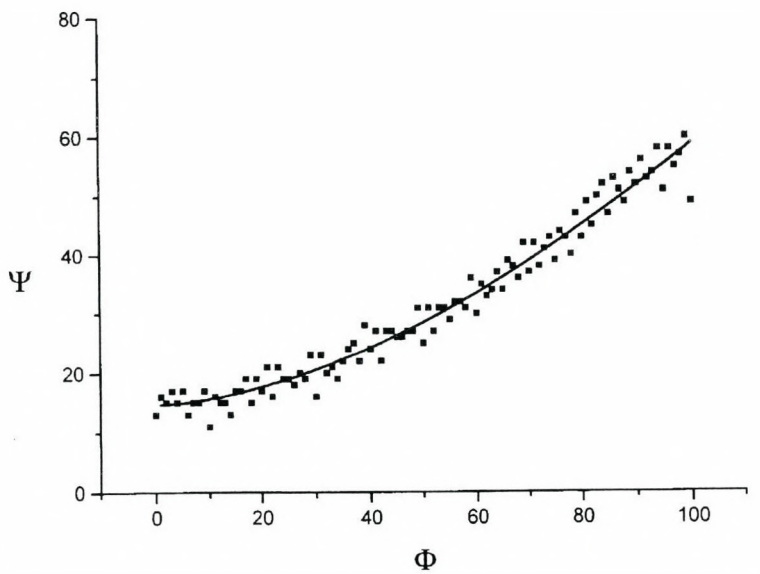

Fig. 1. Coordinate graph of the *slow* (1 ml/sec) gut distension values of the 0 to 100-span (ϕ-values on the x-axis) plotted against the gut-feeling numbers (ψ-values on the y-axis) as expressed according to the scale of the VAS sliding potentiometer numbers. Each of the 100 small black square points of the ψ-cluster represents the grand average of momentary gut-sensation detected and tuned by all 6 subjects on the VAS, which accompanied every step of 1% balloon inflation of the 0 to 100-span. The fitted curve of the Fechner–Stevens equation is superposed on the 100-point ψ-cluster. Further explanation in the text.

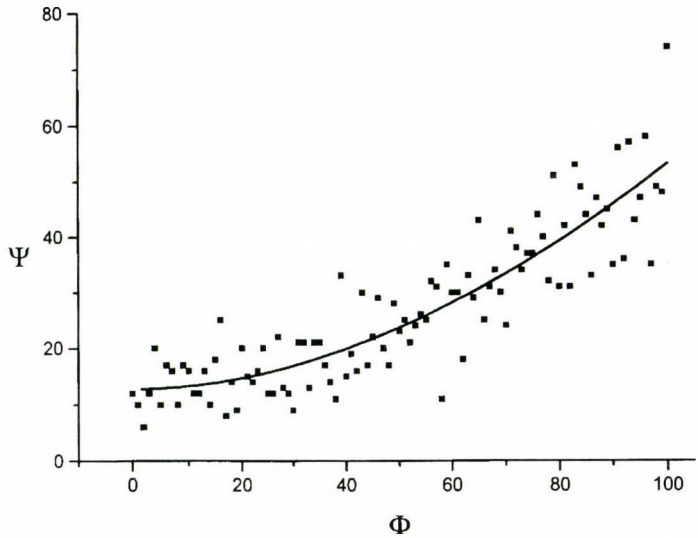

Fig. 2. The same type of coordinate graph as in Figure 1 which represents in this case the *rapid* (5 ml/sec) gut distension (ψ) data. Further explanation in the text

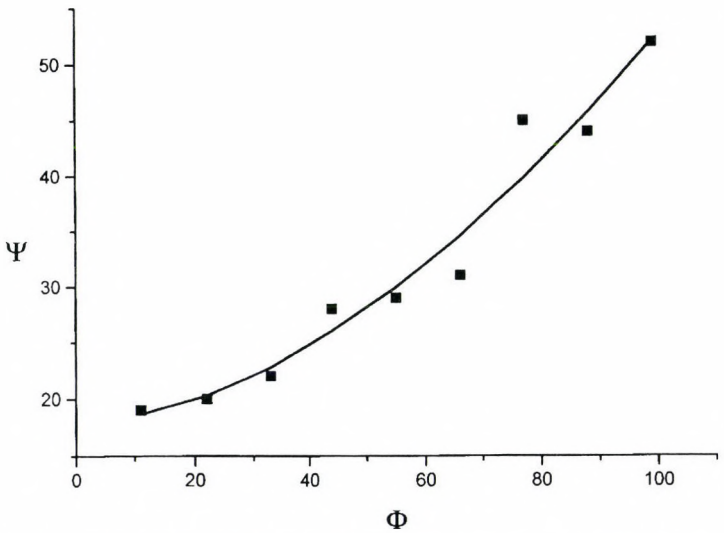

Fig. 3. Coordinate graph of the stroke-like 9 gut distension *("gut-impulse")* values of the 0 to 100-span (ϕ-numbers on the x-axis) plotted against the detected nine gut-feeling values tuned on the VAS (ψ-numbers on the y-axis). Each of the 9 black squares represents the grand average of the ψ-values of all corresponding trials on the six subjects during repeated and random balloon inflations starting with 11% up to 99% of the 0 to 100-span. The graph displays also the superposed Fechner–Stevens curve fitted to the row of ψ-values similarly to Figs 1 and 2

ence appears even more convincing in Figure 3, where the ascending nature of the nine ψ-values of the "gut-impulse" tracking trials and the relation with the fitted Fechner–Stevens curve seems evident.

DISCUSSION

The results outlined above seem to reinforce the validity of the Fechner–Stevens power law in the field of the visceromechanical modality, more precisely in the domain of the distension of the human large intestines. The linear relation between the volume of air in the inflated balloon inserted into the gut and the estimated "feeling" of distension displayed on the VAS-device seems evident in analyzing the three Figures of the present study. Consequently the data confirm our presumption about the possibility to extend the force of this power law to the sphere of visceral sensation and perception. We have found merely sporadic data in the recent literature which seem to be in agreement with our results [5, 8]. Thus the statement of Stevens [9] cannot be denied: "The psychophysical power law now stands as the most pervasive and perhaps the best supported quantitative generalization in psychology. There appears to be no exception to the rule...". It seems that the above data confirm the veracity of this statement: the internal milieu of the organism and the rather "dim feelings" arising from that domain do not constitute "exception to the rule".

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THE ELECTROGENIC SODIUM PUMP ACTIVITY IN *APLYSIA* NEURONS IS NOT POTENTIAL DEPENDENT*

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We have investigated the potential dependence of the electrogenic sodium pump in *Aplysia* neurons by recording the potential and current induced by sudden change of the artificial sea water from one containing K^+ at various concentrations to K^+-free sea water in the presence or absence of ouabain. Both K^+-free sea water and ouabain block sodium transport and result in a significant depolarization due to removal of a maintained outward current that is a result of transport of more Na^+ out of the cell than K^+ into the cell during pump operation. In the presence of ouabain there is, however, an inward current induced by changing external K^+ concentration from zero to some value between 1 and 20 mM, and this current is greater with a greater K^+ concentration gradient. The current induced by change from zero to 1 mM K^+ does not show any potential dependence, although those currents induced by higher K^+ concentrations are potential dependent. We conclude that the activity of the electrogenic sodium pump is not potential dependent, but that the potential independence is obscured if higher concentrations of K^+ are used to activate the electrogenic sodium pump.

Keywords: Sodium pump – electrogenic pump – *Aplysia* neurons – potential dependence – ouabain – potassium-free seawater

INTRODUCTION

All living cells must control ionic concentrations gradients. This is particularly significant in electrically excitable cells, such as nerve and muscle, which depend upon the concentration gradients for Na^+ and K^+ for action potential generation. The maintenance of the low intracellular Na^+ and high intracellular K^+ is accomplished by the activity of the sodium pump, which extrudes Na^+ and accumulates K^+ through activity of the enzyme, Na^+-K^+-ATPase [6, 7]. However, this process does not transport equal numbers of sodium and potassium ions, and therefore the difference, a transport of more Na^+ than K^+, constitutes a net outward current [1]. While the sodium pump is electrogenic in all cells, the net current generated by the sodium pump is pro-

* Dedicated to Professor János Salánki for his 70th birthday.

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nounced and physiologically more important in those cells that have high membrane resistance, especially in neuronal cell bodies [3].

While the characteristics of the electrogenic sodium pump have been investigated for many years, there remains uncertainty as to whether or not it is a potential-dependent process. It is not easy to approach this question experimentally because of the fact that most manipulations which will turn the pump on and off, such as temperature, drugs or alteration of the ionic composition of the medium, also alter other processes. We have applied a rapid microperfusion system to study this old question, and have induced rapid changes in pump activity by removal of external K^+. Our results suggest that the electrogenic sodium pump is not potential dependent.

MATERIAL AND METHODS

Over 70 medial pleural neurons were recorded from the right or left pleural ganglia of *Aplysia californica* (Pacific Biomarine) and studied under current or two-electrode voltage clamp as previously described [8, 9]. All data were collected on a Gould pen recorder. A fast microperfusion was applied through a thin (1.0 diameter) perfusion tube placed close to the neuron from which the recording was being made [9], which allowed a complete change of the external solution within 100 ms.

The ganglia were perfused with an artificial sea water containing (in mM) NaCl, 480; KCl, 10; $MgCl_2$, 20, $MgSO_4$, 30, $CaCl_2$, 10, HEPES, 5, pH 7.8. In many experiments a K-free sea water was used in which KCl was replaced with additional NaCl. In some experiments ouabain (Sigma) was added and was prepared just before use. All experiments were done at room temperature (23–24 °C).

RESULTS

Figure 1 shows a current clamp recording from a medial pleural neuron upon rapid microperfusion going from normal sea water to one without K^+ (Fig. 1A) or to one containing 10^{-4} M ouabain (Fig. 1B). Both manipulations cause a rapid depolarization of the cell. Of 70 neurons studied, with resting membrane potentials between –40 and –85 mV, in 53 the depolarization in going from normal to K^+-free sea water was between 1 and 15 mV, in 8 neurons it was between 15 and 20 mV and in 9 neurons it did not significantly change. None of the neurons demonstrated a hyperpolarization in spite of the fact that the potassium concentration gradient was increased by this manipulation, and on the basis of the Goldman–Hodgkin–Katz equation this would tend to cause a hyperpolarization of the membrane potential. Ouabain caused a similar but somewhat greater depolarization. Since ouabain is a relatively specific inhibitor of Na^+-K^+-ATPase, and since Na^+-K^+-ATPase requires external K^+ for activity, we attribute the depolarization to be due to the blockade of an electrogenic sodium pump. The observation that the depolarization in ouabain is greater than that in K^+-free solution is consistent with the hyperpolarizing effect expected by the

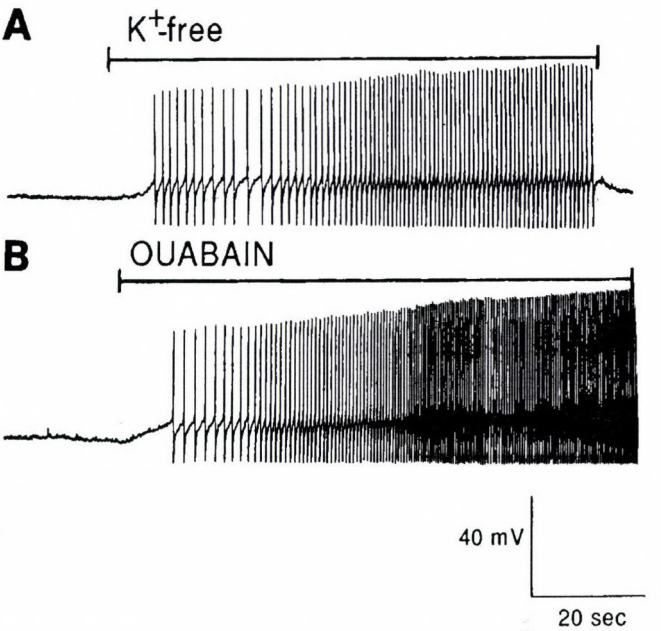

Fig. 1. Current clamp recordings from a neuron upon perfusion of K^+-free sea water (A) and ouabain (10^{-4} M) (B). Note that both depolarize the cell

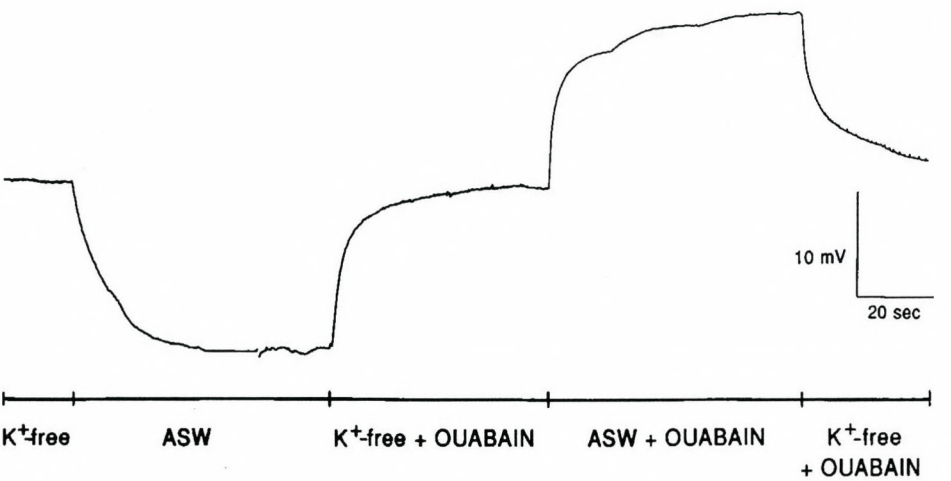

Fig. 2. Current clamp recording from another neuron upon changing the sea water composition as indicated below from K^+-free to normal artificial sea water (ASW) to K^+-free plus ouabain to ASW plus ouabain to K^+-free plus ouabain. The total potential produced by the pump is thus the difference between potential is ASW and ASW plus ouabain

increase in the potassium concentration gradient, which partially counteracts the effect of pump inactivation.

In order to determine the total potential contribution of the electrogenic sodium pump, we explored the interactions among K^+-free sea water, ouabain and normal sea water, as shown in Figure 2, from another current clamp recording. These recordings were obtained from one of 18 neurons which showed only membrane depolarization, not spike discharge, upon exposure to K+-free sea water and/or ouabain. In this cell the membrane potential increased by about 15 mV in going from K^+-free to normal sea water. When perfused with K^+-free sea water containing ouabain the membrane potential returned to about the same level as in K^+-free, indicating that the K^+-free sea water resulted in a total blockade of the electrogenic pump. When then perfused with normal sea water containing ouabain the membrane potential depolarized an additional 15 mV, and this depolarization reflects the pump contribution that offsets the effect of the increase in K^+ concentration gradient. Thus in this neuron the electrogenic sodium pump contributes current that generates about 30 mV.

In our next series of experiments we attempted to determine how the electrogenic pump activity was altered by membrane potential and external K^+ concentration. These experiments were done under voltage clamp conditions, and pump current was induced by measurement of the current induced when the microperfusion was changed from K^+-free to sea water containing various concentrations of K^+. Figure 3 shows how these experiments were done, and contrasts the effects of activation of the electrogenic pump to those of activation of a transmitter current. In the records at the

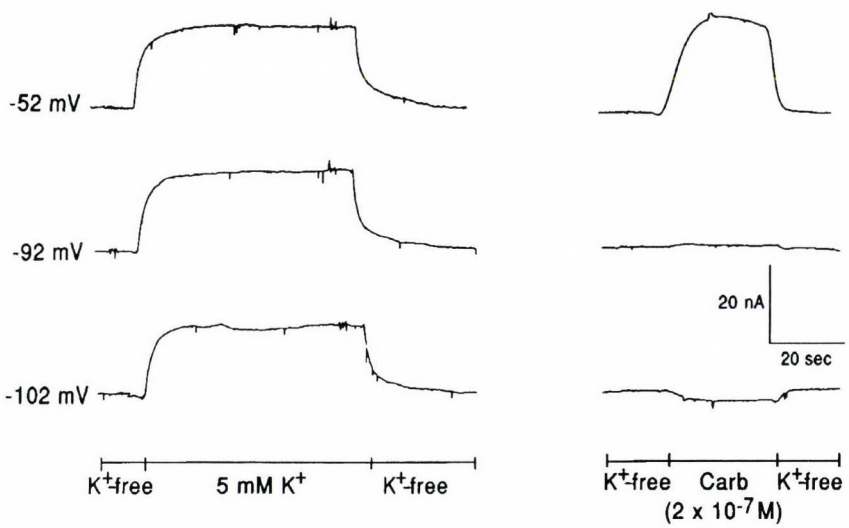

Fig. 3. Voltage clamp recordings from a neuron. The current produced by going from K^+-free to 5 mM K^+ sea water has a different voltage dependence than that produced by application of carbacol (Carb) (2×10^{-7} M), in that the latter current reverses at the potassium equilibrium potential, whereas the former does not

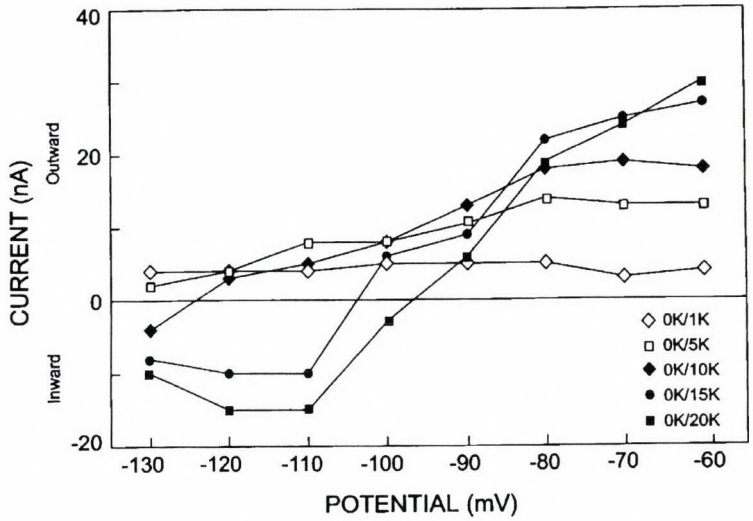

Fig. 4. Potential dependence of the current induced by changing the perfusion solution from K^+-free sea water to sea water containing various amounts of K^+. Note that there is little or no potential dependence in going from K^+-free to 1 mM K^+, and that the degree of potential dependence increases with increasing K^+

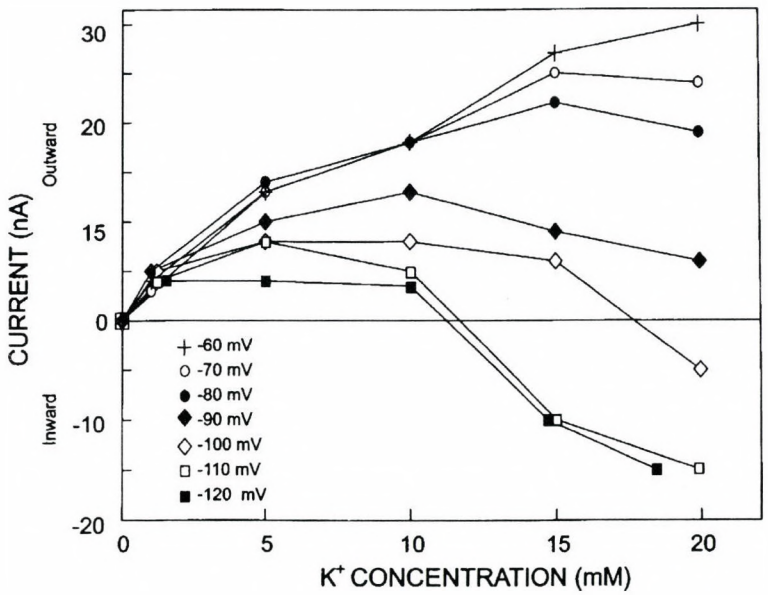

Fig. 5. Magnitude and direction of the current induced in going from K^+-free to various concentrations of K^+ in the ASW. This is the same data as in Figure 4, but plotted against the K^+ concentration. Note that the degree of potential dependence increases as the K^+ concentration increases

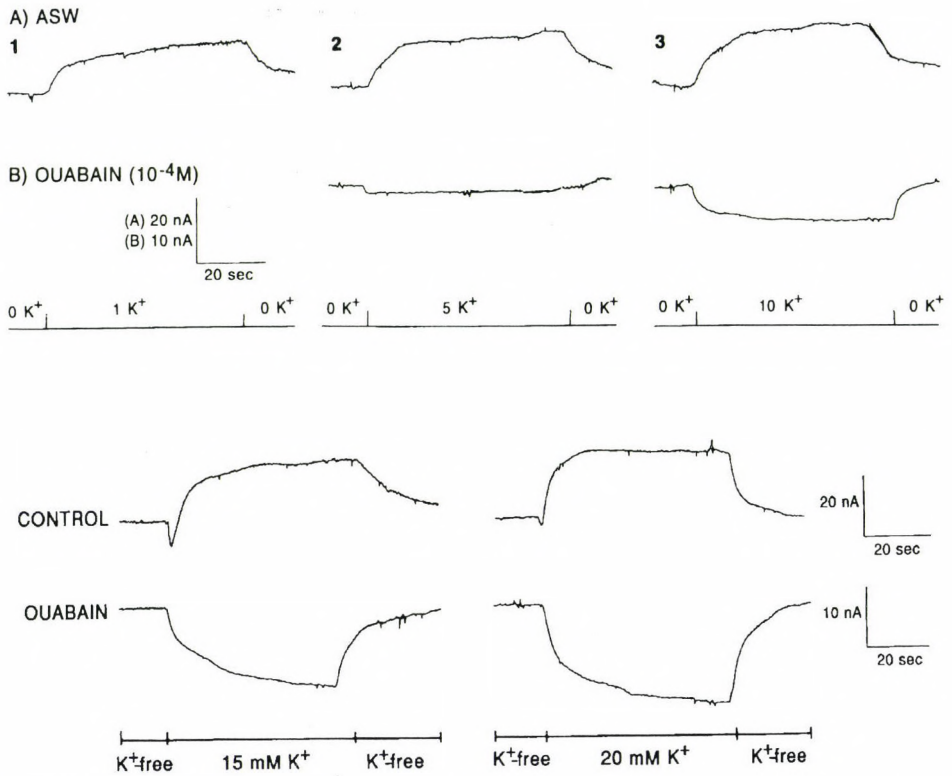

Fig. 6. Voltage clamp recordings of the current induced by going from K^+-free to 1, 5, 10, 15 and 20 mM K^+ in the control (arteficial sea water, ASW) and presence of ouabain. Note that in presence of ouabain at higher K^+ concentrations there is an inward current produced from only the alteration in K^+ concentration, indicating that the total pump current is the sum of the control outward current and the inward current in the presence of ouabain

left pump current was induced by switching the microperfusion from K^+-free to 5 mM K^+ at three different holding potential. Note that the current magnitudes are not greatly altered with increasing holding potential, although there is a slight decrease at –102 mV. This is in dramatic contrast with the voltage dependence of a response to carbacol, shown at the right. In this experiment, microperfusion of carbacol in K^+-free sea water produces a large outward current at –52 mV, no net current at –92 mV and a small inward current at –102 mV. This response is secondary to an specific increase in membrane potassium conductance, and reverses at E_K, which under the circumstances of our experiment was at about –92 mV.

The results of studies designed to explore the dependence of pump current on holding potential and K^+ concentration are shown in Figures 4 and 5, which are the same data presented in two different ways. Figure 4 shows the induced current plot-

ted against the K^+ concentration. At lower membrane potentials the current was outward and increased with increasing K^+ concentration. This almost certainly reflects an increased electrogenic pump activation induced by increasing the external K^+ concentration. While there was little potential dependence of the electrogenic current induced by 1 mM K^+ between –60 and –130 mV, at 5 and 10 mM K^+ there was a clear reduction of pump current at higher holding potentials. At the higher holding potentials the outward current declines with increasing K^+ concentration, and actually reverses at holding potentials of 100 or more with the higher K^+ concentrations. These data are shown with current plotted against holding potential in Figure 5. Note that there is no obvious voltage dependence when K^+ is changed from 0 to 1 mM, and that with increasing K^+ concentrations the apparent voltage dependence grows with the K^+ concentration gradient.

Figure 6 shows voltage clamp recordings of the currents induced by varying K+ concentration in the absence and presence of ouabain. This is thus the voltage clamp equivalent of Figure 2. Note that in the presence of ouabain the inward current in going from K^+-free to K^+ containing solution increases with the K^+ concentration gradient.

DISCUSSION

There are two components to membrane potential – that determined by the ionic concentration gradients and the relative ionic permeabilities, and that resulting from electrogenic transport processes, primarily the electrogenic sodium pump [2, 4]. Both processes are dependent upon the external potassium concentration. Because the neuronal membrane is much more permeable to K^+ than to any other cation, membrane potential approximately that of a K^+ electrode at high external K^+ concentrations [5]. However, operation of the sodium pump requires external potassium. There is no pump activity in absence of K^+, and pump activity increases with external K^+ concentration as clearly shown in Figure 2, where the total pump current is the sum of the outward current obtained in going from K^+-free to K^+-containing sea water. The net current is increased even when comparing 15 mM with 20 mM K.

It has been difficult to determine the variation of sodium pump activity with potential because of the fact that almost every manipulation which blocks sodium pump activity also affects other parameters of cell function which influence potential, or the manipulations are effectively irreversible or very slowly reversible, leading to accumulation of sodium intracellularly and disruption of the ionic concentration gradients. In the present studies we were able to overcome some of these problems, but not all. Use of the rapid microperfusion system between K^+-containing and K^+-free sea water allowed us to rapidly remove sodium pump activity for brief periods of time that did not result in significant accumulation of sodium. However, in this protocol we are changing the K^+ concentration gradient, which imparts a voltage-dependent current source which increases with the concentration of K^+ in the sea water. However, the magnitude of this current can be determined by performing the same

variation in external K^+ concentration in the presence of ouabain, which totally blocks pump activity.

On the basis of our observations we conclude that there is no significant potential dependence of the electrogenic sodium pump over the potential range of –60 to –130 mV in *Aplysia* neurons. The evidence for this conclusion is most clear from studies where the pump was activated by changing the microperfusion system from zero to 1 mM K^+, a change which causes the least shift of the K^+ equilibrium potential (Figures 4, 5). In this experiment the pump current was constant over the full voltage range. In contrast when the microperfusion system was changed to higher K^+ concentrations there was a voltage dependence of the currents induced, becomes smaller and inverting at hyperpolarized potentials and becoming greater at depolarized potentials. We ascribe this potential dependence to the passive membrane properties. The obvious experiment to do in order to prove this conclusion – comparing potential dependence to the same sequence of K^+ changes in the presence and absence of ouabain – is not realistic because of the alteration of concentration gradients induced by prolonged ouabain exposure.

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ALUMINUM ENHANCES THE VOLTAGE ACTIVATED SODIUM CURRENTS IN THE NEURONS OF THE POND SNAIL *LYMNAEA STAGNALIS* L.*

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1. The effects of aluminum on voltage activated sodium currents (VASCs) were investigated by using the conventional two-electrode voltage clamp technique in *Lymnaea stagnalis* L. neurons. The peak amplitude, kinetics, and voltage-dependence of activation and inactivation of the sodium currents were studied in the presence of 5–500 μM $AlCl_3$, at pH = 7.7.
2. There was a significant concentration-dependent increase in the peak amplitude of sodium currents after Al treatment, ED_{50} = 67 μM. The threshold concentration of the enhancement was 50 μM. The maximal peak increase of 143% was caused by a 500 μM aluminum. The action of aluminum on VASCs developed slowly, and it is not recovered by washing within 20 min.
3. There was little alteration of the voltage-dependence of the current. It was not a significant effect on the activation- and inactivation time constants of I_{Na}, but the steady-state inactivation curve shifted to negative direction on the voltage axis in the presence of Al.
4. The leak currents were not influenced by aluminum up to the highest concentration applied.

Keywords: Aluminum – sodium-currents – voltage clamp – snail neurons

INTRODUCTION

Although aluminum is the most common metal and third most abundant element on the Earth, it has no biological function. This metal is characterised as a toxic element which causes morphological, physiological, and biochemical alterations in prokaryotes, fungi, plants [3] and animals [6]. In the case of animals and humans, many of these effects occur in the nervous system. Aluminum injections into various experimental animals evoked seizures [19], neurofibrillar degradation [2, 16] and age-related accumulation in the CNS in close correlation with behavioural changes [5]. In humans, the exact role of aluminum in the amyotrophic lateral sclerosis/Parkinson dementia of Guam [15], and Alzheimer's disease [1, 14] is controversial and unknown.

* Dedicated to Professor János Salánki for his 70th birthday.

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Some observations report that the passive and active membrane properties [22] are changed by extra- and intracellularly applied aluminum, which can finally caused an altered excitability of the neurons [8]. The action of Al on membrane potential, input resistance of snail neurons and biphasic modulation of the evoked excitatory postsynaptic potentials and current have already described [22]. These results suggest that aluminum may be effective on certain voltage and ligand activated ion channels. Marked inhibition was found in the peak amplitude of voltage activated Ca currents (VACCs) on cultured rat dorsal root ganglion [17, 18], and an alteration of the kinetics of VACCs was reported on snail neurons [7]. Aluminum also inhibits the mitochondrial, voltage dependent anion selective channels (VDACs) [4] and the fast phase of voltage-dependent calcium influx into synaptosomes [11]. However, there are conflicting results as relates the action of aluminum on the voltage-activated sodium channels (VASCs). A non-significant change of VASCs after Al treatment was found by Platt and Büsselberg in cultured rat dorsal ganglion [17], but Kanazirska et al. found an Al-evoked VASCs inhibition in hippocampal neurons [9]. Oortgiesen described a novel type of ion channel-activated by Pb^{2+}, Cd^{2+}, and Al^{3+} in cultured mouse neuroblastoma cells [13]. Al activated current was eliminated by sodium-free solution.

The aim of the present study was to analyse the influence of aluminum on voltage-activated sodium channels in certain neurons of the pond snail, *Lymnaea stagnalis* L. at physiological pH values, where the $Al(OH)_4^-$ is the active molecule.

MATERIALS AND METHODS

The conventional two-electrode voltage clamp study was carried out on identified and on some unidentified neurons of the pond snail, *Lymnaea stagnalis* L. Animals were kept under natural light-dark cycle at 22–25 °C, and fed *ad libitum.*

The CNS was dissected and incubated in 0.1% solution of Protease (SIGMA) in normal HEPES-buffered saline (HBS) for 5 min. After incubation, the CNS was rinsed twice and pinned down in a dish containing HBS. The sheet of connective tissue covering the anterior lobe of the right cerebral ganglion was removed using scissors, after which the lobe was severed from the ganglia. The ganglia were regenerated in 4 °C for two hours, then stored at room temperature for 30 min. The biggest, identified (RPeD1, RPaD1, VV1 and VV2) neurons [21], and some unidentified large neurons were used in the experiments. All experiments were performed at room temperature.

The composition of standard saline (HBS) was (in mM): NaCl 45, KCl 1.7, $CaCl_2$ 4, $MgCl_2$ 1.5, HEPES 5; pH 7.7 set by 2.5 mM NaOH. For isolation of sodium currents we used a bathing solution of (in mM) NaCl 45, $CaCl_2$ 1, $MgCl_2$ 4, HEPES 5, TEA-Cl 15, 4-AP 0.2, and for Ca current elimination, $CdCl_2$ 0.03. The $AlCl_3 \cdot 6H_2O$ was dissolved in the sodium current isolating solution at concentration ranging from 5 to 500 μM. The pH was adjusted by using 1M HCl or 1 M KOH. Each compound was purchased from SIGMA Co. The application of aluminum solution to neurons

and the change of bathing solution (3 ml) were carried out by a peristaltic micropump (1.5 ml/min, Peripump D, Hungary).

The conventional two-electrode voltage clamp technique was employed to measure the sodium currents. A DAGAN 8500 amplifier (DAGAN Co., USA) was used to record the currents. Microelectrodes were made from borosilicate glass (Clark Co., England), and pulled on a gravity puller (NARISHIGE PC-10, Japan.) The intracellular microelectrodes, filled with 2.5 M KCl, had 2–4 MΩ resistance. The voltage, and current signals were monitored on a Tektronix oscilloscope. For control of the experiment, and data acquisitions an UAM3216 AD/DA converter (Hungary) was connected to an IBM 286 personal computer which was run with a home made voltage clamp software (VCL/OCL) developed by Attila Szűcs [20].

Data are presented as means and standard deviation, and *n* refers to the number of preparations in all cases. The statistical significance of the alterations was determined by unpaired *t*-tests. The level of significance was accepted at p value <0.05.

RESULTS

The effects of aluminum on voltage-activated sodium channel currents were studied at physiological pH (pH = 7.7) in 9 neurons composed of identified [6] or unidentified [3] cells. Figure 1 illustrates a representative example of control and aluminum-modified sodium currents, elicited by a voltage jump from –55 to –10 mV membrane

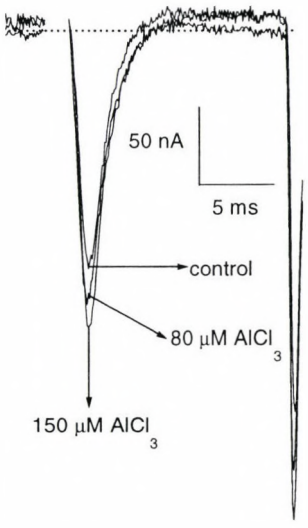

Fig. 1. Facilitatory actions of aluminum on isolated sodium currents under voltage clamp. Holding potential –55 mV, command potential –10 mV. Calibration: 50 nA, 5 ms. RPeD1 neuron

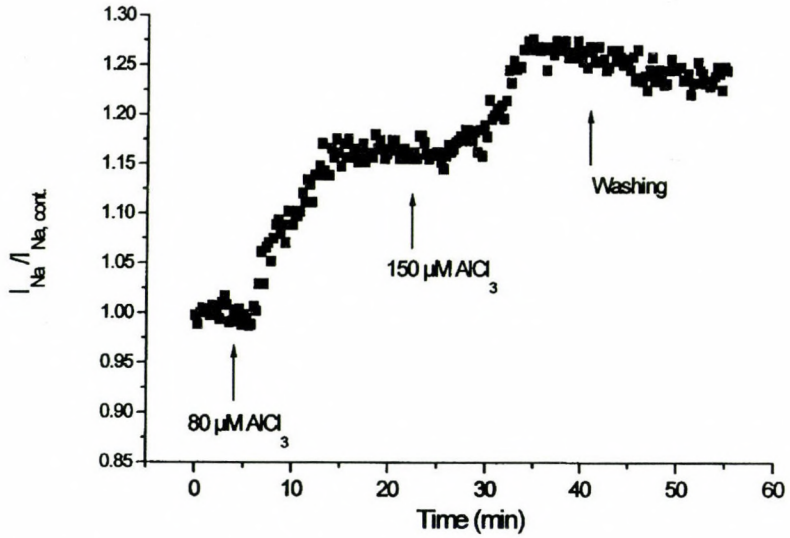

Fig. 2. Normalised sodium currents activated with a depolarising voltage step from –55 mV holding potential to –10 mV membrane potential versus time plot. 80 and 150 µM Al increased the amplitude of sodium currents stepwisely with by 17.5 and 28.2 per cent, respectively

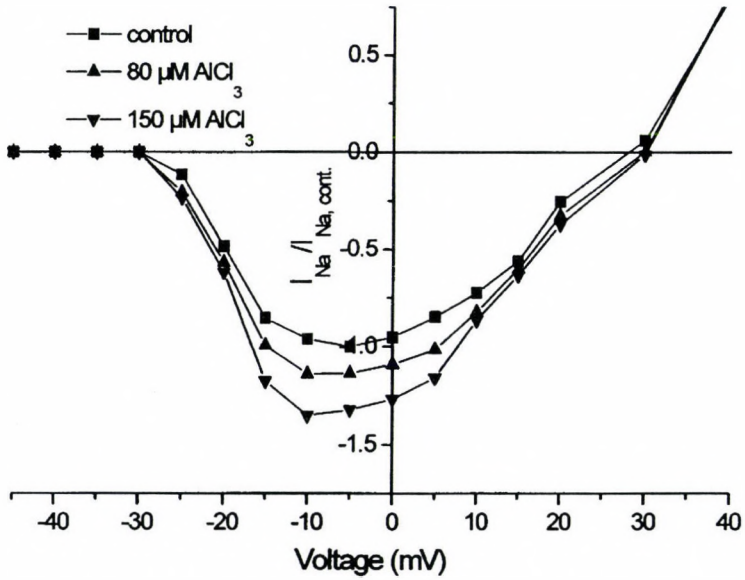

Fig. 3. Current-voltage curves of I_{Na} in control (squares), 80 µM (triangles peak up) and 150 µM (triangles peak down) Al containing media, respectively. Al treatment increased the amplitude of I_{Na} over the whole voltage range and slightly shifted the curves to the left

potential in the RPeD1 neuron. The elevated current after application of 80 and 150 μM Al are superimposed. The time to peak of the sodium current was 1 ms and had a peak amplitude of –60.1 nA in the control solution. Al treatment caused an alteration of the current amplitude in dose-dependent way. Al increased the peak current by 15.4%, and 26.1% after application of 80 μM and 150 μM aluminum, respectively, in the experiment presented in Figure 1.

The time course of the aluminum action on sodium current amplitude was relatively slow (Fig. 2). A significant increase of the current amplitude was observed after 8 min of application of 80 μM Al to the bath. Additional extracellular application of 150 μM Al led to a further 10.7% increase. There was little or no recovery after washing out of Al (max. 10%) with Na- or normal HBS. All actions were independent of advanced depolarisations.

Figure 3 shows the current-voltage (I–V) relationship before and after application of 80 and 150 μM aluminum, respectively. The leak-corrected sodium current reached a maximum at –5–0 mV membrane potential in control circumstances. Aluminum increased the VASCs over the entire voltage range, and shifted slightly the curve to hyperpolarized potentials by about 5 mV.

The effect of aluminum on voltage-activated sodium currents was concentration dependent. Figure 4 shows the changes in the current amplitude versus $AlCl_3$ concentrations graphically. In Figure 4, mean values are given and the standard devia-

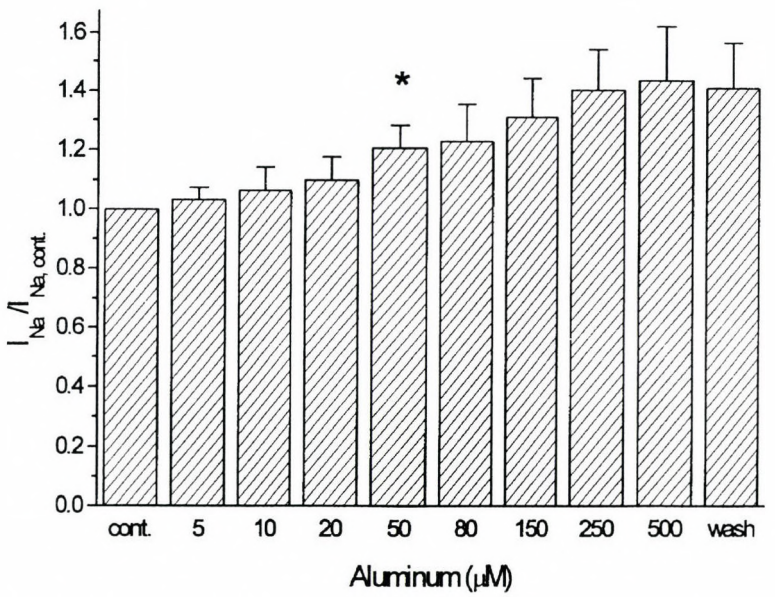

Fig. 4. Normalised amplitude of Na currents versus aluminum dose plot shows dose-dependent actions. Significant enhancement of I_{Na} can be observed from 50 μM which is marked with a star and the binding site is saturated with 200 μM Al. The calculated EC_{50} is 67 μM Al

tions are indicated by bars. The threshold aluminum concentration was 50 μM as it is indicated by a star in Figure 4 which altered the current amplitude significantly. The maximum effect was a 1.43-fold increase on the peak amplitude of the sodium currents. The calculated ED_{50} was 67 μM.

The Al treatment did not alter activation- and inactivation time constants, but the steady-state inactivation was slightly modified, as shown in Figure 5. Namely, the current amplitude was significantly elevated between –47 and –80 mV membrane potential values in the aluminum containing solution, followed by a decrease which dominated between –20 and –47 mV membrane potential values, respectively.

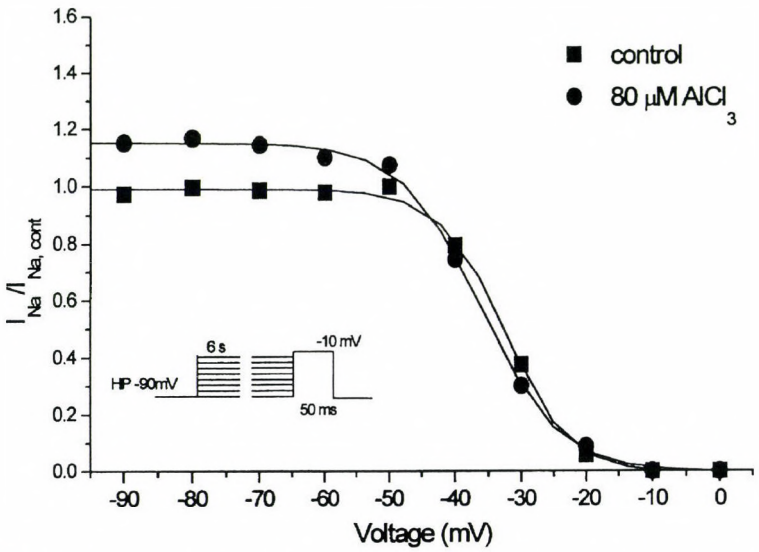

Fig. 5. Effects of Al on the steady-state inactivation of voltage activated sodium currents. Conditioning prepulses (6 s duration) ranging between –90 mV and –20 mV in 10 mV increments were applied, which followed by 50 ms test pulse to –10 mV from –90 mV holding potential. Steady-state inactivation with 80 μM Al (circles), and without Al (squares). Al increased the amplitude of I_{Na} at more negative potential values then the resting membrane potential and slightly decreased that between –50 and –20 mV membrane potential range

DISCUSSION

The data presented in this paper demonstrate that aluminum significantly increased the amplitude of the isolated sodium currents in identified and some unidentified neurons of the snail *Lymnaea stagnalis* L., at pH 7.7 where negative charged aluminum complexes are present in the bath solution.

Contrary to previous observations which declared Al as inactive [17] or inhibitory [9] metal ion on the neuronal sodium channel of various mammals at pH 7.4 we

found a clear dose-dependent facilitatory actions of aluminum on the neuronal sodium channels of the pond snail (ED_{50} = 67 μM).

It is known that divalent and trivalent cations have a strong effect on the gating properties of voltage-dependent ion channels. These effects are usually explained by the surface change theory [10]. However, this theory is not adequate for aluminum compounds, because a mixture of negatively charged aluminum compounds are present in the physiological solution at pH 7.7.

As it is known, aluminum chloride in water will turn into aluminum trihydroxide ($Al(OH)_3$). This compound is amphoteric. Thus, the aqueous solution of aluminum chloride contains various aluminum hydrate forms (aluminates) which can be charged positively or negatively depending on the pH of the given solution. The dominant form is the Al tetrahedral ($Al(OH)_4^-$) [12] at physiological pH (pH 7.5–7.8). Our results presented evidence that the current-voltage relationship shifted to the hyperpolarized direction in the aluminum solution, which might be caused by a binding of the negative charged Al complex on the voltage sensor area of the sodium channel protein. It is interesting that $Al(OH)_4^-$ did not modify significantly the activation and inactivation kinetics of the sodium current in the snail neurons. Thus, extracellularly acting anion complex may increase the negative charge on the channel protein which, in turn, can facilitate the Na^+ translocation.

Further research is needed to investigate the effects of the aluminum compounds on snail neurons at lower pH, where other, positively charged aluminum ions are present.

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EFFECTS OF NALOXONE ON C-JUN/AP-1 IN MET-ENKEPHALIN- AND FMRFAMIDE-IMMUNOREACTIVE NEURONS OF A GASTROPOD SNAIL*

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1. Opioid- and FMRFamide (FMRFa)-ergic systems are believed to play antagonistic behavioral roles in both higher and lower animals. In our previous experiments on a snail, behavioral choice has been demonstrated to be dependent on a balance between FMRFa and enkephalins [7]. Here, we examined if the disturbance of the balance causes changes in the activity of both systems. Opiate receptor blocker naloxone was applied and its effect on c-jun expression of met-enkephalin (MEnk)- and FMRFa-ergic neurons was examined immunocytochemically in terrestrial gastropod snail *Cepaea nemoralis*.
2. In control, untreated snails, central neurons with c-jun/AP-1-like-immunoreactivity were found to occur. These included MEnk-, FMRFa- and 5HT-immunoreactive (-ir) neurons, as was revealed by double-labelling.
3. After treatment with naloxone for 4 h, the following changes were observed: (i) increase in the number of MEnk-ir neurons; increase in the number of neurons showing c-jun/AP-1 and MEnk double-labeling; (ii) disappearance of c-jun/AP-1-immunoreactivity from some FMRFa-ir neurons.
4. It is suggested that immediate early genes are involved in the mechanisms responsible for the reciprocal regulation of the opioid and antiopioid neuropeptide systems.

Keywords: FMRFamide – enkephalins – naloxone – immediate early genes – invertebrates – snail

INTRODUCTION

FMRFamide (FMRFa) and enkephalin-like peptides belong to evolutionary old signal molecules. Opposite effects of FMRFa and enkephalins were observed in many cellular and behavioral experiments arousing interest in their relationships suggested to be antagonistic [17–20, 28, 29, 33]. Their antagonistic relationships have been confirmed by Kavaliers and Yang [21] who demonstrated antinociceptive, opioid-like effects of antisera against FMRFa in mammals. Similarly, in our previous experiments on a snail, behavioral choice has been demonstrated to be dependent on a balance between FMRFa and enkephalins [7].

* Dedicated to Professor János Salánki for his 70th birthday.

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0236-5383/99/$ 5.00

Little is known on how a balance between antagonistic systems can be controlled. The only evidences for existence of a link between FMRFa and enkephalinergic systems seem to have been a report on changes in neuronal FMRFa immunoreactivity under methionine-enkephalin (MEnk) treatment in the snail *Achatina fulica* [32] and demonstration of increase in the FMRFa content under morphine treatment in rats [22].

Among the factors determining a balance between the antagonistic neuropeptides, rates of their synthesis and release are essential. These are known to be manifested by the activity of immediate early genes (IEGs). The genes of fos and jun families have found a wide use as markers of both electrical and synthetic activities in studies on mammal neurons [14, 16, 25]. These genes are activated by various cellular events. In their turn, they control the activity of other genes through binding of their protein products in the form of homo- or heterodimers, termed AP-1 complex, to the untranslated regions of the target genes [23, 24]. Specifically, transcription of the preproenkephalin gene has been shown to be regulated by AP-1 in mammals [31]. A few comparative studies suggest that fos and jun could be old in evolutionary terms and their sequences preserved in several phyla [13, 27].

In the present study, we utilized a double-staining technique to examine if expression of c-jun in FMRFa- and MEnk-like immunoreactive (FMRFa-ir and MEnk-ir, respectively) neurons is changed under pharmacologically produced shift in a balance between the two neuropeptide systems. To disturb the balance, an opiate receptor blocker naloxone was used. 5-HT-like-immunoreactive (5HT-ir) neurons were examined as a control. The study was performed on the terrestrial gastropod snail, *Cepaea nemoralis*. The distribution of FMRFa-ir, MEnk-ir and 5-HT-ir central neurons was earlier described in this or closely related species [8–10, 15, 30].

MATERIALS AND METHODS

Animals

Mature specimens of *Cepaea nemoralis* were collected locally in Lund, Sweden. The snails were maintained at room temperature with free access to food. Twenty untreated specimens were used for immunocytochemical mapping of immunoreactive products of IEG elements in the CNS. Forty specimens (20 treated and 20 control) were double stained in experiments with naloxone.

Naloxone treatment

Animals were activated by rehydration in a humid container and anaesthetized by injection of 0.2 ml 0.2 M $MgCl_2$ into the haemocoel. The CNS was dissected and incubated for 4 h in the snail saline containing 0.05 mM naloxone (Sigma, USA). Control CNS preparations were incubated for 4 h in saline only.

Immunocytochemistry

Ganglia were fixed in 4% formaldehyde in 10 mM sodium phosphate buffer (PBS), pH 7.4, for 2 h at 4 °C, washed overnight at 4 °C in PBS, and immersed in 20% sucrose in PBS. They were then embedded in Tissue-Tek O.C.T. and frozen in liquid nitrogen. Serial sections were cut at 20 μm with a Reichert-Jung frigocut E 2800 cryostat and placed on chrome-alum-gelatin coated glass slides. After a brief rinse in PBS with 0.25% Triton-X (PBS-TX), the slides were incubated with primary antisera diluted 1 : 1000 in PBS-TX with 0.25% bovine serum albumine (BSA) for 12 h at 20 °C. After 3×15 min rinses in PBS, location of the primary antibody was visualized either by PAP method or by a 3 h incubation with rhodamine-conjugated swine anti-rabbit Ig-G diluted 1 : 40 in PBS. For double staining, further binding to secondary antibody was blocked by incubation with rabbit IgG diluted 1 : 25 in PBS. This was followed by 3×15 min rinses in PBS and a second staining cycle in which the sections were incubated for 24 h in biotinylated antiserum [2] that, after 3×15 min rinses in PBS, was visualized by treatment with streptavidin-FITC at 1 : 20 (for the biotinylated antibodies from rabbit). Sections were rinsed in PBS, mounted in phospate buffered glycerol and examined in a Leitz Aristoplan fluorescence microscope.

Specificity of Jun-like immunostaining was controlled by incubating the sections with primary antibody solutions preadsorbed with 10 μg/ml control peptide. Control of the method was performed by the substitution of primary antibody with 1% normal rabbit serum. Anti-Jun immunostaining was completely abolished after these control experiments. Antibodies to the IEG products raised in rabbits were all from Santa Cruz Biotechnology, Inc. Antisera against MEnk, 5-HT, and FMRFa raised in rabbits were from Incstar, Minnesota. Rhodamine-conjugated swine anti-rabbit IgG, as well as unconjugated rabbit IgG and streptavidin-FITC, were from Dakopatts, Denmark.

RESULTS

IEGs

In preliminary experiments, four IEG antisera were tested, specifically those against c-fos (K-25), c-fos (4), c-jun/AP-1 and jun B. The results were considered unspecific with the antisera against c-fos (K-25). Specific immunoreactive patterns were found in the CNS of *Cepaea* using other three antisera.

The IEG reaction products were not restricted to the nucleus but appeared in the cell body and its extensions.The c-jun/AP-1 antiserum was characterised with distinct results, intensive staining and large number of reactive neurons. This antiserum was therefore chosen for a further detailed study.

The c-jun/AP-1-like immunoreaction occurred in the entire cell body and its extensions. Immunoreactive nerve cells were situated at all levels of the ganglia from their surface to interior. Some of the immunoreactive cells and cell groups were

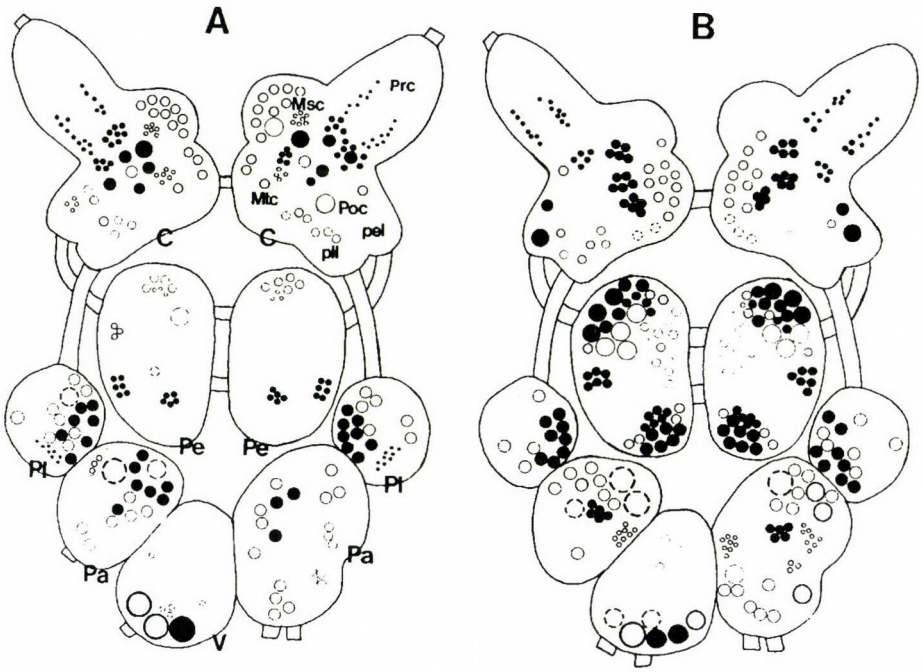

Fig. 1. c-jun/AP-1 immunoreactive neurons. Diagrammatic representation of the central nervous system of *Cepaea nemoralis,* dorsal view (redrawn after Sakharov et al. 1993) showing. (A) Dorsal portion. (B) Ventral portion. *Filled circles:* neurons showing "consistent" immunoreactivity; *open circles:* neurons showing "less consistent" immunoreactivity; *dashed circles:* neurons showing "low consistent" immunoreactivity. C – cerebrum, Msc – mesocerebrum, Mtc – metacerebrum, Pa – parietal ganglia, Pe – pedal ganglia, Pel – pedal lobe of postcerebrum, Pl – parietal ganglia, Pll – pleural lobe of postcerebrum, Poc – postcerebrum, Prc – procerebrum, V – visceral ganglion

stained in all individuals examined thus representing a "consistent" subpopulation. Other appeared in more than 50% specimens ("less consistent"). A few neurons reacted in less than 50% individuals ("low consistent"). Figure 1 shows the c-jun/AP-1 pattern in the central ganglia of *Cepaea.*

Colocalization: Untreated animals

A number of neurons that demonstrate colocalization of c-jun/AP-1 and FMRFa, or MEnk, or 5-HT immunoreactivity was found in the snail CNS. Figure 2 shows the pattern of double-stained neurones. See also Fig. 3a, Fig. 3b and Fig. 4 illustrating colocalization of c-jun/AP-1 plus FMRFa in the left parietal ganglion and c-jun/AP-I plus 5HT in the visceral ganglion, respectively.

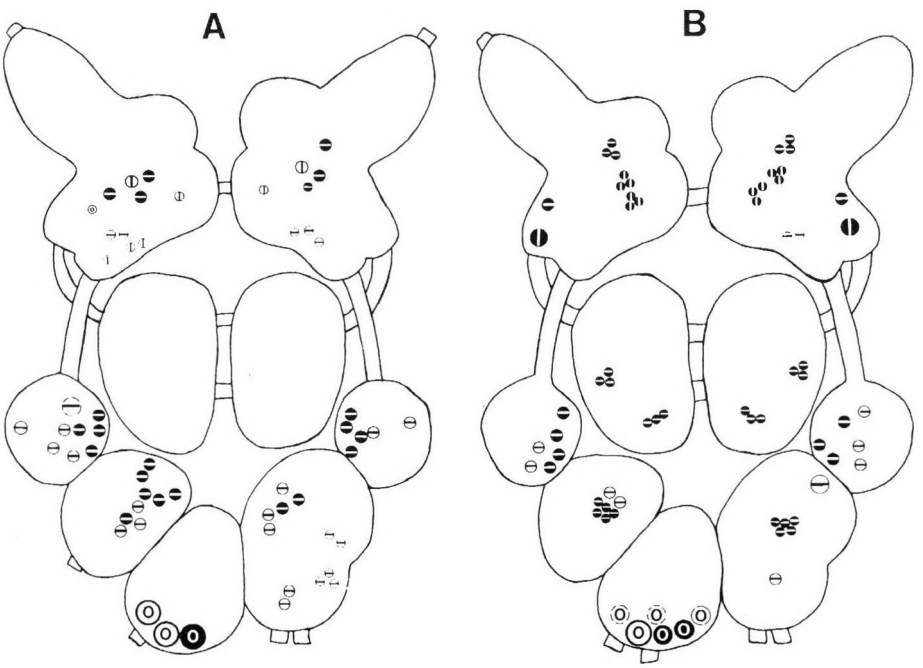

Fig. 2. Coexistence of c-jun/AP-1 immunoreactivity with that of MEnk (vertical bars inside circles), FMRFa (horizontal bars inside circles), and 5-HT (double circles). The same view and symbols of the central nervous system as in Fig. 1

Colocalization: Naloxone-treated animals

c-jun/AP-1 plus FMRFa

Naloxone caused disappearance of c-jun/AP-1 immunoreactivity from some of the FMRFa-immunoreactive neurons, which showed coexistence in untreated animals. FMRFa immunoreactivity by itself was not seen affected by naloxone (Fig. 5, compare to Fig. 2).

Decrease in the number of neurons showing coexistence of FMRFa plus c-jun/AP-1 immunoreactivity was seen in the pleural and parietal ganglia.

In the pleural ganglia this occurred to:

1. some of the large "consistent" and "less consistent" double-stained c-jun/AP-1 plus FMRFa neurons (4 ± 3 neurons in each left and rigth ganglion of naloxone treated snails, 14 ± 7 in controls, $p = 0.034$).
2. the giant "low consistent" neuron situated dorsally in the left ganglion.

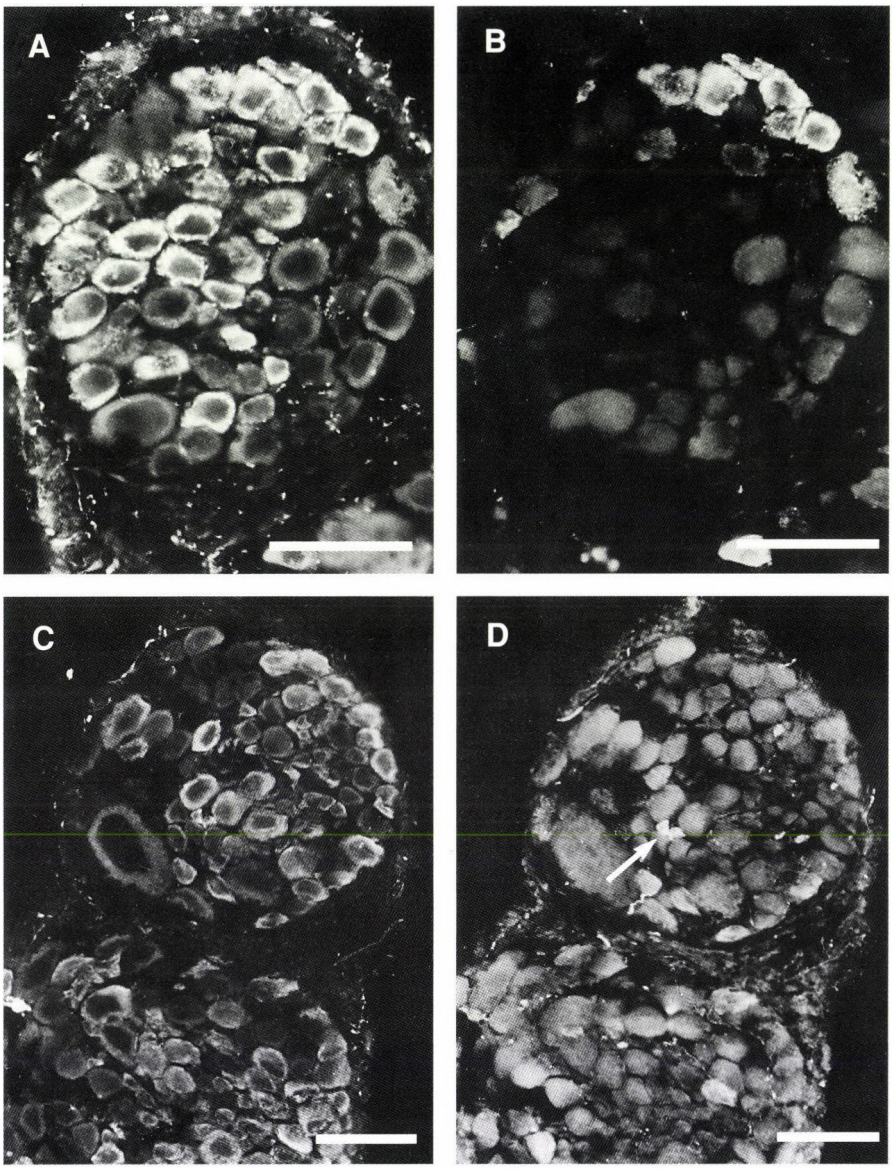

Fig. 3. Colocalization of FMRFa and c-jun/AP-1 immunoreactivities in the left parietal ganglion of control (A, B) and naloxone-treated preparations (C, D). FMRFa (A) and c-jun/AP-1 (B) immunoreactivity after CNS incubation in the normal snail saline. Colocalization is visible in 10 neurones of the anterior portion of the ganglion. FMRFa (C) and c-jun/AP-1 (D) immunoreactivity after incubation in naloxone. In D, no specific c-jun/AP-1 staining is seen in the anterior portion, two small neurons with specific staining are marked by arrow, the background fluorescence of neuron perikarya is stronger due to longer exposition time. Scale bars = 200 μm

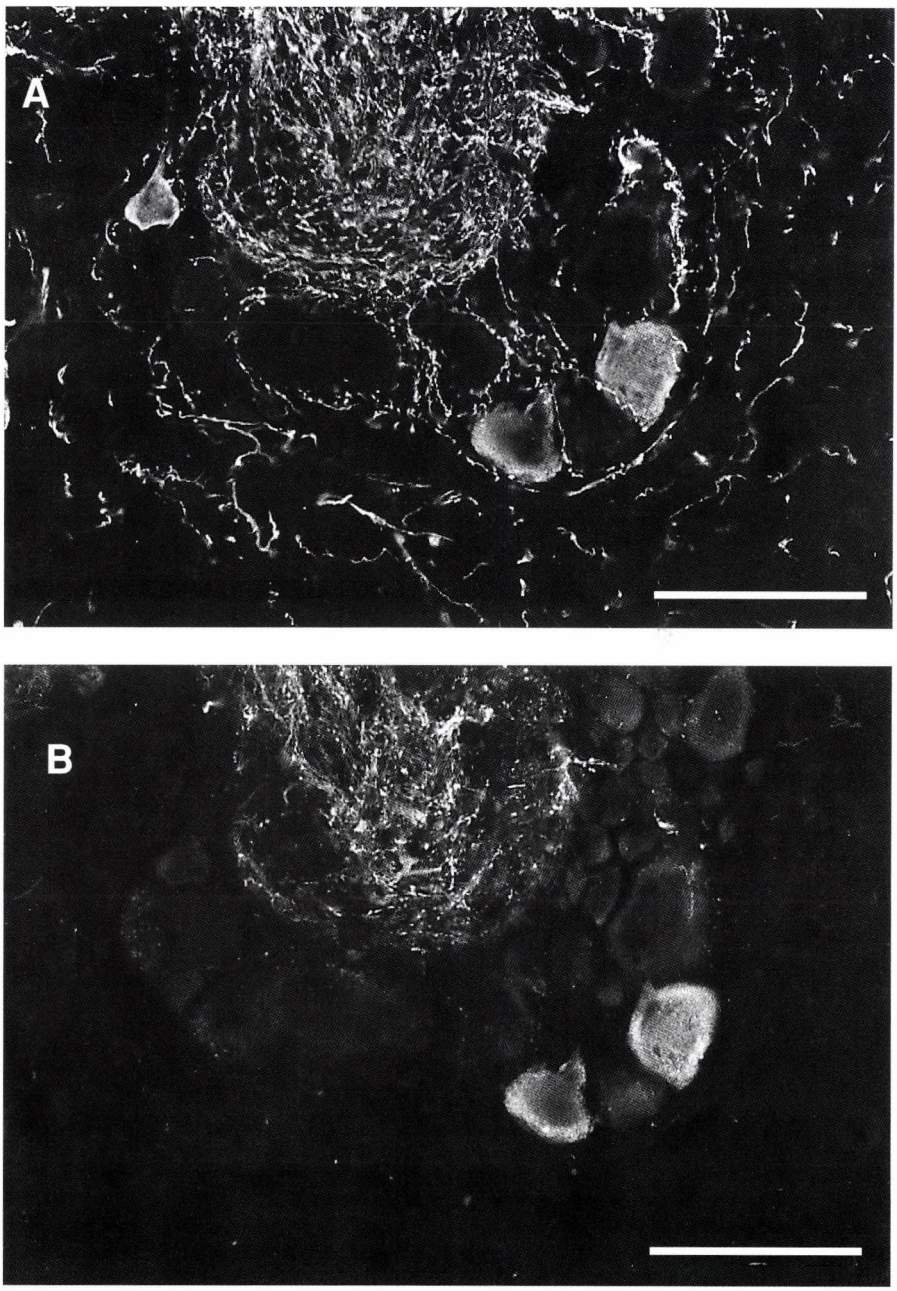

Fig. 4. 5-HT (A) and c-jun/AP-1 (B) immunoreactivity in the visceral ganglion of *Cepaea.* Two neurons immunoreactive to both 5-HT and c-jun/AP-1 are visible in the posterior portion of the ganglion. Scale bars = 200 mm

In the right parietal ganglion, similar changes occurred to:

1. some of the large "consistent" and almost all of the "less consistent"neurons situated along the dorsal medial border (4 ± 5 in naloxone treated snails, 13 ± 5 in controls, $p = 0.036$);
2. the anterior of two groups of medium-sized neurons in the dorsal portion of the ganglion;
3. one large "less consistent" neuron on the border with the right pleural ganglion, and one medium-sized "less consistent"neuron posteriorly on the ventral side.

In the anterior portion of the left parietal ganglion, a marked decrease in the number of c-jun/AP-1 immunoreactive neurons was observed (5 ± 2 in experimental animals, 13 ± 6 in controls, $p = 0.022$), see also Fig. 3.

c-jun/AP-1 plus MEnk

Naloxone caused appearance of new c-jun/AP-1 plus MEnk-double-stained cells unseen in untreated snails. New double-stained neurons were seen in the right and left cerebral and pedal ganglia (Fig. 5, shown by arrows).

In the cerebral ganglia, these were as follows:

1. two-three medium-sized neurons dorsally in each ganglion in the mesocerebrum (Fig. 6) and one centrally in the right ganglion;

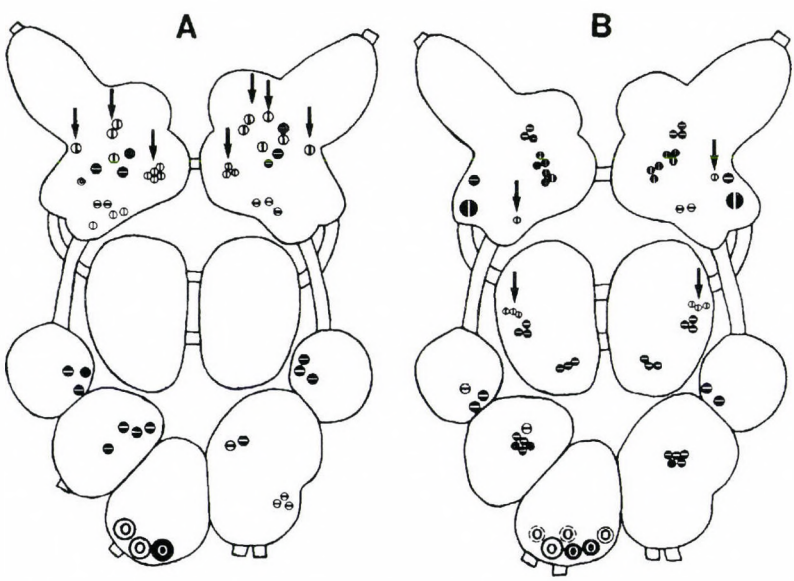

Fig. 5. Coexistence of c-jun/AP-1 immunoreactivity with that of MEnk, FMRFa and 5-HT in the CNS of *Cepaea* after naloxone treatment. The same view and symbols as in Figs 1 and 2. Comparison with Fig. 2 shows that the number of c-jun/AP-1 plus FMRFa immunoreactive neurons have decreased in the pleural and parietal ganglia. Arrows mark the newly appeared c-jun/AP-1 plus MEnk immunoreactive neurons

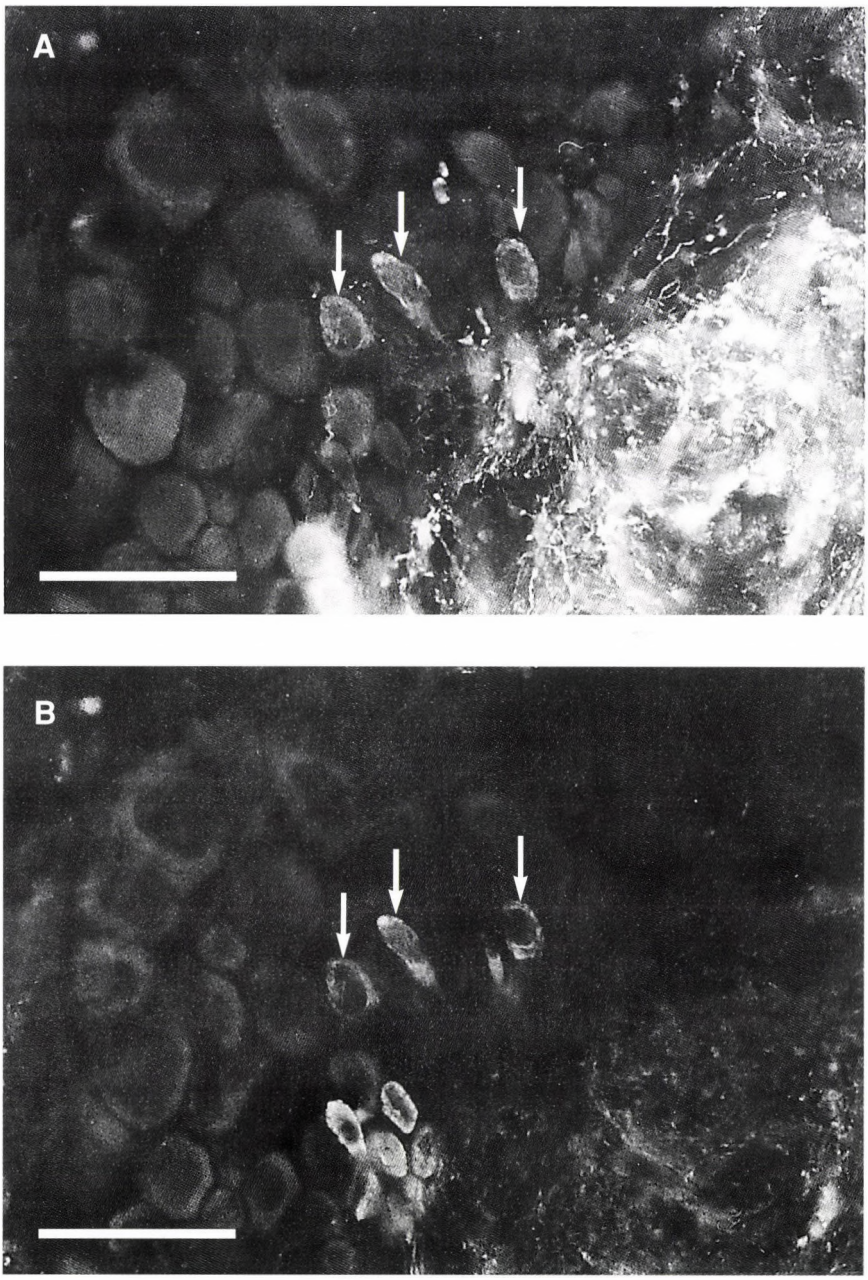

Fig. 6. Met-enkephalin (A) and c-jun/AP-1 (B) immunoreactivity in the right mesocerebrum of *Cepaea* after incubation in naloxone. Three cells showing double-staining only in naloxone-treated specimens are marked by arrows. Scale bars = 50 μm

2. 4 to 5 medium-sized neurones in the dorsal metacerebrum;
3. one medium-sized clearly symmetrical neuron dorsally on the border between pro- and postcerebrum;
4. one small to medium-sized neuron ventrally in the right pedal lobe;
5. one small to medium-sized neuron ventrally in the left pleural lobe.

Generally, the number of MEnk immunoreactive neurons of the postcerebrum seemed larger in naloxone-treated animals than in control.

In the pedal ganglia, a group of medium-sized neurons with coexistence appeared latero-ventrally.

c-jun/AP-1 plus 5HT

No differencies in the distribution of c-jun/AP-1 plus 5HT coexistence were seen between treated (N = 6) and untreated (N = 6) specimens.

DISCUSSION

IEG products have been shown in this investigation to form a specific pattern in the CNS of untreated specimens of the gastropod mollusc *Cepaea nemoralis.* We have found that IEG products are stained in the entire nerve cell including the neurite. This finding agrees with what has been reported for IEG products in a teleost fish [3], the honeybee [11], locust, beetle [34] and cricket (M. Hoerner, personal communication), but differs from staining in the mammals known to be restricted to the nucleus only. Speculation concerning the reason for this difference is premature but, as far as the present knowledge goes, it might manifest an evolutionary trend.

In this immunocytochemical investigation, the opioid antagonist naloxone was found to affect the c-jun/AP-1 and MEnk production of some neurons. The efficacy of the antagonist points to the existence of a tonic activity of the endogenous opioid system in molluscs. Such tonic activity was also evidenced by previous behavioral and electrophysiological findings [5, 6]. Similarly, changes in Fos-expression have been found in some brain regions of rats treated with another opiate receptor antagonist, naltrexone [4].

Naloxone caused an increase in the number of MEnk-immunoreactive and double-stained MEnk plus c-jun/AP-1 neurons. This might suggest that the tonic activity of the opioid system is controlled by inhibitory autoregulation. Again, this agrees with findings of a feedback inhibition of the opioid system at the level of proopiomelanocortin gene expression [12] and peptide release [26] in the mammals.

Our results have also shown that, upon naloxone treatment, some of the FMRFa-ir cells lose their c-jun/AP-1 immunoreactivity. In general, c-jun and c-fos expressions are thought to be associated with neuron activation. We are not familiar with any results that demonstrated involvement of AP-1 factor in the regulation of FMRFa gene expression. Partial disappearance of c-jun/AP-1 reactivity from FMRFa-ir neurons in our experiments might suggest a decrease in their electrical activity.

Some of the MEnk- and FMRFa-ir neurons described here were previously characterized in the same or related species. In *Helix,* the giant pleural neuron, cell Pl-1, and several large neurons on the dorsal surface of the parietal ganglia have been identified as FMRFa-ir neurons involved in control of the defensive behavior [1, 8]. It seems that, in *Cepaea nemoralis,* we have been dealing with homologous neurons.

In a previous behavioral study, we found that the choice between active and passive avoidance is determined by a balance between the enkephalinergic and FMRFa-ergic systems [7]. This may explain a marked increase in the expression of passive avoidance observed in naloxone-treated snails [5, 6]. In the present study, naloxone-produced suppression of the opioid system affected both the enkephalinergic and FMRFa-ergic neurons, thus suggesting their reciprocal regulation. Our results thus indicate that IEGs may be involved in the mechanisms responsible for reciprocal regulation of the opioid and antiopioid neuropeptide systems.

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MIP-IMMUNOREACTIVE INNERVATION OF THE SNAIL, *HELIX POMATIA,* HEART. AN ULTRASTRUCTURAL STUDY*

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The ultrastructural characteristics of the innervation established by MIP-(*Mytilus* inhibitory peptide) immunoreactive neurons was investigated in the heart of the snail, *Helix pomatia,* applying correlative light- and electron microscopic pre-embedding immunocytochemistry on Vibratome-slices. In both the auricle and ventricle, the muscle fibers receive a rich innervation by MIP-immunoreactive (IR) varicose fibers. However, the innervation is seasonally changing in the two parts of the heart. The varicosities, containing a morphologically uniform population of large (120–150 nm) electron-dense granules, can be found in three different positions in relation to the muscle fibers: (i) close (15–20 nm) but unspecialized membrane connections between MIP-(IR) varicosities and muscle fibers; (ii) MIP-IR varicosities located relatively far (0.5-several μm) from the muscles fibers; (iii) MIP-IR profiles localized freely in the extracellular space among the loosely arranged muscle fibers. A general modulatory role of MIP in regulating the heart activity of *Helix* is suggested.

Keywords: *Mytilus* inhibitory peptide – neuromuscular contacts – heart – electron microscopic immunocytochemistry – snail, *Helix* pomatia

INTRODUCTION

The myoactive role of neuropeptides identified in the nervous system of invertebrates has widely been demonstrated [see e.g. 3, 15, 16, 18, 19, 21]. In molluscs, FMRFamide, the catch-relaxing peptide (CARP), myomodulin, buccalin, and the recently fully sequenced members of the *Mytilus* inhibitory peptide (MIP)-family are the best known and most studied representants of myoactive peptides.

MIP has first been isolated from the pedal ganglia of the bivalve, *Mytilus edulis* [9, 11]. Later additional members of the MIP-family have been sequenced [9, 10]. To date, the MIP-family consists of altogether seven members in *Mytilus* [8], and additonal MIP-family members have been discribed in the gastropods, *Helix pomatia* [13], *Lymnaea stagnalis* [17] and *Aplysia californica* (Fujisawa, personal com-

*Dedicated to Professor János Salánki for his 70th birthday.

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munication). All MIP-family members have been shown to affect the muscle activity when applied in bioassays.

In the course of a detailed light microscopic immunocytochemical study, the wide distribution of MIP-immunoreactive (IR) neurons in the central nervous system as well as the MIP-IR innervation of different peripheral muscle tissues of *Helix* and *Lymnaea* have been demonstrated [4]. At the periphery, a particularly rich MIP-IR innervation has been found in the heart of *Helix pomatia.* In general, our observations suggested an overall significant role of MIP in different peripheral tissues, regulating muscle functions.

The aim of the present study was to reveal the nature of neuro-muscular contacts established by MIP-IR elements in the heart of *Helix,* by means of correlative light- and electron microscopic immunocytochemistry. In this way, we want to furnish the first ultrastructural data, supporting the regulatory role(s) of MIP in muscle function, and also, a basis for broader ultrastructural immunocytochemical studies of MIP-innervated peripheral tissues of *Helix* and *Lymnaea.*

MATERIAL AND METHODS

Adult specimens of the snail, *Helix pomatia* L., were used.

Fixation

The heart was quickly dissected, pinned out in a Sylgard-coated dish, and fixed overnight at 4 °C with 4% paraformaldehyde and 0.08% glutaraldehyde diluted in 0.1 M phosphate buffer (PB). After washing 2×10 in PB, the auricle and the ventricle were separated. The auricles and ventricles were embedded in a mixture of gelatin (Sigma) and albumine (Sigma) at 40 °C, using a flat embedding mold. After 15 min in refrigerator, the gelatin/albumine cubes, containing the preparations, were removed from the mold and fixed overnight at 4 °C in 4% paraformaldehyde diluted in PB.

Immunocytochemistry

Following brief washing for 2×15 min in PB, series of 50 μm thick slices were cut on a Vibratome (Type Pelco 101,TPI) from the auricles and ventricles, respectively. The series of slices were processed for correlative light and electron microscopic MIP-immunocytochemistry as follows: (i) Incubation for 30 min with 1% H_2O_2 diluted in phosphate buffered saline (PBS); (ii) Washing for 10 min in PBS, then 2×10 min PBS containing 0.25% Triton X-100 (TX); (iii) Incubation for 60 min with 5% normal goat serum diluted in PBS-TX; (iv) Incubation overnight (ca. 20 h) with anti-MIP antiserum diluted 1 : 1000; (v) Incubation for 5–6 h with goat anti-rabbit IgG (Human) diluted 1 : 50; (vi) Incubation overnight with peroxidase anti-peroxidase IgG (Dako) diluted 1 : 200.

All antisera were diluted in PBS-TX containing 0.25% bovine serum albumine. All antiserum incubations were performed at room temperature and were followed by 3×10 min washing with PBS-TX.

The immunocytochemical reaction was developed in 0.05% DAB (Sigma) diluted in 0.05 M Tris-HCl by adding 0.01% H_2O_2. The time of development varied between 10 and 20 min, and was monitored under a stereomicroscope.

After development the slices were washed in Tris-HCl and PB, and postfixed for 30 min at 4 °C in 0.5% OsO_4 diluted 0.1 M Na-cacodylate. Postfixation was followed by dehydration in graded ethanol and propylene oxide, in the course of which block staining was performed in 70% ethanol saturated by uranyl acetate. Following dehydration and infiltration with 3 : 1, 1 : 1 and 1 : 3 mixtures of propylen oxide and Araldite (Durcupan ACM, Fluka), the slices were mounted on slides in Araldite. After 24 h polymerization, the slices were analyzed light microscopically, then re-embedded for ultrathin sectioning. Serial sections cut with a diamond knife were stained with lead citrate and viewed in a TESLA BS 500 electron microscope.

RESULTS

Light microscopic immunocytochemistry

At the light microscopic level, the *Helix* heart showed a rich MIP-IR innervation (Fig. 1a), however, with a seasonally different distribution. During spring and summer the auricle was innervated very densely whereas the ventricular musculature revealed MIP-IR fibers only rarely. In autumn, this situation has changed, and mainly the ventricle was heavily innervated. At the same time, the pattern of MIP-IR innervation was the same throughout the year. The innervating MIP-IR fibers were, without exception, varicose, and originated from bundles which consisted of several axon processes. The varicose MIP-IR elements ran along the muscle fibers for longer distances and innervated them by several varicosities (Fig. 1b, c).

Electron microscopic immunocytochemistry

The axon bundles, running between the muscle fibers, consisted mainly of unlabelled axon profiles, among which MIP-IR axon processes could be found (Fig. 2a). The diameter of the immunolabelled axon profiles varied between 1–4 μm, and contained large (120–150 nm) highly electron-dense granules. Some of the MIP-IR axon processes were located on the surface of the axon bundles and faced the extracellular space (Fig. 2b). Along their free membrane surface, omega shaped configurations occurred. Many unlabelled axon profiles contained also electron-dense granules of various size, mixed with 80–100 nm granular (dense-core) vesicles. Other axon processes revealed only neurotubules and mitochondria.

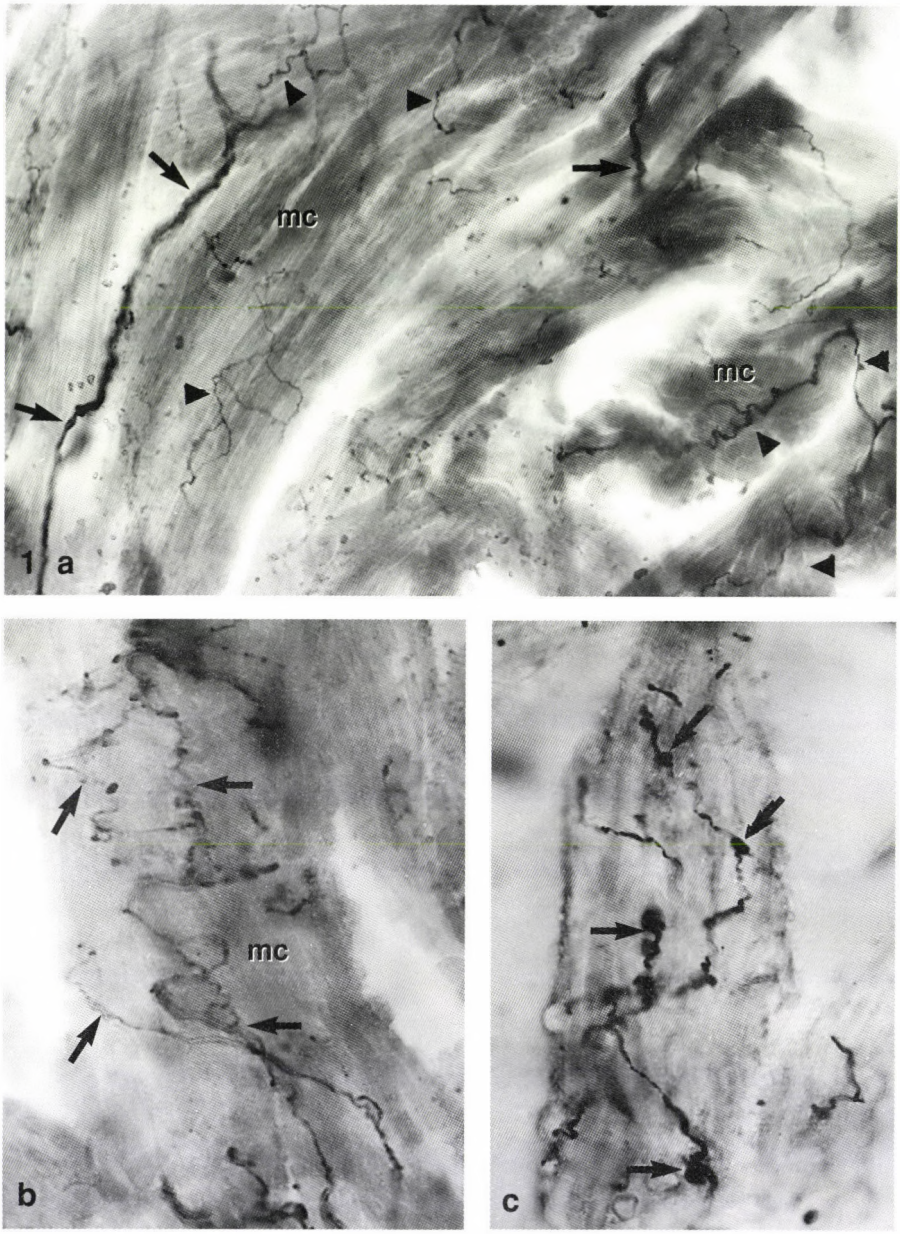

Fig. 1. Innervation of *Helix* heart by MIP-IR fibers at the light microscopic level, seen in Vibratome-sections. **a.** A relatively thick axon (arrow) shows several branchings (small arrows) which form varicose arborizations (arrows) over the muscle fibers (mc). Ventricle. ×250 **b.** Fine varicose processes (arrowheads) over a muscle fiber in the auricle. ×500 **c.** Immunoreactive processes with large varicosities (arrows) over a muscle fiber in the auricle. ×500

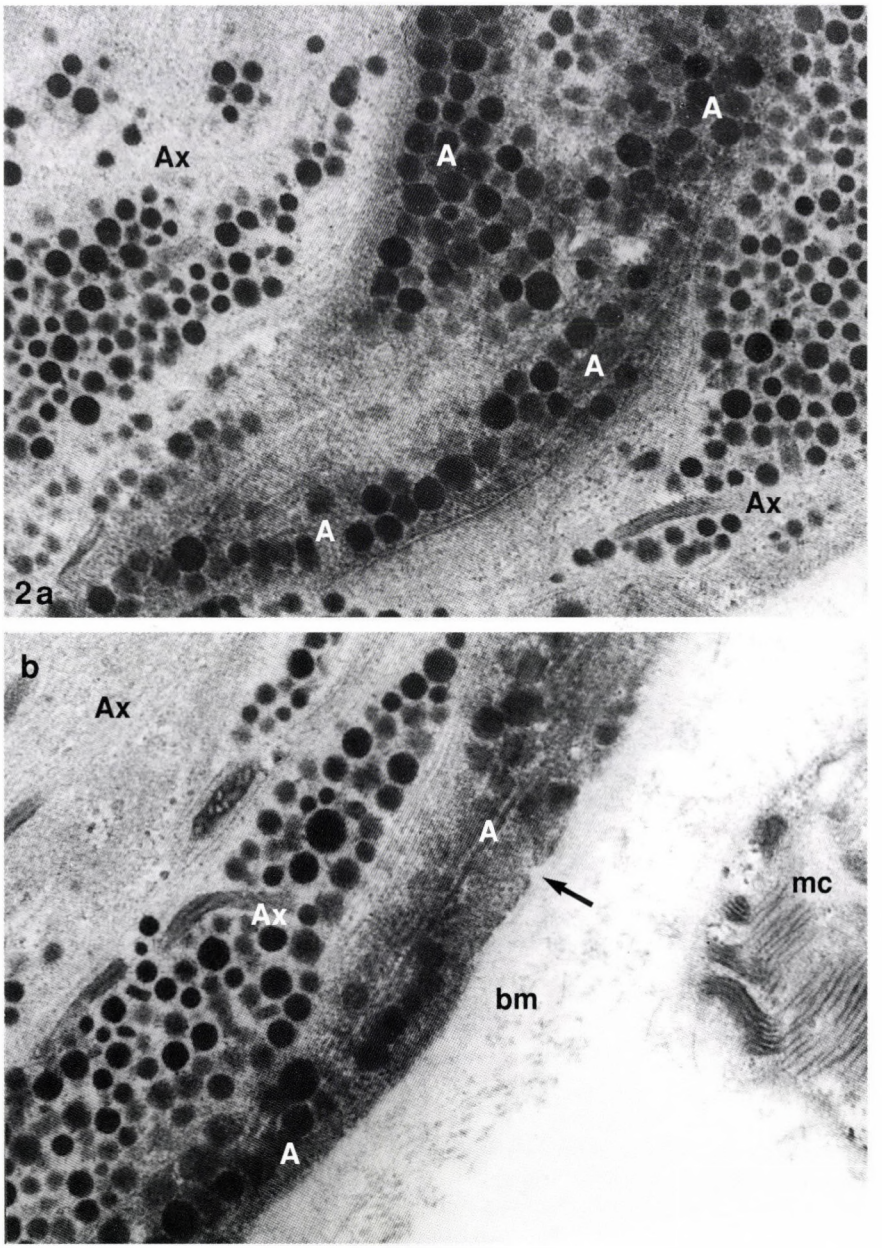

Fig. 2. **a.** MIP-IR axon profiles (A) in a small nerve innervating the auricle. The labelled profiles contain numerous large (120–15 nm) granules. Ax – unlabelled axon profiles. ×28 000 **b.** Thin MIP-IR axon profile (A) located on the surface of an axon bundle in the auricle. Note that the profile faces the wide extracellular space. Arrow – omega-shaped membrane configuration; Ax – unlabelled axon profiles; bm – basal membrane; mc – muscle fiber. ×28 000

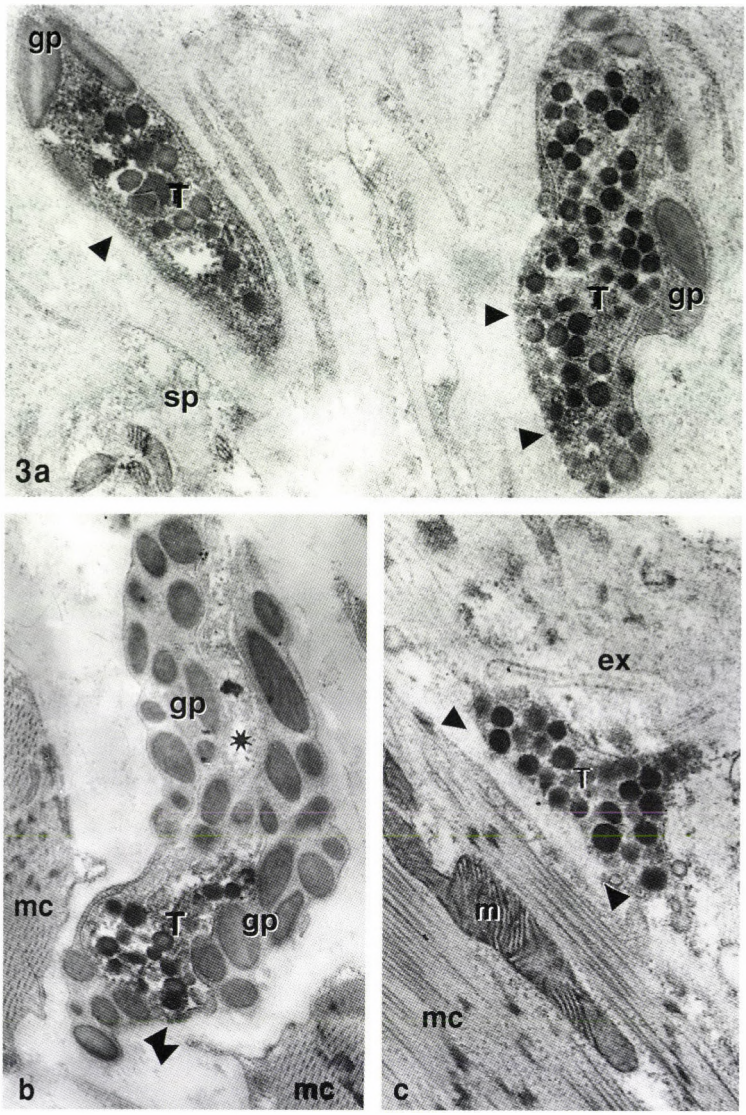

Fig. 3. Remote contacts of MIP-IR varicosities with heart muscle fibers. **a.** Two MIP-IR varicosities (T), located in the extracellular space within the muscular wall of the auricle. Numerous large electron-dense granules are seen in them. The profiles are only partly surrounded by glia processes (gp), a long segment of their axolemma faces freely the extracellular space (arrowheads). sp – sarcoplasma process. ×31 500 **b.** MIP-IR axon profile, ending up in a varicosity (T), is shown in the auricle. The varicosity and its preterminal segment (asterisk) are surrounded by glia processes (gp). Note that a small segment of varicosity membrane faces freely towards a fine sarcoplasmic process (arrowheads). mc – muscle fiber. ×21 500 **c.** MIP-IR varicosity (T) located nearby a muscle fiber (mc) is seen. The width of the "synaptic cleft" (arrowheads) is, however, still increased (40–130 nm). m – mitochondrium, ex – extracellular space. ×31 500

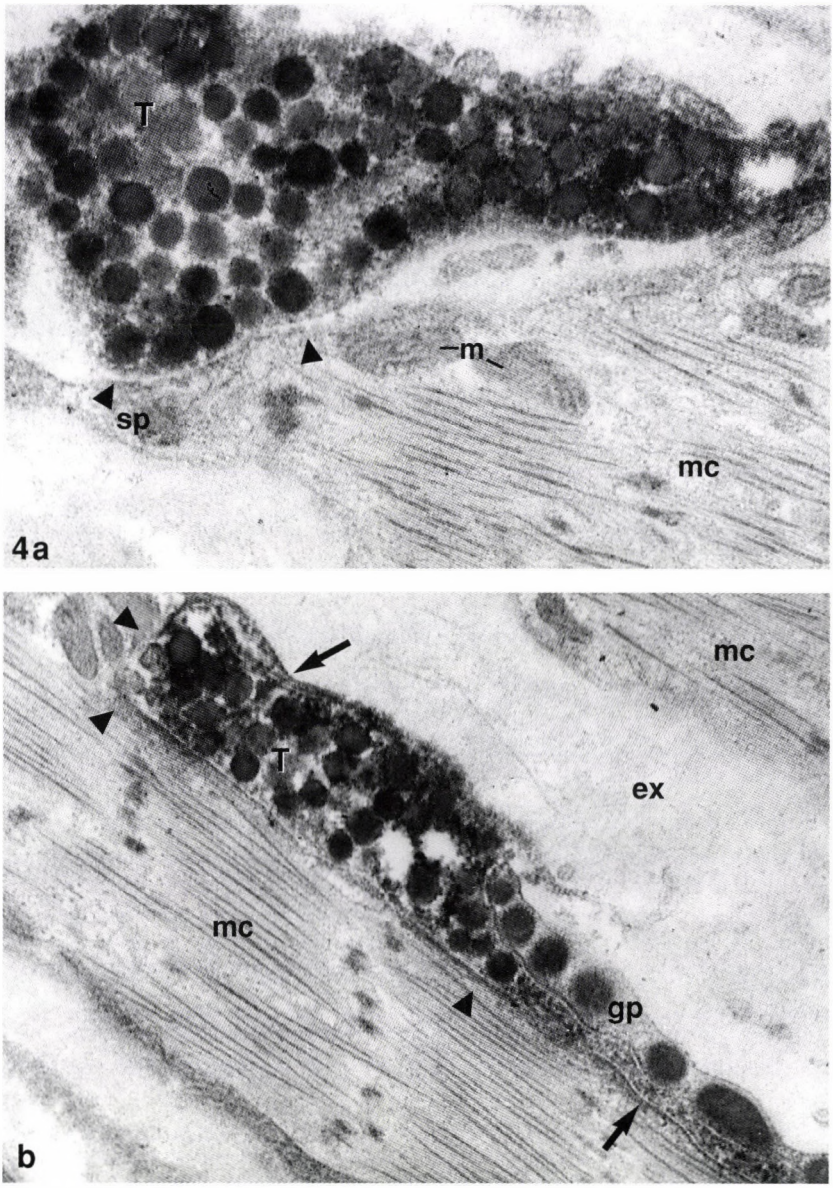

Fig. 4. MIP-IR neuromuscular contacts in the auricle of *Helix* heart. **a.** A varicosity (T) filled with 120–150-nm electron-dense granules forms a close unspecialized membrane contact (between arrowheads) with a small sarcoplasmic process (sp). mc – muscle cell, m – mitochondria. ×48 000 **b.** A varicosity (T), containing numerous 120–150 nm granules, contacts a muscle cell (mc) along an unspecialized membrane segment (arrowhead), whereas its "other" side is covered by a fine sarcoplasmic process (double arrow). The intervaricosity axon segment is also tightly connected to the muscle cell (arrow). m – mitochondria, gp – glia process, ex – extracellular space. ×36.000

MIP-IR varicosities were found in three typical localizations in the heart musculature: (i) Varicosities located relative far (0.5-several μm) from the muscle cells (Fig. 3a, b); (ii) varicosities in the vicinity of the muscle fibers, separated by a wide (30–40 nm) cleft from the muscle fibers (Fig. 3c); and (iii) varicosities establishing close (16–20 nm cleft) but unspecialized neuromuscular contacts (Fig. 4). The varicosities were characterized uniformly by the presence of 120–150 nm electron dense granules, and occasionally small 50–60 nm clear agranular vesicles were also seen in them. In the first two cases, the MIP-IR varicosities were partly or almost entirely covered by glia processes, and a short or a longer segment of the axolemma remained free, facing the extracellular space or the opposing muscle fiber. In the case of unspecialized close neuromuscular contacts, the MIP-IR varicosities were seen in different forms of interactions (Fig. 4). They were either partly surrounded by sarcolemmal protrusions of the sarcoplasm, or formed several contacts with small sarcoplasm processes (Fig. 4a), or contacted the sarcolemma with a long "presynaptic" membrane segment (Fig. 4b). No membrane specializations could be observed at any of these close neuromuscular contacts. The contacting MIP-IR varicosities were accompanied regularly by glia processes.

DISCUSSION

The present findings unequivocally show that the MIP-IR innervation of the heart of *Helix pomatia* is a typical modulatory innervation, according to the ultrastructural characteristics of the innervating elements. The different types of modulatory, non-specialized innervation described, that is the close and the remote contacts, suggest that MIP may have a double role in regulating the activity of the heart muscle fibers: (i) a direct effect onto the muscle fiber throughby forming close but unspecialized parallel membrane contacts, and (ii) a typical modulatory effect, resembling that described for octopamine in the somatic and visceral muscles of arthropods [7, 12]. In these latter case, MIP-IR profiles are located in a distance of 0.5–several μm from the muscle fiber. Investigating the FMRFamidergic innervation of different peripheral tissues, including the heart, of *Helix* by postembedding immunogold labelling, it has been demonstrated that, except the tentacle, the immunolabelled elements formed exclusively close unspecialized neuromuscular membrane contacts [6].

On the other hand, MIP-IR profiles were not found in neurohaemal areas, described earlier by Cottrell and Osborne [2] on the inner surface of the *Helix* heart. So, to our present knowledge, a typical neurohormonal role for this neuropeptide, pumped out from the heart through the circulation towards the periphery, seems to be unlikely. The MIP-IR axon processes, localized freely on the surface of the axon bundles running within the heart muscle, may, however, be indicative of some kind of intracardial neurohormonal role of this substance, excerting a fine regulating effect on the muscle fibers. To test these hypotheses and to specify the modulatory effect(s) of MIP, bioassays and microelectrophysiological studies are needed. Effects of MIP

on muscle activity, including the byssus retractor muscle of *Mytilus* and the calcium release from the muscle cells, have been demonstrated [8–10].

The granules seen in the MIP-IR profiles, independently whether the profiles were found in the innervating nerves or in the varicosities, showed a highly uniform ultrastructure. The 120–150 nm diameter granules were spheroid and, in great majority, of high electron density. No immunolabelling was seen in axon profiles containing other types of granules or granular vesicles. In an earlier immunogold electron microscopic study performed on different peripheral tissues, including the heart, of *Helix pomatia* [6], a great variety, seven types, of ultrastructurally different FMRFamide-IR granules have been demonstrated. Five of them were present in the heart. Also, the axon profiles in the *Lymnaea* heart immunoreactive to antisera raised against different members of the peptide family encoded by the FMRFamide-gene of *Lymnaea* [1] revealed a wide variety of granule/vesicle content (Dobbins, Thorpe, Elekes, Benjamin, unpublished). The MIP-family originally identified from *Mytilus* has been shown to consist of seven members, whereas that from *Helix* has two members, and all possess a highly conserved C-terminal structure [13, 14, and see in 8]. Therefore, and contrary to that suggested for the granular localization of FMRF-amide-immunoreactivity in *Helix* [5, 6], it appears that the ultrastructural localization of MIP in *Helix* heart is rather bound to a single form of granule. Although the possibility of intragranular colocalization of additional signal molecules cannot be excluded either. The MIP-IR axon profiles contained mostly large granules, agranular synaptic vesicles could be seen in a very small number in them. Even along the "presynaptic" membrane the agranular synaptic vesicles were not dominating at all. The FMRFamide-IR varicosities in different peripheral tissues of *Helix pomatia* [6], and the buccalin and SCP containing elements in the accessory radula closer muscle of *Aplysia californica* [20] have been demonstrated to contain numerous agranular synaptic vesicles, many of them localized along the presynaptic membrane. Further ultrastructural studies on the MIP-IR innervation of other peripheral tissues of *Helix* are in progress, partly to reveal and compare the different forms of neuro-muscular contacts, and partly to analyze the composition of granule population of the immunolabelled profiles.

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GUAIACOL AND VANILLOID COMPOUNDS MODULATE THE A-TYPE POTASSIUM CURRENTS IN MOLLUSCAN NEURONS*

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The actions of guaiacol (2-methoxy-phenol), vanillin (4-hydroxy-3-methoxy-benzaldehyd) and other vanilloid compounds such as zingerone (4-/4-hydroxy-3-methoxyphenyl/-2-butanon) and eugenol (2-methoxy-4-/2-propenyl/phenol) were investigated on the fast outward potassium currents (A-type currents) in molluscan neurons.

Guaiacol (0.01–0.1%, w/v) moderately decreased the peak amplitude but increased the rate of inactivation of the A-currents in dose-dependent way (Kd = 0.06% 4 mM, nH = 0.8). Vanillin (5 mM) slightly decreased the peak amplitude of the A-currents in *Helix* neurons but its action was more pronounced in dialysed *Lymnaea* nerve cells. However, vanillin similarly decreased the time-to-peak and the time constant of decay of the A-currents both in the faster and the slower inactivating *Lymnaea* and *Helix* neurons (Kd = 5 mM, nH = 0.6). The voltage-dependence of activation and inactivation of the A-currents were not significantly influenced by guaiacol and vanillin in *Helix* or *Lymnaea* neurons. Vanillin hardly influenced the delayed outward currents, but decreased the leak currents in the identified LPa and RPa 2,3 neurons. A structure-activity analysis clearly showed that increasing alkyl tail length from the aldehyde side of the vanillin molecule increased the efficacy of the various compounds on the amplitude of the A-currents and modified the kinetical influence on the A-current channel. Furthermore, an attenuation of the late outward currents and an increase of the leak conductance also developed in the presence of zingerone or eugenol.

Excitatory actions of the studied vanilloids predominated on the various molluscan neurons.

Keywords: Vanilloid compounds – molluscan neurons – A-type current – voltage clamp

INTRODUCTION

The fast outward potassium current or A-current is a significant component of the outward membrane ionic currents in a number of identified *Helix* neurons as it was first reported by Gola and Romey [9] and Neher [12]. A-current can be activated by membrane depolarization after a conditioning hyperpolarization pulse and this cur-

*Dedicated to Professor János Salánki for his 70th birthday.

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rent component plays an important role in regulation of subthreshold events such as interspike interval and several aspects of synaptic transmission [1, 2].

Guaiacol and eugenol are widely used as dental medicaments. Zingerone is a natural pungent-testing, oral irritant which can be found in ginger. Some cosmetic products also contain eugenol as natural ingredient. Eugenol and its metabolite (quinone methide) can evoke cytotoxic actions on certain cell lines [16]. Contrary to the cytotoxic actions of eugenol, a neuroprotective activity of the molecule was also reported on cultured cortical neurons against neurotoxic and oxidative injuries [17]. Vanillin is widly used as food additive for various cakes, chocolates or vanilla ice cream and it is an effective drog on the ion channels of the neuron membrane [6]. The capsaicin related structure makes the mentioned vanilloid molecules scientifically important. While, the actions of capsaicin on potassium currents of vertebrate and some invertebrate neurons or nerves are extensively studied, those of the basic molecules on isolated A-currents are poorly understood [3, 7, 11, 13, 15].

Earlier, the actions of hydroxybenzenes, methoxybenzenes and capsaicin have already been reported on the A-currents of molluscan neurons as related compounds [5, 7, 8]. This report describes the actions of guaiacol, vanillin and some other vanilloids such as zingerone and eugenol on the isolated fast outward potassium currents of molluscan neurons. The structure-activity studies may help to understand how related compounds act on a given target site.

MATERIALS AND METHODS

Experiments were performed on in vitro ganglion preparation of the land snail *Helix pomatia* L. or enzymatically isolated and dialysed neurons of the pond snail *Lymnaea stagnalis* L.

Experimental animals were collected locally and prior to the experiments, they were kept in an active state under laboratory conditions. The ganglionic complex was removed and isolated from various specimen of *Helix pomatia* L. The connective tissue capsules covering the neurons were removed under binocular microscope and the preparation then pinned to a Silastic-covered (Sylgard 184, Dow Corning Corp., Midland, Mi. USA) floor of a 5 ml Plexiglass recording chamber. The studied neurons were identified left and right parietal 2, 3 cells (LPa and RPa 2,3) located on the dorsal surface and upper edge of the ganglia (Fig. 1A, 14). They had –50, –60 mV (–55 + 4.5 mV; mean + S.D., n = 30) resting membrane potential and characteristic A-currents which could be studied under spike threshold voltages in normal bathing solution. The A-current had 4-aminopyridine sensitivity and they were 70% resistant in high concentration of tetraethylammonium medium [4]. The physiological solution contained (mM) NaCl, 80; KCl, 4; $CaCl_2$, 7; $MgCl_2$, 5; Tris-HCl, 5; (pH = 7.4). In some experiments Na- and Ca-free, Tris-solutions were also used with the following composition (mM) Tris-HCl, 80; KCl, 4; $MgCl_2$, 12; (pH = 7.4). The ganglionic complex was removed and isolated from specimen of *Lymnaea stagnalis* L. and further processed by enzymatic treatment. 3.5 mg/ml pronase E (SERVA) for 30 min at

room temperature was used in an isolation solution contained (mM) NaCl, 86; KCl, 1.6; $CaCl_2$, 1.5; $MgCl_2$, 4; Tris-HCl, 4 (pH = 7.5). After enzymatic treatment the individual neurons were isolated mechanically. In most of the cases the left parietal large cells were selected for experiments. Polyethylene pipettes were used for intracellular dialysis and voltage-clamping [10]. The extracellular and intracellular solutions contained (mM) NaCl, 86; KCl, 1.6; $CaCl_2$, 4; $MgCl_2$, 1.5; Tris-HCl, 4 (pH = 7.5) and KCl, 74; $CaCl_2$, 0.02; EGTA, 1; Tris-HCl, 20 (pH = 7.2), respectively. Guaiacol, vanillin and vanilloid compounds were applied in the extracellular solution and added to the organ bath by changing the reservoir from which the bathing solution was drawn. Experiments were performed at room temperature 22–24 °C.

A home-built voltage-clamp amplifier based on the design of Wilson and Goldner [18] was used to record the membrane voltage and currents. The series resistance and leak current were electronically compensated in most of the experiments. Low resistant microelectrodes were used (2–4 MOhm) when single electrode voltage-clamp measurement took place without cell dialysis. The normal sampling frequency was 3.5 kHz at 50% duty cycle. The potential and current signals were recorded on a Hitachi V-6025 digital storage oscilloscope and photographed from the screen. An IBM AT 286 base personal computer equipped with Labmaster DMA, TL-1-125 kHz interface and software (Axon Instruments) were also used to process the data and construct plots.

The A-currents were isolated under spike threshold voltages in most of the experiments (see Fig. 1B), but in some cases the TEA^+-resistant component of the A-currents was also studied when TEA^+ substituted for NaCl. The double pulse method in combination with the conditioning hyperpolarization protocol was used to study the activation and inactivation characteristics of the A-currents. The recorded currents were corrected for other small contaminating currents before plots were made (mainly outward currents which activated without conditioning hyperpolarization). Two files were constructed with and without conditioning hyperpolarization pulses and the difference of currents was studied as A-current. Delayed outward potassium currents were activated from a holding potential of resting level (–50 mV) by using long depolarizing command pulses (500 ms or longer) with and without extracellular Ca^{++} or in the presence of Ca-channel blockers. Leak currents were activated from a holding level by use of hyperpolarizing command pulses.

For statistical analysis, the conventional Students's *t*-test was used.

RESULTS

Figure 1A shows the position of the identified L and RPa 2,3 neurons on the dorsal surface of the left and right parietal ganglia (L and RPaG). These cells were used in most of the experiments discussed in this paper. *Helix* L and RPa 2,3 neurons have a significant A-current under spike threshold voltages what can be recorded in normal, Na-free or Na- and Ca-free solutions in voltage-clamp conditions (Fig. 1B). Figure 1B presents a fast outward (a), a complex late outward potassium (b) and a leak cur-

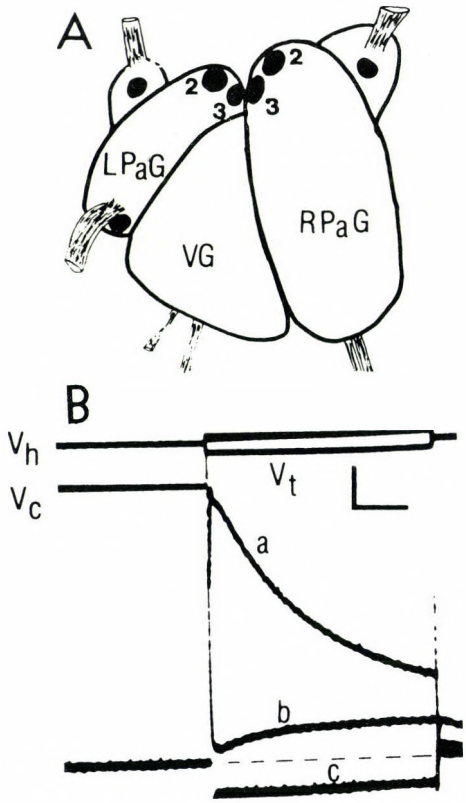

Fig. 1A. Ganglionic complex of *Helix pomatia* L: dorsal surface. Identified left and right parietal ganglion cells (L and RPaG) are numbered. *B.* Isolation of the A-currents in Na-fee (Tris) solution. Three current traces are shown (a–c) which were activated from –50 mV holding potential (V_h) by use of depolarizing command pulse (V_t) preceded with (a) or without (b) conditioning hyperpolarization (V_c) to –100 mV for 500 ms and with a hyperpolarizing command pulse (c). Calibration: 50 mV, 50 ms and 25 nA

rent trace (c) as they were photographed from the screen of a dual beam storage oscilloscope. Fast outward, complex late outward and leak currents were activated from –50 mV holding potential (V_h) by use of 30 mV depolarizing command step (V_t) with and without conditioning hyperpolarizing pulse (V_c) of –100 mV for 500 ms and a –30 mV hyperpolarizing command step ($-V_t$), respectively. However, A-currents can be isolated by use of a computer technique in the following way: two files are recorded with and without conditioning hyperpolarization from a given holding potential (near the resting membrane potential) with increasing amplitude of several depolarizing command pulses. The difference of the two current files will give a family of isolated A-currents.

Actions of guaiacol on the A-currents and action potential

Guaiacol (0.01–0.1%) induced characteristic and reversible actions on the A-type potassium currents and action potentials when it was applied extracellulary in normal solution as it is illustrated in Fig. 2A and B. Figure 2A includes families of A-currents elicited by the activation protocol and the inset shows the resting membrane potential level and action potential pattern in each phase of the experiment. Figure 2B shows other families of A-currents recorded by use of the inactivation protocol. The (a), (b) and (c) panels of Fig. 2A and B present the recorded currents and action potentials in control, in 0.1% guaiacol containing (5 min) and in guaiacol-free washing media (15 min), respectively. The following events are prominent in the presence of 0.1% guaiacol (b records): 1. the time-dependent activation and inactivation of the A-currents are faster, 2. the peak amplitude of the A-currents are moderately reduced, 3. the action potential duration is prolonged and the hyperpolarizing afterpotential is higher in amplitude and finally; 5. the guaiacol-induced alterations can be reversed by washing. The peak amplitude of isolated A-currents versus membrane potential plots (Fig. 3A) and the normalized steady-state activation and inactivation versus

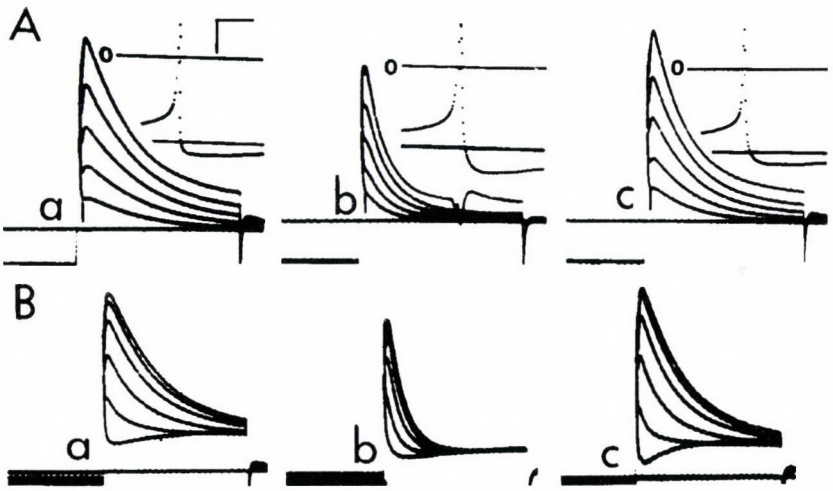

Fig. 2. Activation of A-currents (A) in control (a), in 0.1% guaiacol (5 min) containing solution (b) and recovery after 15 min washing (c). Inset shows the resting membrane potential level and spike pattern in each phase of the experiment in current clamp circumstances. A-currents were activated by depolarizing command voltage steps from a holding potential of –50 mV to –30 mV in increments of 5 mV with preceding hyperpolarizing pulses of 500 ms duration to –100 mV. LPa2 cell. Inactivation of A-currents (B) in Na-free (Tris) solution (a), 0.1% guaiacol (5 min) containing medium (b) and after 15 min washing (c). Inactivation was studied with depolarizing step to –25 mV from conditioning prepulse level of –100 mV to –45 mV. LPa3 neuron. Calibration: 25 nA, 100 ms (for voltage clamp) and 20 mV, 20 ms (for the inset current clamp records)

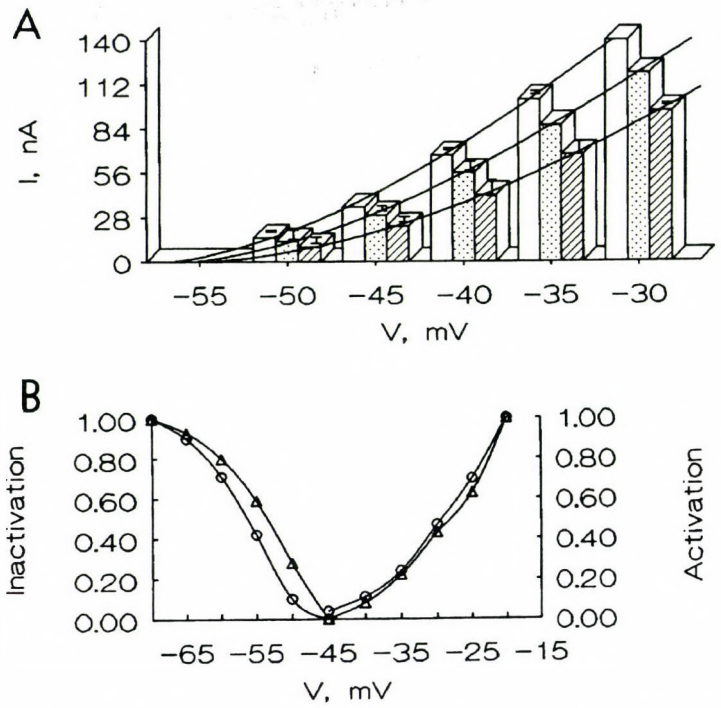

Fig. 3A. Peak amplitude of isolated A-currents versus membrane potential plots shows dose-dependent suppression of the current amplitude in 0.04% (spotted column) and 0.08% (stripped column) guaiacol containing normal solution. The differences are mathematically significant at various significance level; $p>0.05$–0.001 from left to right (mean ± S.D. indicated, $n = 6$). RPa3 cell. *B.* Normalized steady-state activation and inactivation versus membrane potential curves plotted in control (triangles) and 0.1% guaiacol containing medium (circles). The mid-point potentials of activation and inactivation did not shift significantly in the presence of guaiacol. LPa2 cell

membrane potential curves (Fig. 3B) illustrate that 0.04% and 0.08% guaiacol decreased the peak amplitude of the A-currents in dose-dependent way in RPa3 neuron (A). The maximal effect was 16 and 24 per cent decrease at –30 mV membrane potential. However, a decrease of the amplitude of the A-currents was not in correlation with an action of guaiacol on the activation and inactivation gates of A-current channel because the mid-point potentials of activation and inactivation curves plotted in normal and 0.1% guaiacol-containing solutions were not significantly different (Fig. 3B). A pronounced action of guaiacol on the time-dependent activation and inactivation kinetics predominated. Guaiacol decreased the time constant of decay of A-currents in dose-dependent way as can be seen in Fig. 4A, where normalized amplitudes after peak are plotted against time semilogarithmically, in control (crosses), 0.02% (triangles), 0.04% (circles) and 0.08% (squares) guaiacol containing solutions, respectively. However, the single exponential characteristics of the A-current

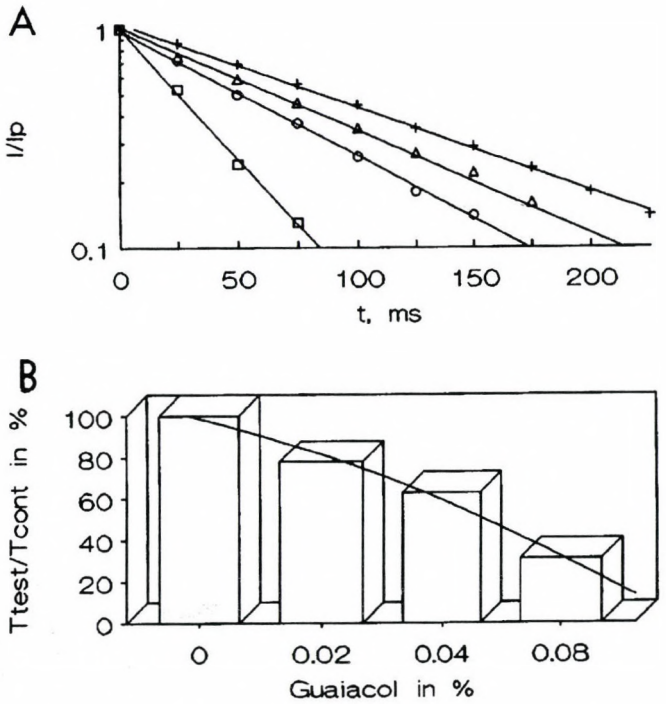

Fig. 4A. Semilogarithmic plot of A-current amplitudes after peak versus time graph shows dose-dependent decrease of the time constant of decay in guaiacol solution. Single exponential decays are presented in control solution (crosses), in 0.02% (triangles), 0.04% (circles) and 0.08% (squares) guaiacol containing media respectively. *B.* Dose-response relationship of guaiacol action on the time constant of decay (Kd = 0.06%, nH = 0.8). RPa3 neuron

decays remained unchanged in the guaiacol media. Figure 4B shows the dose-response relationship of the guaiacol action on the time constant of decay of isolated A-currents in a RPa 3 neuron, where the calculated Kd = 0.06% and the Hill's coefficient, nH = 0.8.

Actions of vanillin on the A-currents and membrane properties

The actions of vanillin on the A-currents were studied in dialysed *Lymnaea* and *in vitro Helix* neurons under current- and voltage-clamp circumstances. As Fig. 5A shows, vanillin (5 mM, 10 min) decreased the peak amplitude, the time-to-peak and time constant of decay of A-currents in the large left parietal ganglion cell of the pond snail. Figure 5Aa presents two families of A-currents recorded by a faster and a slower beam speed to see the time course of activation and inactivation of the A-currents. The peak amplitude versus membrane potential plot shows the vanillin induced sup-

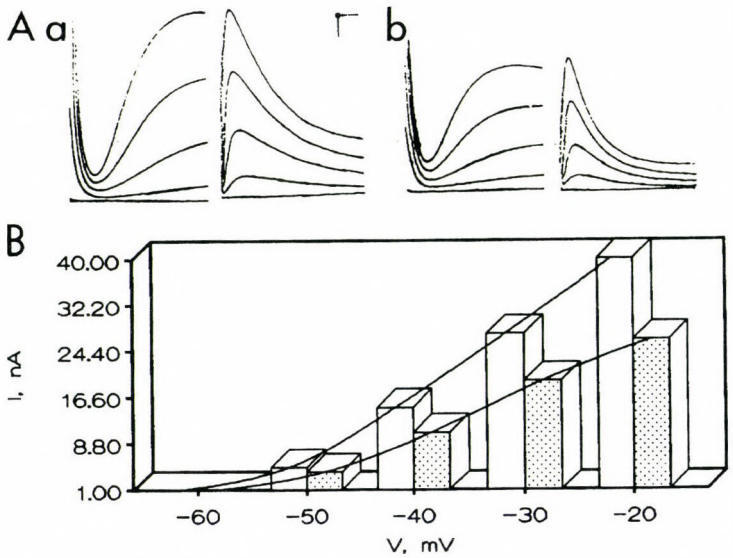

Fig. 5A. Families of A-currents as they were recorded from dialysed *Lymnaea* left parietal large cell in normal solution (a) and in 5 mM vanillin (10 min) containing medium (b). Vanillin acted in the extracellularis solution. A-currents were activated by depolarizing voltage steps starting from hyperpolarized level at –110 mV and going to –60 to –20 mV with 10 mV increments. Conditioning hyperpolarization was held for 500 ms, starting from a holding potential of –60 mV. Vanillin (5 mM, 10 min) decreased the amplitude of A-currents (spotted column in B). Calibration: 1 ms (A), 10 ms (B) and 10 nA

pression in Fig. 5B. While, the actions of vanillin on the potential dependence of activation and inactivation of the A-currents is presented in Fig. 6A. As can be seen, vanillin decreased the peak amplitude of the A-currents but did not influence the potential-dependence of the activation and inactivation. A characteristic effect of vanillin on the rate of inactivation has been found as it is shown in Fig. 6B where semilogarithmic plot of A-current amplitude after peak versus time graph shows a significant decrease of the time constant of decay of A-currents. The single exponential characteristics of the decay, however, remained unchanged in the vanillin-containing solution, as well. A very similar action of vanillin (1–10 mM) was found on the A-currents of identified *Helix* neurons. Figure 7 shows the reversible actions of vanillin on the inactivation (A) and activation (B) of the A-currents recorded in voltage-clamped RPa 3 neurons. In comparison with *Lymnaea* neurons a moderate decrease of the peak amplitude of the A-currents was found in *Helix* nerve cells but the voltage dependence of the steady-state activation and inactivation of the A-currents did not change significantly in the studied neurons of both species (Fig. 7A and B). An enhanced effect of vanillin on the peak amplitude of the A-currents was observed in Na-free (TEA) solution when the TEA-resistant component of the A-currents was studied. Contrary to the slower inactivating A-currents of *Helix* neurons,

vanillin similarly increased the rate of inactivation as in the faster inactivating type of *Lymnaea* neurons (see and compare Fig. 6B and Fig. 8A). The action of vanillin on the time constant of decay of A-currents recorded in *Helix* neurons was dose-dependent as an example is shown in Fig. 8B (Kd = 5 mM, nH = 0.6).

Vanillin decreased the resting membrane potential, increased the membrane resistance and spike duration in all of the neurons studied.

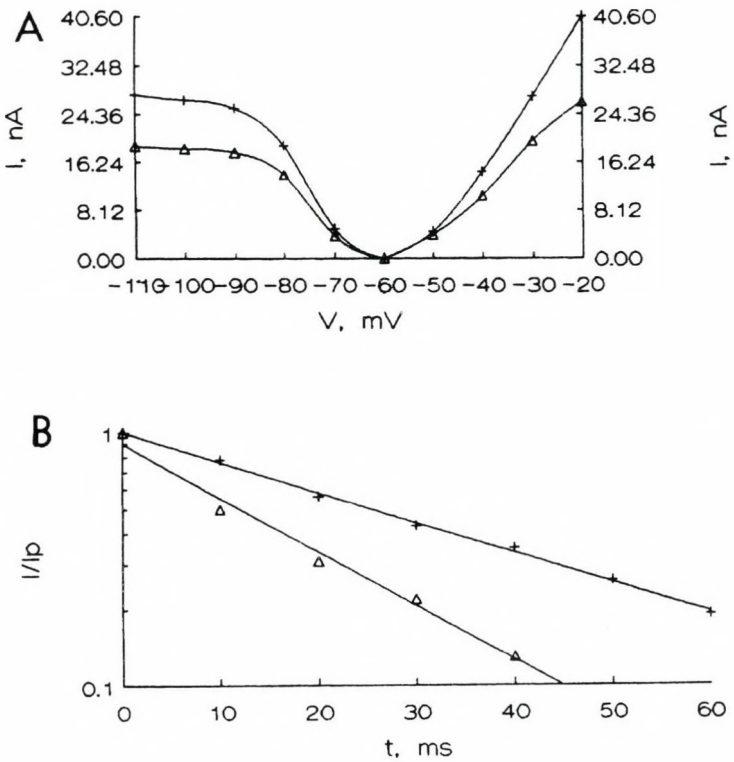

Fig. 6A. Steady-state activation and inactivation curves recorded in control (crosses) or in 5 mM vanillin (10 min) containing solution (triangles). Vanillin did not modify the voltage dependence of activation and inactivation of A-currents. *B.* Semilogarithmic plot of A-current amplitudes after peak versus time graph shows vanillin induced decrease of the time constant of decay (triangles). Crosses mark the control values. *Lymnaea* LPa cell

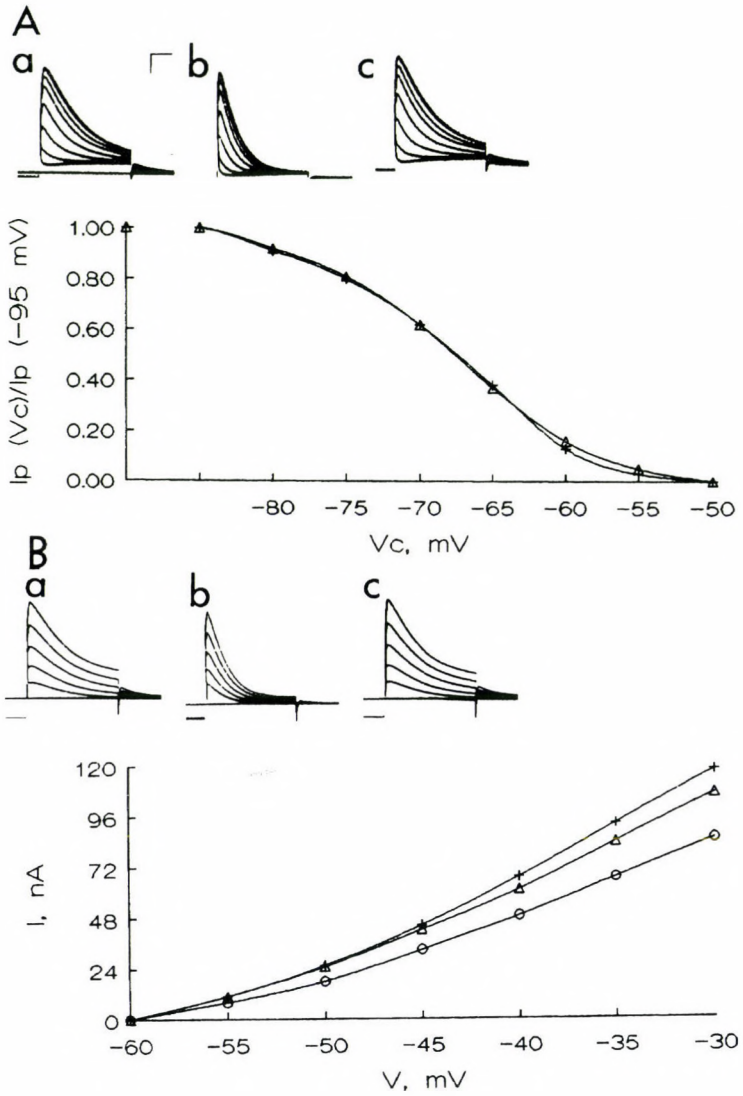

Fig. 7. Inactivation (A) and activation (B) of A-currents as measured in control Na-free (Tris) solution (a), in 7 mM vanillin (10 min) containing medium (b) and after 25 min washing (c). *Helix* RPa3 neuron. Normalized amplitudes versus membrane potential plot present no effect of vanillin on the potential dependence of steady-state inactivation (crosses and triangles are the control and test values, respectively). Vanillin reversibly decreased the peak amplitude of the A-currents (B). Crosses, open circles and triangles are the control, test values and recovery after washing. Calibration: 25 nA and 100 ms

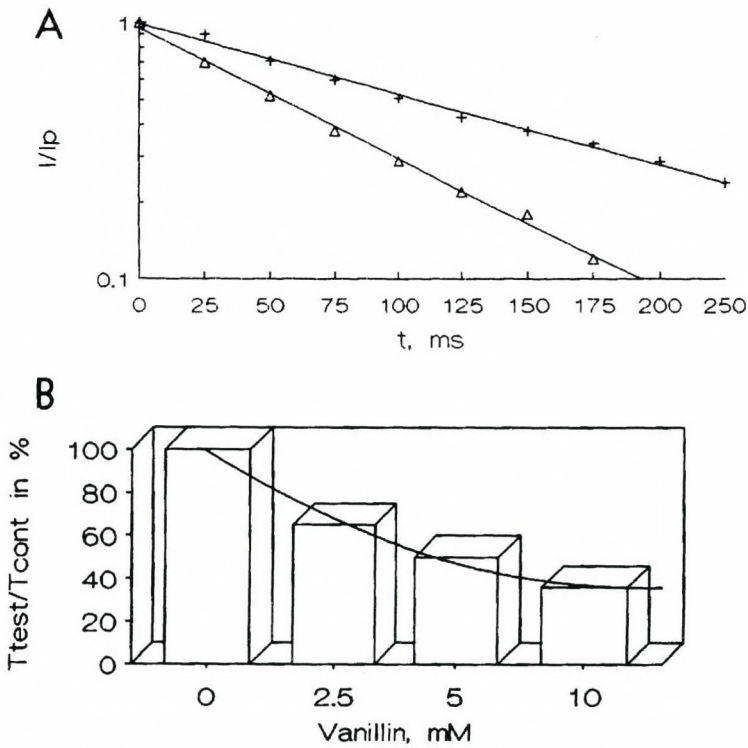

Fig. 8. Vanillin decreased the time constant of decay of A-currents in dose-dependent way (*Helix* LPa3 neuron). *A.* Semilogarithic plot of A-current amplitudes after peak versus time graph presents a decrease of the time constant of decay of A-currents in 5 mM vanillin (10 min) containing solution (triangles) relative to the control (crosses). *B.* Dose-response relationship of vanillin action on the time constant of decay of A-currents (Kd = 5 mM, nH = 0.6)

Structure-activity study with vanilloid compounds

Substitution of the vanillin aldehyde with allyl group modified the effectiveness of the molecule. Eugenol (4-allyl-2-methoxyphenol) decreased the amplitude of the A-currents about three times more than vanillin did and enhanced effect on the rate of inactivation was also prominent. Figure 9A–C show the actions of eugenol both on activation (A), inactivation (B) and voltage-dependence of steady-state activation and inactivation of the A-currents. As shown in Fig. 9A–C the voltage dependence of activation and inactivation of the A-currents did not change as in the presence of guaiacol or vanillin but the peak amplitude of the A-currents was significantly attenuated in the eugenol containing (2 mM, 10 min) solution. Zingerone (5 mM, 10 min) was also more effective relative to vanillin by acting on the studied *Helix* neurons. It looks like that zingerone and eugenol can induce more complex actions on L and RPa

2,3 neurons than vanillin or guaiacol does. Eugenol increased the leak and decreased the late outward potassium currents significantly. The effects of the studied compounds on the A-currents were fairly reversible by giving the possibility to study the action of each compound in one neuron during long experiments. The result of such an experiment is presented in Fig. 10A–C. Figure 10A shows families of A-currents recorded in control Na-, Ca-free solution (a) and in vanillin (5 mM, 10 min., b), zingerone (5 mM, 10 min., c), and eugenol (2 mM, 10 min., d) containing media,

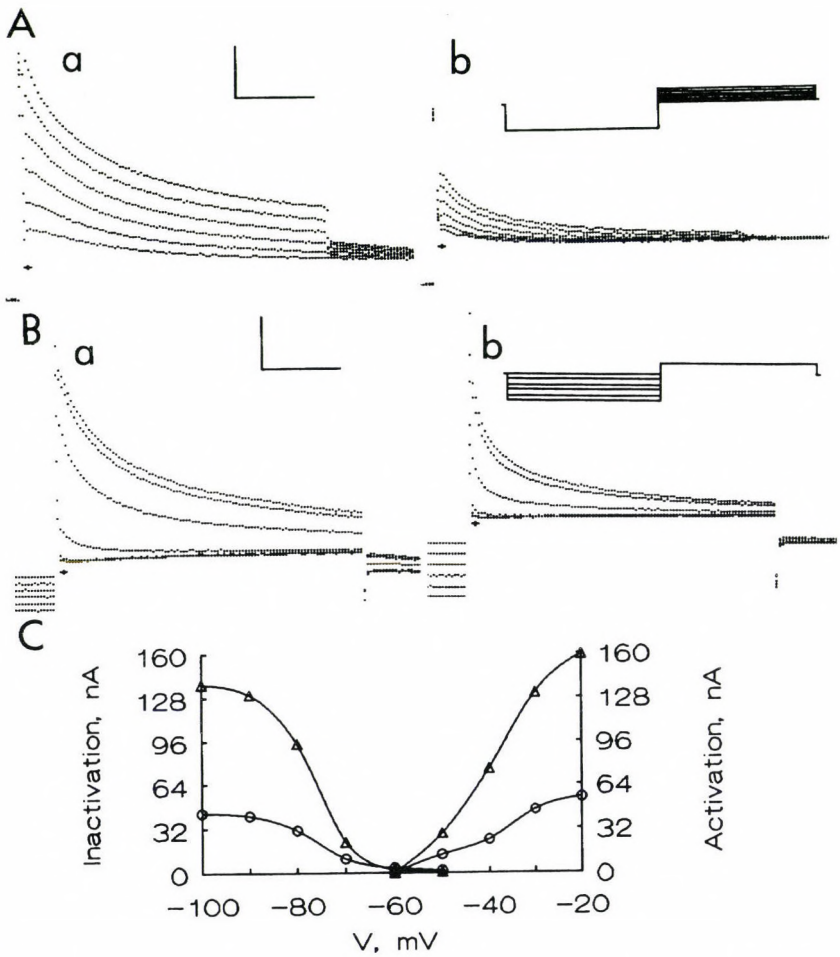

Fig. 9. Activation (A) and inactivation (B) of A-currents in eugenol (2 mM, 10 min) containing Na-, Ca-free solution. Families of A-currents are in control (a) and test solutions (b), respectively. C. Eugenol decreased the peak amplitude of A-currents but did not influence the voltage-dependence of activation and inactivation of A-currents. Triangles and circles present control and test values, respectively. Calibration: 40 nA and 80 ms

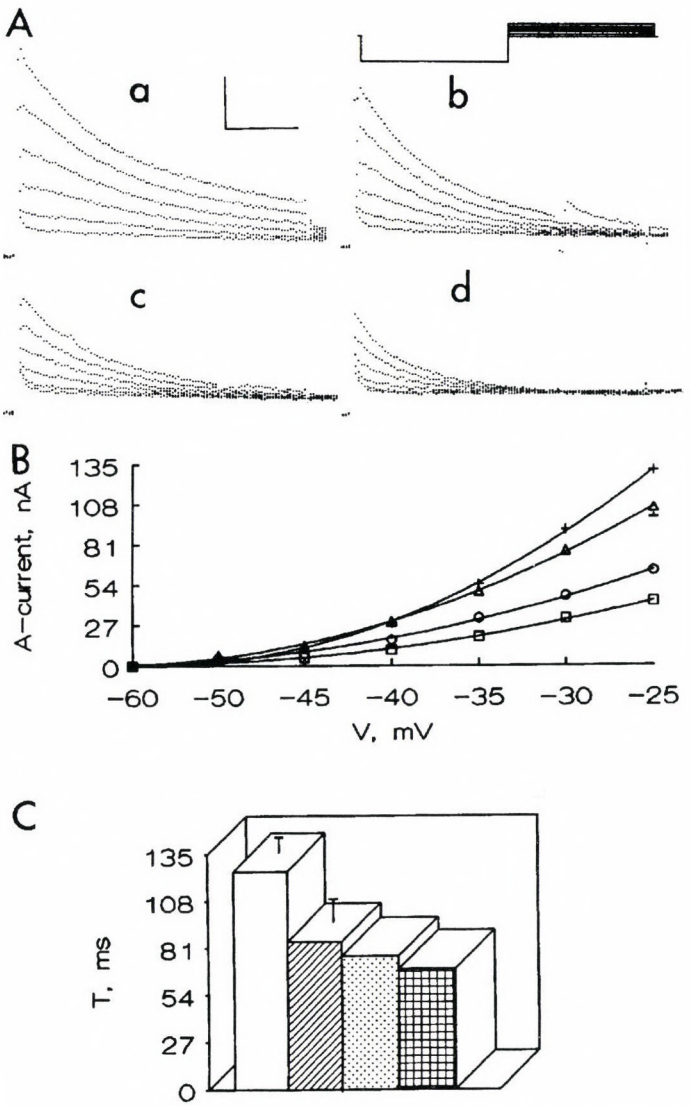

Fig. 10. Families of A-currents as they were activated in normal (a), 5 mM vanillin (b), 5 mM zingerone (c) and 2 mM eugenol (d) containing solution for 10 min. *Helix* RPa2 neuron. B. Peak amplitudes of A-currents versus membrane potential plot constracted from the A records show significant decrease of the measured parameters between –35 and –25 mV membrane potentials relative to the control in the increasing order: vanillin (triangles), zingerone (circles) and eugenol (squares). The significance increased from –35 to –25 mV membrane potential ($p < 0.01$–0.001). Values are means of six measurements. C. The time constant of decay (T) of A-currents decreased relative to the control (open column) in the following order: vanillin (stripped column), eugenol (spotted column) and zingerone (squared column). Relative to the control the values are mathematically significant but there is no significant difference between those of eugenol and zingerone (Mean ± S.D., n = 6). Calibration: 40 nA, 100 ms

respectively. The actions of the compounds on the amplitude of the A-currents were statistically significant as could be seen in Fig. 10B where peak amplitude of A-currents were plotted against membrane potential. The sequence of effectiveness of the studied molecules was on the amplitude of the A-currents at –25 mV membrane potential as follows: vanillin (triangle), zingerone (circle) and eugenol (square). The kinetics of the A-currents were similarly influenced (Fig. 10C). Namely, a decrease of the time constant of decay of A-currents was mathematically significant in the presence of vanillin. Zingerone and eugenol also decreased the time constant of decay of the A-currents relative to the control value significantly, but the difference between zingerone and eugenol was not statistically significant.

DISCUSSION

A dose-dependent and nontoxic, reversible modulatory action of guaiacol, vanillin and other vanilloid compounds such as zingerone and eugenol on the fast outward potassium currents recorded in *Lymnaea* or *Helix* neurons was demonstrated. The studied compounds acted differentially in equipotential dose on the inactivation kinetics of the isolated A-currents. Also, the effectiveness of the hydroxy-methoxy benzene derivatives increased on the amplitude of the A-currents with increasing 4-allyl or alkyl hydrophobic tail substitution. The most potent vanilloid molecule being the capsaicin on the amplitude of the A-currents recorded in RPa 3 *Helix* neurons (IC_{50} = 130 μM), as it was reported earlier [7].

It is interesting that, the dihydroxybenzene catechol preferably attenuated the amplitude and scarcelly influenced the inactivation kinetics of the A-currents, while mono- and dimethoxy benzenes predominantly decreased the time constant of decay of the A-currents in the same neurons in which the actions of the vanilloid compounds were studied [5, 8]. All these observations point to a possible action of the hydroxy-methoxy benzene molecules on a target site situated at a membrane lipid-hydrophobic protein interface of the A-current channel. This molecular interaction can theoretically modulate both the amplitude (or conductance) and kinetics of the A-currents depending on the head substituents of the hydrophobic benzene ring. A further hydrophobic tail substituent on the benzene ring may promote the lipid permeability of the molecule which can help it to reach the binding site on the lipid-protein interface. Furthermore, a hydrophobic tail in cooperation with the methoxy group can weaken the ionised state of the OH-substituent which may modulate the binding potency of the molecule to the recognition site.

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IN VITRO AND *IN VIVO* EFFECTS OF FORMAMIDINES IN LOCUST *(LOCUSTA MIGRATORIA MIGRATORIOIDES)**

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In vivo and *in vitro* experiments were used to study the effects of formamidines in the locust, *Locusta migratoria migratorioides. In vivo* the lethal and the antifeeding effects, *in vitro* the inhibition of the binding of a selective ^{3}H-ligand to the receptors of octopamine, tyramine, dopamine, serotonin and γ-amino butiric acid were studied. We have demonstrated that demethylchlordimeform is specific agonist to octopamine receptor, having high affinity to octopamine receptor, a moderate affinitiy to tyramine receptor and a low affinity to dopamine, serotonin and to γ-amino butiric acid receptors. The demethylated chlordimeform analogoues, demethylchlordimeform and didemethylchlordimeform have higher affinity to the octopamine receptor than the parent compound.

The formamidines had a toxic and an antifeeding effects when injected into the locust. The half lethal doses (LD_{50}) and the feeding inhibition were correlated with the affinity of the compounds (K_i). The ring substitutions of the mulecule have alterated the both affinity and *in vivo* effect of the compounds. The most effective ring substitution pattern is 2,4-disubstitution with a combination of methyl groups or halogens. Our results suggest that the lethal effect of formamidines is mediated through the octopamine receptor.

Keywords: Octopamine – receptor – formamidine – toxicity – insect *Locusta migratoria migratorioides*

INTRODUCTION

The substituted formamidines have an extremely broad spectrum of useful biological activity. The activity ranges from bactericidal, antiprotozoal, antihelminthic, fungicidal, herbicidal to insecticidal actions [8]. It is generally accepted that the insecticidal action of formamidines is due to the perturbation of octopamine (OA) mediated processes in the target species. Experiments with the most studied formamidine derivatives, chlordimeform (CDM), [N'-(4-chloro-o-tolyl)-N,N-dimethyl formamidine] and demethylchlordimeform (DCDM), [N'-(4-chloro-o-tolyl)-N-methyl for-

*Dedicated to Professor János Salánki on the occasion of his 70th birthday.

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mamidine], suggest that these compounds are OAergic agonists [3, 4, 5, 9, 10, 15, 17]. Formamidines have been shown to stimulate the adenylate cyclase activity in both nervous and nonnervous tissues [3, 4, 5, 10, 15, 17], to modulate the neuromuscular transduction [5], and to inhibite the binding of OA to the receptor [7]. At the same time, their possible effect on the ion channels [10, 14] or on other transmitter (serotonergic, dopaminergic, GABAergic) systems cannot be excluded either [16, 18, 20, 21]. Biochemical studies have shown that the *in vitro* potency of DCDM was higher than that of CDM, and it was found that *in vivo* the CDM was demethylated to DCDM [1, 11, 12].

The aim of the present study were to compare the affinity of DCDM to the receptors of OA, dopamine (DA), tyramine (TA), serotonin (5HT) and γ-aminobutitiric acid (GABA) and to analyse the structure-activity relations for the affinity of substituted phenyl formamidines and demethylated formamidines and the toxic effect as well as the feeding inhibition of the fomamidines were also studied.

MATERIALS AND METHODS

The effect of formamidines was analyzed on OA, TA, DA, 5HT and GABA receptors, investigating the binding properties of the selective ^{3}H-labelled ligands to the appropriate receptors. The ligands were the following: ^{3}H-OA (32.4 Ci/mmol), ^{3}H-TA (40 Ci/mmol), ^{3}H-haloperidol (14 Ci/mmol), ^{3}H-5HT (28 Ci/mmol) and ^{3}H-flunitrazepam (90 Ci/mmol). For the binding studies, membrane from the brain of the locust, *Locusta migratoria migratorioides* R.F. were used. Two-three weeks old adult male and female animals were taken from colonies maintained in our laboratory. The membranes for the study of the OA, TA, DA and 5HT binding were prepared as described before [7]. For the flunitrazepam binding, the membranes were prepared by the method of Robinson et al. [19]. The membrane fraction corresponding to 20 mg wet tissue was incubated at 30 °C for 20 min in 2 ml 50 mM Tis buffer, pH 7.4, containing 2 nM of ^{3}H-labelled ligands and test substances as indicated. The ^{3}H-flunitrazepam binding was measured at 4 °C for 30 min and the incubation mixture contained additionally 5 mM $CaCl_2$. The incubation was started by adding the tissue to the incubation medium and terminated by rapid filtration under vacuum through Whatman GF/C filter with three 5 ml rinses of ice-cold buffer. Radioactivity on the filter was counted after overnight extraction with a Triton X-100-toluene based scintillation solution. Specific binding was defined as the excess over blanks containing 10 μM of OA, TA, DA, 5HT and 100 μM of diazepam, respectively. Experiments were performed in triplicate and drug inhibitions of the ^{3}H-ligands were determined at six different concentrations of each drug. The IC_{50} values were estimated by a computer program [13] and the Ki values were calculated by the equation of

$$K_i = \frac{IC_{50}}{1+[S]/K_d}.$$

To estimate the LD_{50} values, drugs were dissolved in physiological solution and a volume of 100 µl were injected into the abdomen of locusts. The control animals received 100 µl of physiological solution. The mortality was counted 48 h after the injection. Ten animals were injected with a single dose and 5 different doses were used to estimate the mortality. The LD_{50} values were estimated by the computer program [13].

Investigating the feeding inhibition, a sublethal dose of the drugs, the half amount of the LD_{50} values, was injected into the abdomen of the animals. The injected volume was 100 µl and the control animals received the same volume of the vehicle. After the injections, the animals were fed by fresh wheat seedling. The consumed amount of wheat seedling was measured after 6 h and its amount was given in percent of the control.

The GraFit computer program [13] was used to analyze the pharmacological data.

The formamidine derivatives were synthetized as described earlier [8].

RESULTS AND DISCUSSION

The *in vivo* and *in vitro* effects of CDM and DCDM suggested that not the CDM but its demethylated derivative (DCDM) is the actual toxicant *in vivo* [11, 12]. We have found that the affinity of DCDM to the OA receptor is much more higher than that of CDM, and even more higher than that of OA itself (Fig. 1). This is in a good agreement with other results, showing that DCDM is a potent agonist of the OA receptor and is more effective compound than CDM [3, 4, 5, 7, 10, 15, 17]. The further demethylation, didemethylchroldimeform (DDCDM), however, does not result a more active compound. The affinity of DDCDM is lower than that of DCDM (Fig. 1) but higher than that of OA. The activity of formamidines on ion chanels and on other transmitter systems has also been demonstrated [10, 14, 20, 21], and it was suggested that these effects contribute to the insecticide effect. Ozoe and Matsumura [18] suggested that the lethal effect of the chlordimeforms could be related to an interaction with central diazepam binding sites. They have found that chlordimeform at a concentration of 100 µM inhibited the diazepam binding by 55% [18]. We have also found that the DCDM inhibited the flunitrazepam binding to the GABA receptor, however, a 10,000 times higher concentration was needed to the 50% inhibition of GABA (100 µM) than for that of the OA (10 nM) receptor. Our results suggest that DCDM is specific to OA receptor, having a high affinity to OA receptor, a moderate affinity to TA receptor, and a low affinity to DA, 5HT and to GABA receptors (Fig. 2). The formamidines had a toxic effect when injected into the locust (Fig. 3). Analysing the toxic effect of these compounds, we have found a close correlation between the lethal dose (LD_{50}) and the affinity of the compounds (K_i) to the OA receptor. Those compounds which possessed high affinity to the OA receptor *in vitro,* revealed high toxicity *in vivo* (Fig. 3). These results are in good agreement with the findings showing a correlation between the lethal activity of the formamidines and the stimulation of the adenylate cyclase activity [10]. An antifeeding effect of for-

mamidines were described by Hiriata and Sogawa [6] which led to death through starvation. In locust, the sublethal dose of formamidines inhibited also the feeding. There is also a good correlation between the feeding inhibition of the compounds and their affinity to the OA receptor (Fig. 4). These results clearly suggest that in locust the target site of the formamidines are the OA receptor rather than the GABA receptor.

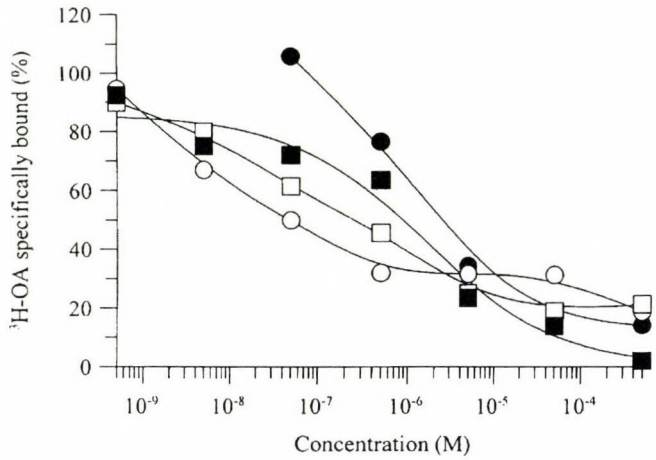


Fig. 1. The effect of CDM (●), demethyl-CDM (○), didemethyl-CDM (□) and octopamine (■) on ^{3}H-OA binding

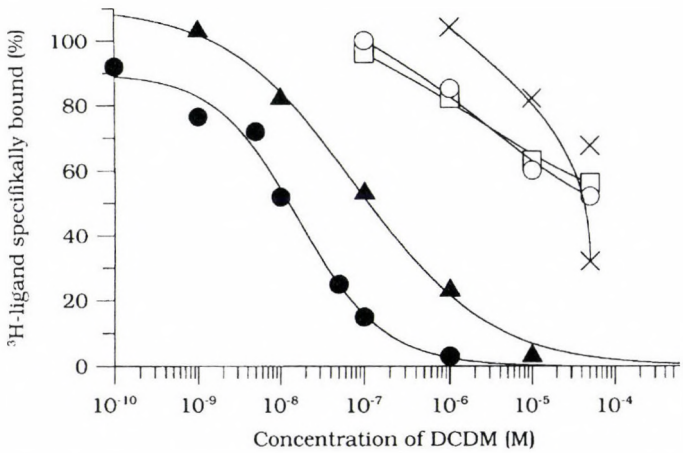


Fig. 2. The inhibition of DCDM on the binding of ^{3}H-OA (●), ^{3}H-TA (■), ^{3}H-Haloperidol (□), ^{3}H-5HT (○) ^{3}H-Flunitrazepam (×)

The alteration of the affinity to the OA receptor as well as the toxicity of the compounds have been investigated after ring substitution of the molecule (Table 1). The substitution with methyl or Cl groups resulted 10 times (2-methyl-DCDM, 4-Cl-DCDM) and 100 times (2-Cl-DCDM), lower affinity compounds than the parent compound, respectively. The disubstitution at 2,4 positions did not influence (2,4-

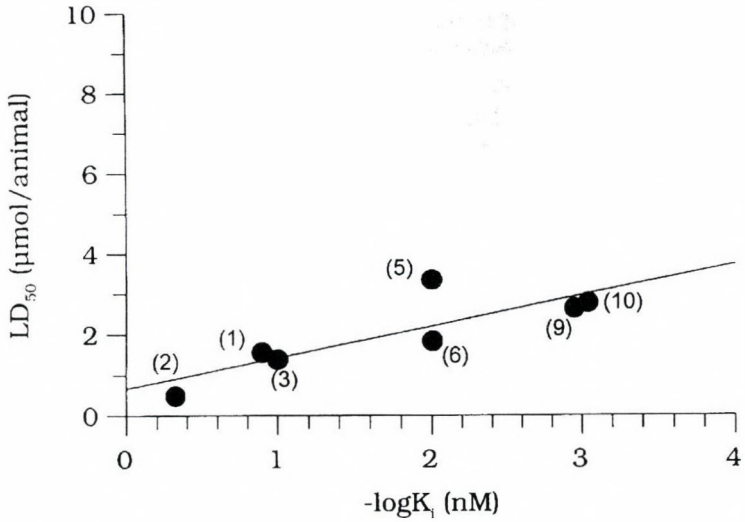


Fig. 3. Correlation of LD_{50} values and the affinity ($-\log K_i$) of formamidine analogues (numbers 1–10, Table 1). (r = 0.82)

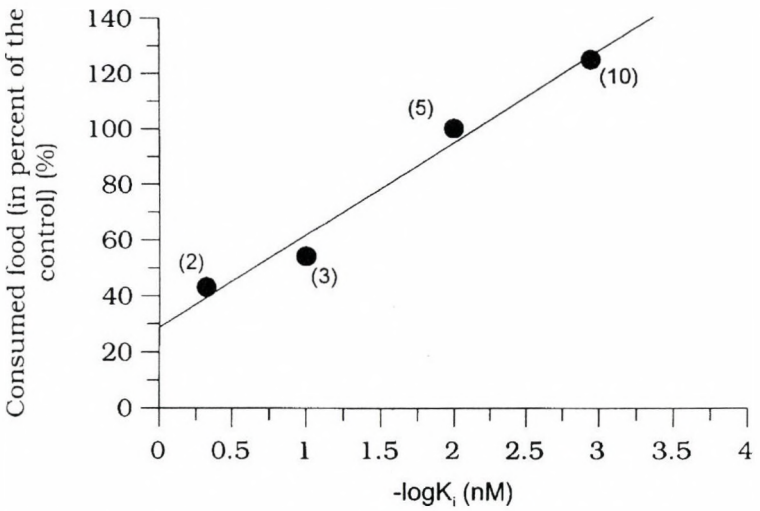


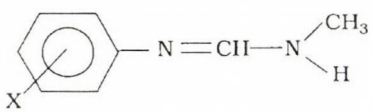
Fig. 4. Correlation of affinity ($-\log K_i$) of formamidine derivatives (numbers 2, 3, 5, 10, Table 1) with the consumed food by the animals injected with sublethal dose of drugs (r = 0.99)

diCl-DCDM, 2-methyl-4-Br-DCDM) or increase (2,4-dimethyl-DCDM) the affinity of the new compound. The disubstitution at 2,5 or 3,4 positions (2-methyl-5-Cl-DCDM, 3,4-di-Cl-DCDM) resulted a 10 times decrease in the affinity. The trisubstitution (2,4,6-tri-Cl-DCDM) resulted a 100 times decrease in the affinity (Table 1). Similar results were found for the adenylate cyclase stimulation [10]. Both the literature data [10] and our present results suggest that the most effective ring substitution pattern is 2,4-disubstitution with a combination of methyl groups or halogens. In each case, the 2,4-dimethyl is the best, followed by 2,4-dichloro and 2-methyl,4-halogen combination. Monosubstitution or 3,4- or 2,5-disubstitution as well as the trisubstitution gave less effective derivatives.

The present results support the hypothesis that the lethal effects of formamidines in locust are mediated through the OA receptor.

Table 1

Structure-activity relations for *in vitro* (K_i) and *in vivo* (LD_{50}) activity of substituted phenyl formamidines



X =	Ki (nM)	Ld50 (μmol/animal)
1. 2-methyl-4-Cl	7.9	1.57
2. 2,4-dimethyl	2.1	0.5
3. 2,4-diCl	10	1.4
4. 2-methyl-4-Br	13	3.07
5. 2-methyl	100	3.35
6. 4-Cl	100	1.84
7. 2-methyl-5-Cl	110	–
8. 3,4-diCl	200	–
9. 2,4,6-triCl	890	2.63
10. 2-Cl	1100	2.77

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DEVELOPMENT OF A MONITORING NETWORK FOR THE ANALYSIS OF ELEMENTS IN AEROSOL SAMPLES COLLECTED AT LAKE BALATON*

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Determination of different toxic elements in aerosol and precipitation samples collected at Lake Balaton were carried out. A simple sequential leaching procedure was applied for the determination of the distribution of elements. The distribution of elements was determined among environmentally mobile, bound to carbonates and oxides, and bound to silicates and organic matters (environmentally immobile) fractions. Particular attention was paid to distinguish between environmentally mobile and environmentally immobile fractions because these represent the two extreme modes by which the metals are bound to the solid matrices. Aerosol samples were weekly collected in Tihany, Siófok and Keszthely on 5 cm diameter Teflon filters with a membrane pump. While Cd-compounds have been found enormously in the environmentally mobile fractions, As-compounds accumulated almost evenly among portions. The results of sequential leaching give an indication of the mobility of the elements once the aerosol is mixed directly into natural waters on during scavenging of the aerosol by wet deposition. Based upon the data it can be concluded that the effect of anthropogenic sources is minor in this area.

Keywords: Monitoring – chemical speciation – aerosols – wet and dry deposition

INTRODUCTION

In environmental sciences the development of a monitoring system is of importance. Monitoring is done in order to gain information on the present level of harmful or potentially harmful pollutants in discharges to the environment, within the environment itself, or in living creatures, that may be effected by these pollutants. Objectives of monitoring are manifold [1]. Along with the assessment of pollution effects on man and his environment; it is necessary to obtain a historical record of environmental quality and provide a data base for future use. For a reliable monitoring program the sampling of analytes from the environment is fundamental [2]. In the environmental sampling the act of sample removal from its natural environment can dis-

*Dedicated to Professor János Salánki for his 70th birthday.

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turb stable or metastable equilibrium and, after the measurement, we may characterise the entire population.

Speciation of trace metals in the atmosphere is somewhat different from those applied to speciation in aqueous media. This comes mainly from two considerations: the mechanism of interaction of the biosphere and the atmosphere, and the mechanism of transport in the atmosphere. Metals are transported in the atmosphere primarily on aerosols which can be removed by wet and dry deposition process. The deposition, transport and inhalation processes are controlled predominantly by the size of the atmospheric aerosols. Thus, the primary type of speciation is the fractionation by size. Once deposited however, chemical speciation both in terms of the dissolved/particulate distribution of the metals in precipitation and the inorganic or organic complexes that the metal may form, play an important role in controlling the environmental impact of atmospherically deposited metals. Wet deposition provides a mechanism by which the metals in aerosol particles can be solibilised and the pH of rainwater is a major control on metal solubility in precipitation [3, 4]. Before aerosol particles are removed in precipitation they are cycled within the atmosphere through clouds and subjected to repeated wetting and drying cycles. The final pH of precipitation (which usually ranges between 4 and 6) may not reflect the pH conditions to which the aerosol is subjected in the atmosphere.

Lake Balaton is situated in the western part of Hungary. Its surface area is 596 km^2, the mean depth is 3.25 m and the volume is about $1.9*10^9$ m^3, so the lake is a shallow lake having a large surface area.

The aim of the work was to apply a 3-stage sequential leaching procedure for the determination of the distribution of elements in atmospheric aerosols collected on filters. The distribution of metals was determined among environmentally mobile *(1),* bound to carbonates and oxides *(2),* and bound to silicates and organic matters (environmentally immobile) *(3).* The classification of metals in aerosol matrix according to particle size and chemical bonding was carried out as it has been defined as a fractionation.

MATERIAL AND METHODS

Sampling

Aerosol samples were collected in Tihany, Siófok and Keszthely on 5 cm diameter Teflon filters (pore size 0.45 μm) with a membrane pump of 1.2–1.5 m^3/h air. In the two day periods 55–72 m^3 air was sampled and collected by weekly. The moderately polluted Tihany is situated in the peninsula of the Lake Balaton. The average elevation of the city is 290 m and the sampling was carried out on the top of a small hill, 35 m above the street level. Keszthely is located at the west part of the lake, it is a small city with no industrial activities. Siófok is the capital of the south part of the Lake Balaton, the most frequently used resort area having unusual high number of tourists in summer.

In the same survey period precipitation samples were also collected in Tihany by wet-only sampling device. The collector consisted of a funnel with a diameter of 23 cm and a beaker of 2 L, both made from polyethylene. The precipitation was transferred to a polyethylene bottle, acidified by nitric acid to pH = 2, and kept in refrigerator. The sampling apparatus was placed close to the aerosol sampler.

Aerosol leaching

A 3-stage sequential leaching procedure was applied for the fractionation of metals among 3 fractions. Environmentally mobile fraction, bound to carbonate/oxide fraction, as well as bound to organic matter/silicate fraction were separated The leaching experiments were carried out as follows [5]:

Stage 1. **Environmentally mobile fraction:** filters were folded and placed in centrifuge tubes. Extraction was carried out at room temperature for 15 min with 25 mL of 1 mol/L NH_4OAc at pH 7 then the suspensions were centrifuged for 15 min at 3000 rpm. The supernatant phase was pipetted out and stored for analysis.

Stage 2. **Bound to carbonate and oxide fraction:** residues from stage 1 were extracted at room temperature for 6 h with 25 mL of 1 mol/L hydroxyl-amine hydrochloride +25 v/v% acetic acid and, after leaching, suspensions were centrifuged for 15 min at 3000 rpm. The supernatant liquid was removed and stored for analysis.

Stage 3. **Bound to silicate and organic fraction:** residue was transferred to a PTFE beaker and 10 mL of cc. HNO_3 was added to each sample. The beakers were placed on a water-bath at a temperature of 95 °C and after decomposition of the filter residues 2 mL of cc. HF was added. The beakers were placed on the water-bath and left until all the particles had dissolved. Four mL cc. of HNO_3 were repeatedly added for the evaporation of the HF, and the cold solutions were transferred to a 50 mL volumetric flask and made up to volume with 0.1 mol/L HNO_3 and stored for analysis (4 °C). Before the analysis the PTFE beakers were treated by 10 v/v% HNO_3 for 48 h and rinsed by double distilled, deionized water.

Solutions were stored at 4 °C prior to analysis. Analytical blanks were performed on filters from the same batch, on centrifuge tubes and on PTFE beakers. The 3-stage sequential technique was run on unexposed filters in the same way as the exposed samples. The blank obtained is a reagent, + beaker + filter blank and the values have been subtracted from the atomic absorption measurements. Amount of a single sample was found to be small, so it was not possible to perform parallel measurements. Nor was it conceivable to directly verify the accuracy of the analysis since appropriate filter-collected aerosol standards have not been available yet. Before extraction, all glassware and plastic vessels were treated in a solution of 10 v/v% HNO_3 for 24 h and washed with double-distilled water.

Atomic Absorption Spectrometry measurements

Elemental concentrations of the fractions were determined by atomic absorption spectrometry (AAS) in an electrothermal atomization mode using Perkin-Elmer 5100 PC, HGA 600 graphite furnace (GEM software) and Perkin-Elmer 303, HGA 70 graphite furnace instruments. Standard solutions were prepared from 1 g/L of each metal (Merck standard solutions) and freshly diluted before use. Cu, Fe, Mn, Zn were determined by flame AAS in acetylene/air flame. The concentration of an element was measured with an RSD of <5% from solutions.

RESULTS AND DISCUSSION

The chemical speciation data of elements in aerosols are presented as a geometric mean concentration being calculated by the sum of the concentrations determined in each fraction of an individual element found in 92 measurements at Tihany and 23 at Keszthely and Siófok. From the average concentration of elements dry deposition budget (mg/m2*y) was calculated. The average geometric concentration of elements in aerosol samples collected at the three stations, and the fractionation by chemical bonding of elements is summarized in Table 1. In a two year sampling period it has been observed that there were not serious pollution from the dry deposition on the Lake Balaton. Since practically there is no industrial activity in the close neighborhood of the lake and the catchment area, and samples are collected on the beach, the toxic metals are transported from foreign sources with a long range transport.

Table 1
Elemental content of aerosol samples collected at the Lake Balaton (ng/m^3), and distribution of elements among fractions (%). (T = Tihany, K = Keszthely, S = Siófok)

Element	Tihany (T)	Keszthely (K)	Siófok (S)	Mobile, T-K-S (%)	Carb.+oxide T-K-S (%)	Org.+silicate T-K-S (%)
Fe	82.2	95.3	150	19-16-30	41-43-28	40-41-42
Mn	53	35	74	6-13-30	4-3-28	90-84-42
Al	51.4	38.2	47.3	22-28-30	46-46-28	32-26-42
As	6.8	5.3	7.0	26-29-30	19-50-28	55-21-42
Cd	0.67	0.55	0.74	51-39-44	29-38-29	20-23-37
Cr	1.11	1.7	0.78	18-12-26	28-12-31	54-76-43
Cu	4.9	3.8	4.4	33-34-33	46-48-28	21-18-39
Ni	5.1	3.3	3.0	37-50-17	40-26-44	23-24-39
Pb	29.5	29.8	26.4	46-52-52	22-29-23	32-19-25
Zn	37.0	8.7	14	26-53-25	43-16-25	31-31-43

In natural systems manganese has two dominant oxidation states, Mn(IV) that is insoluble in water, and Mn(II) that is water soluble. A knowledge of oxidation state is important since it can govern either the biological availability or the toxicity of an element once it enters the natural waters. The amount of manganese compounds in aerosols was found much less compared to a recent survey in Hungary (35–74 ng/m^3) [6]. The variance among the samples collected at different sites is either due to the resuspension of the dust from the street by traffic and from the upper soil layer or to the effects of combustion. Manganese ions are highly concentrated in the stable fractions (bound to organic matter/silicates) and do not have any direct impact on the environment. Recent chemical speciation studies showed that aerosol samples collected at the north part of the Lake Balaton contained 90% of Mn in the bound to organic matter/silicate fractions [6]. The data show that the Al and Fe can mostly be found in the bound to carbonate-metal oxide fractions and bound to organic matter-silicate fractions. These findings agree well with our earlier results of the investigation at Veszprém and Kabhegy [6].

Based upon the fraction by particle size Pb showed an unimodal distribution at a maximum of <1 μm indicating unambiguously anthropogenic sources [7]. The pollution around the lake is in average 28 ng/m^3, and it is at least one or two magnitudes less due to smaller traffic and long-range transportation. Emissions from vehicle exhausts have dominated to the lead contribution to the atmosphere, although smelting operations also contribute to this atmospheric lead load, emitting both Pb^0 and PbO [8]. Since there is no smelting activities close to this area, the pollution is mostly originated from the traffic. The distribution of Pb-compounds among the three-fractions is rather even, the environmentally mobile fraction is about 46–52%. The relative significance of lead sources in the atmosphere is currently changing as a result of the decline in the use of leaded vehicle fuels. The actual lead compounds in a particular aerosol will depend on the other constituents in the atmosphere and the age of the aerosol. The predominant inorganic aerosol-phase lead species was identified as $PbSO_4*(NH_4)_2SO_4$ by XRD [9, 10]. Weathering of this compound resulted in the production of $PbSO_4$, the most frequently observed crystalline form of lead in street dust [11].

The average geometric mean concentration of Cd was found as <0.75 ng/m^3 and an unimodal distribution pattern was obtained [7]. The concentration of Cd in aerosols depends considerably on the location, pollution sources, time, meteorological conditions, etc. and ranges among 1–300 ng/m^3 in major cities [12]. The fractionation of cadmium resulted in the association with the environmentally mobile fractions (51% at south part), and less amount of Cd-compounds was found at the other two locations, 29 and 20%, respectively. Lum et al. [13] reported that Cd in urban aerosol was found to be almost completely in exchangeable form. Recently high enrichment factor (EF) values indicated that Cd was emitted from anthropogenic sources (EF = 4665 for Veszprém, and EF = 2404 for Kabhegy) [6]. Cadmium, liberated during combustion processes, has been shown to occur in elemental and oxide forms, whereas emissions from refuse incineration were dominant-

ly as $CdCl_2$. The cadmium species in aerosols are probably Cd, CdS, CdO, $Cd(OH)_2$ and mixed oxides with Cu and Zn [14, 15].

Enrichment factor values of Cr and Cu indicated that these elements were also emitted by anthropogenic sources, however, the factors were somewhat lower than they has been calculated to Budapest and the regional background monitoring station (K-puszta) [16, 17]. Third of the total Cu was found in the environmentally mobile fraction and half in the bound to carbonate/oxide fraction. Samples collected at Siófok behaved differently, 39% bound to organic matter/silicate fraction, indicating that Cu-compounds were mainly located in stable form. At least one magnitude less Ni was identified in aerosol samples collected around the lake compared to other domestic stations (Budapest, Veszprém, Kabhegy) (16). Lum et al. [13] identified high proportions of Ni and Cu in soluble and/or exchangeable forms and of Cr in bound to silicate fraction in urban particulate matter analysed by sequential extraction procedure. Distribution of Ni-compounds depended upon the location sampled and 50% of the total amount on Ni was found in mobile fraction at the west part of the lake.

Somewhat higher concentrations of As (6.3 ng/m^3) were found in the aerosol samples collected around the lake compared to the other monitoring stations in Hungary (1.9–2.4 ng/m^3) [17]. Arsenic has been shown to occur as both As(III) and As(V) in natural samples. Recently, it was found that As-compounds were connected to coarse particles having a maximum value of 2.8 μm [7].

For evaluation of the atmospheric budget for the lake and the environmental effects of trace metals on biosphere the calculation of the dry and wet depositions is of vast importance. The dry deposition rates were calculated by means of the dry deposition velocities and the amount of annually deposited elements was estimated for the 600 km^2 area. Dry deposition velocities were calculated by Molnár et al. [18] using the size distributions of metals measured with a cascade impactor at Veszprém. Results are summarized in Table 2.

The dry deposition rates obtained for the three sampling sites show a variable picture. It can be seen that along with the main elements (Fe, Mn, Al), less amount of toxic metals has been deposited into the lake. The yearly 12 kg of Cd and 18 kg of Cr are much less than the lake receives from the rivers and creeks coming from the water catchment area. Lead, Zn and Cu were deposited into the lake between 618 and 900 kg/year, respectively and the solution of compounds did not affect the water quality greatly.

Atmospheric removal occurs by dry deposition of aerosol particles to water, soil, buildings or plants, or by wet deposition of aerosol particles and gases in rain, fog, hail and snow. Wet deposition is a very important removal process for those elements associated with small particles and is predominantly anthropogenic in origin. Approximately 80% of the atmospheric removal of elements, like Pb, Cd, Cu, Ni and Zn, to the ocean has been taken place by wet deposition, while 40% of that process for iron and aluminum is related to the dry deposition. This is mostly size-dependent, so the size fractionation is an important control on removal processes.

Table 2
Dry deposition rates (D_d, mg/m^2*y), total dry deposition (kg/y) for samples collected at Lake Balaton

Element	Tihany	Siófok	Keszthely	Mean	D_d (kg/y)
Fe	24.0	43.8	27.8	32.9	19 740
Mn	11.3	15.9	7.5	11.6	6 960
Al	15.0	13.8	11.1	13.3	7 980
As	0.24	0.25	0.19	0.023	138
Cd	0.02	0.02	0.02	0.02	12
Cr	0.02	0.02	0.04	0.03	18
Cu	0.14	0.21	0.11	0.15	900
Ni	0.35	0.21	0.23	0.26	156
Pb	1.06	0.95	1.07	1.03	618
Zn	2.44	0.41	0.25	1.03	618

As it was pointed out earlier, the deposition of Mn is extremely high at Kabhegy (31.7 mg/m^2*y) or at Veszprém (49.8 mg/m^2*y), compared to other part of Hungary, due to the close mining activities [18]. However, much less Mn compound was found in aerosol samples collected around the lake (11.6 mg/m^2*y), which indicated that there were no anthropogenic pollution sources nearby. Deposition of Pb, Cd, Cu, and Cr does not significantly differ from the other values on samples collected at different locations based on a different approach [19, 20]. Wet deposition rates are based upon the concentration of trace metals in precipitation samples collected by a wet-only sampler in Tihany. Weighed mean concentrations were calculated by taking into account the volume of precipitation collected at each case, and the annual precipitation amounts. Results are shown in Table 3.

Wet deposition data are usually lower than they have been published for Hungary and other areas like marine, rural and urban sites [21, 22]. Number of factors influences the level of deposition in any area, such as the locality i.e. remote, rural, urban or industrial. Wet precipitation depends on the existence of rain, the volume, its duration and intensity. In 1996, there was 735 mm rain and this proved to be much higher than it was experienced in 1995 and 1997. Except Ni, other elements were deposited in sometimes one magnitude less amount (Fe, Zn) than that in other two years. So, the dilution effect was observed and only minor pollution was deposited into the lake.

The comparison of wet and dry depositions shows a very exciting picture (Table 4). It has been published recently [19] that the D_{wet}/D_{dry} is significant in particular for Pd and Zn and usually higher for the others (V, Cr, Ni, Cu, As). In our case it has been found that for elements like Fe, Al, and Cu, the ratio of the two depositions gives an opposite appearance; namely, the dry deposition plays an important role in the pollution of the environment. On the other hand, the D_{dry}/D_{wet} ratios

entirely indicated that elements like Mn, As, Cd, Pb, and mainly Zn, were deposited in much higher amount by wet deposition than dry one. The environmentally mobile fractions of Cd and Pb are higher than the other fractions indicating a more soluble part in rain water. Wet deposition provides a mechanism by which the metals in aerosol particles can be solubilised.

Table 3
Wet deposition rate from samples collected at Tihany, Lake Balaton (D_w, mg/m^2*y)
June, 1995–December 1997, 92 samples

Element	1995	1996	1997	Mean	D_w
	435 mm	735 mm	490 mm		kg/y
Fe	60.69	5.81	21.68	29.39	13 482
Mn	2.73	0.91	24.72	9.45	17 634
Al	18.48	15.61	33.31	22.47	5 670
As	0.20	0.11	2.61	0.97	582
Cd	0.015	0.016	0.11	0.09	54
Cr	<0.7	<0.7	<0.7	<0.7	–
Cu	1.11	0.21	0.35	0.56	336
Ni	0.18	0.69	1.70	0.86	516
Pb	1.80	1.19	3.87	2.29	1 374
Zn	7.41	0.72	19.9	9.34	5 604

Table 4
Dry (D_d, kg/y) and wet (D_w, kg/y) deposition and dry and wet deposition ratio (D_d/D_w, kg/y) of elements of samples collected at Lake Balaton

Element	D_d	D_w	$D_d + D_w$	D_d/D_w
Fe	19 740	13 482	33 222	1.46
Mn	6 960	17 634	24 594	0.39
Al	7 980	5 670	13 650	1.4
As	138	582	720	0.23
Cd	12	54	66	0.22
Cr	18	–	–	–
Cu	900	336	1 236	2.68
Ni	156	516	672	0.30
Pb	618	1 374	1 992	0.45
Zn	618	5 604	6 222	0.11

CONCLUSION

The sequential leaching technique has been successfully applied to filter-collected aerosols for determination of the distribution patterns of elements in three fractions. Particular attention was paid to distinguish between the environmentally mobile and environmentally immobile portions because these represent the two extreme modes by which the metals are bound to the aerosols. The fine fractions are characteristic to the anthropogenic pollution sources, and if the samples are collected continuously at one particular site, the data make a possibility to assess the dynamic processes taken place in the atmosphere. While Cd-compounds have been found largely in the environmentally mobile fractions, As-compounds accumulated almost evenly among portions. The environmentally mobile elements being most susceptible to release into aqueous solution after deposition of the aerosols to surface of lakes. The results of sequential leaching give an indication of the mobility of the elements once the aerosol is mixed directly into natural waters on during scavenging of the aerosol by wet deposition. Aerosols sampled within the urban environment usually exhibit a greater solubility than aerosols with a crustal origin and this is important to interpret the results of sequential leaching. The aim of collecting precipitate and aerosol samples in the same site at the same period was to determine the distribution of elements in two depositions. Nevertheless, calculations are affected by the dry deposition velocities and some emission sources along with the air trajectory. Further investigations are necessary to confirm these data; precipitate has to be collected at the other two sampling sites.

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INFLUENCE OF $HgCl_2$ ON THE OSPHRADIAL MULTISENSORY SYSTEM OF *LYMNAEA STAGNALIS* L.*

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The osphradial multisensory system of *Lymnaea stagnalis* L. (Pulmonata, Basommatophora) was used to demonstrate the modulation of chemosensory information both at periphery and central nervous system (CNS) following heavy metal treatments.

A semi-intact preparation including osphradium, CNS and the right inner parietal nerve (r.i.p.n.) connecting them was used to record both extracellular activity of nerve and intracellular activity of central neurons receiving information from osphradium. The ion currents of osphradium were recorded using patch-clamp method. The changes in nerve and neuronal activity were expressed by averaging of firing frequency and interspike intervals. The chemosensory function of osphradium was shown by application of L-aspartate, urea, saccharose and stagnant water to its surface. The central neurons reacting to the stimulation of osphradium were located to visceral, right parietal, pedal and cerebral ganglia of *Lymnaea.*

Both the acute and chronic treatments with $HgCl_2$ damaged the sensory function of osphradium traced on the flow of information from periphery to central neurons. At the same time, mercury chloride modified the synaptic connections of respiratory pattern generators as well as the Ca- and K-dependent ion currents of osphradial neurons.

The results proved the multisensory role of osphradium sensing the alterations in the environment and its usefulness in monitoring the effects of pollutants at various level of regulation from chemosensory epithelium to CNS.

Keywords: CNS – osphradium – spike frequency analysis – ion currents – $HgCl_2$

INTRODUCTION

The osphradial sensory system of Gastropods has been described in the species inhabited in the aquatic ecosystems. The osphradium is located in the mantle cavity along the path of the respiratory currents and adapted to analyse the physico-chemical properties of the environment. On the other hand, the inhalant respiratory current is the source of various odours exciting directly the osphradial neurons.

*Dedicated to Professor János Salánki for his 70th birthday.

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In *Lymnaea* the osphradium was described as an Y-shaped epithelial canal surrounded with nerve cells [1, 11]. The osphradial ganglion is covered by connective tissue sheet and connected to CNS via the osphradial nerve which is a branch of the right parietal nerve [17]. The osphradial ganglion includes both sensory and ganglionic cells [7, 16, 17].

The osphradium was shown to respond to hypoxia, hypercapnia, mixture of amino acids, different classes of odorants and osmotic pressure [7, 17, 20, 27]. The osphradium as a multisensory organ can be involved into the regulation of a wide variety of behaviours, including respiration, feeding, escape from predators, homing, egg-laying, etc.

Although the osphradium is a well-known chemosensory organ sensing the environmental changes and transferring information to CNS leading to modulation of various forms of behaviour it has not been used for monitoring of the environmental pollutants. For this reason the aim of the present investigation was to characterise the osphradial system of *Lymnaea stagnalis* L. and its modulation under the influence of $HgCl_2$ both at the nerve cells of this peripheral sensory organ and in the CNS.

MATERIAL AND METHODS

The experiments were performed on the adult specimens of the pond snail *Lymnaea stagnalis* L. (Pulmonata, Basommatophora) collected from their natural habitat (Balaton-Minor) during the months May–October, and kept in aquarium at temperature 22 ± 5 °C. The animals were fed with fresh lettuce.

The semi-intact preparation including the central nervous system (CNS) and the osphradium with the inner right parietal nerve (r.i.p.n.) connecting them was isolated from the animals that had been anaesthetised by iced water. In the experiments the semi-intact preparation was fixed to the bottom of the Sylgard-lined dish. The connective tissue of the ganglia was removed by mechanical and enzymatic treatments. The experimental chamber was permanently perfused with snail physiological saline or with the test solutions applied directly to the surface of the osphradial canal. For stimulation of the osphradium urea, L-aspartate, L-glutamate, saccharose, NaCl and the stagnant water were used.

In acute experiments following the first response of the osphradium to the chemical stimuli the osphradium was treated for 10–60 min with $HgCl_2$ (2–20 μM) then the chemical stimuli were applied repeatedly.

In chronic experiments animals were treated with $HgCl_2$ (5–10 μg/l) for 3–20 days and the response of the central neurons was compared to that of the untreated snails.

The recording of nerve and neuron activities

The extracellular activity of the internal right parietal nerve (i.r.p.n.) was recorded using glass suction electrode with a tip diameter of 25–35 μM allowing to record 25% of afferent and efferent impulses running on the intact nerve. The nerve activi-

ty was amplified and separated by amplitude discrimination in two channels corresponding to high (>0.5 mV) and low (<0.5 mV) amplitude potentials. The activity of the identified central neurons was recorded using conventional microelectrophysiological methods, sampled by A/D converter and recorded simultaneously with nerve activity.

The computational method has been developed for statistical analysis of nerve and neuron activity patterns based on the voltage discrimination. Statistical analysis of the data was carried out by computer program "Statistica" for comparison of average spike frequency, spike density and interspike intervals in control and following heavy metal treatments. The same program was used for cluster analysis. T-test was employed and the significance was $p < 0.05$.

Patch-clamp recordings

Patch-clamp experiments were carried out in the whole cell configuration in current-clamp condition [10]. Both sensory and ganglionic cells of osphradium were used in these studies. Electrodes of 5–7 MOhm resistance were made from borosilicate glass (1.3 mm, World Precision Instruments Inc.) using two-stage electrode puller Model 730 Kopf. The patch-clamp arrangement consisted of a patch-clamp amplifier (Axopatch 200 B), the piezo-drive patch-clamp micromanipulator Burleigh PCS-250, the digital storage oscilloscope Tektronix 2214 and a Gould-Brush recorder. Seal resistances were in the range of 3–5 GOhm. Whole-cell breakthrough was achieved by brief suction pulses and ZAP hyperpolarizing voltage (1.3 V) with controlled duration. Data were filtered at 2–3 kHz.

In voltage-clamp condition membrane currents were evoked by square-shaped voltage pulses, and a holding potential more negative than the average resting potential of –50 – –80 mV was usually applied. The command pulse programs were written by the author in "Pascal" language and ran on IBM computer which drive a D/A converter [25]. Leakage current correction was adapted before fitting the recorded current curves. When necessary, smoothing was performed in order to decrease the high-frequency noise component of the currents recorded.

Solutions

The experiments were carried out in conditions of continuous flow of physiological solution washing the osphradium and CNS of *Lymnaea.* Standard physiological solution (SPS) contained (in mM): NaCl 44, KCl 1.7, $CaCl_2$ 4, $MgCl_2$ 1.5, HEPES 10 (pH = 7.3 was adjusted with NaOH). For the patch-clamp experiments in current clamp conditions SPS was used as an extracellular solution, while the patch-pipettes were filled with a solution (in mM): KCl 20, KOH 43.5, aspartate 10, EGTA 5, HEPES 25.7 (pH = 7.3 supported with HEPES). For registration of Ca^{2+}-currents in conditions of voltage clamp the following solutions were used (in mM): extracellu-

lar – TEA 44, HEPES 10, $MgCl_2$ 1.5, $BaCl_2$ 4, TTX 0.001 (pH = 7.3 with TEA-OH); intracellular (in mM) – CsCl 44, $MgCl_2$ 1.5, HEPES 10, EGTA 5, Na-ATP 1 (pH = 7.3 with TEA-OH). For registration of K^+-currents in voltage clamp conditions an extracellular solution (in mM) – KCl 1.7, N-methyle-D-glukamine 87.5, HEPES 10, $CaCl_2$ 4, $MgCl_2$ 1.5, 4-aminopiridine 0.5, TTX 0.001 (pH = 7.3 with NaOH) and an intracellular solution (in mM): KCl 44, $MgCl_2$ 1.5, $CaCl_2$ 4, HEPES 10, EGTA 5 (pH = 7.3 was adjusted with NaOH) was used. Chemicals produced by Sigma and Fluka were used.

RESULTS

Responses of cardiorespiratory central neurons in Lymnaea to the stimulation of osphradium

The results proved that chemical stimulation of the osphradium caused characteristic changes in the resting activity of central neurons (RPeD1, VD4, IP3,A-cluster) and in the right inner parietal nerve (r.i.p.n.)

Analysis of compound spike activity of nerve fibres revealed various types of alterations during chemical stimulation of osphradium. The firing pattern of the nerve was sorted into two subgroups: one included the spikes with low or medium

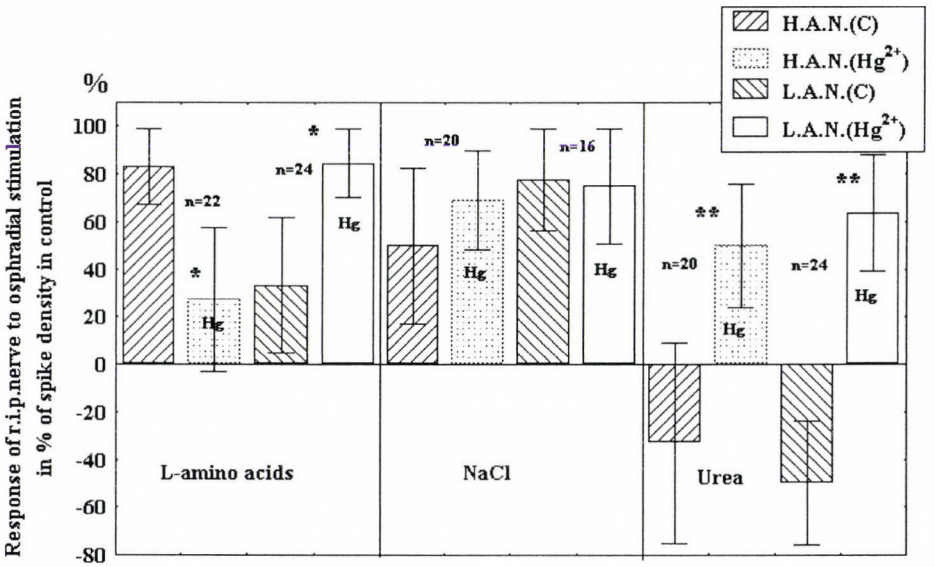

Fig. 1. Modified response of right inner parietal nerve (r.i.p.n.) to the stimulation of osphradium following chronic treatment of *Lymnaea* by mercury. H.A.N. – potentials with high amplitude in firing pattern of the nerve. L.A.N. – potentials with low amplitude in firing pattern of the nerve. C – control. Hg^{2+} indicates the treated samples

size amplitude (L.A.N.) originating from the sensory and ganglionic neurons of the osphradium, and the second one, with high amplitude component (H.A.N.) originated from unidentified sources. Both components showed significant alterations in response to the osphradium stimulation (Fig. 1). The amino acids and NaCl modified both L.A.N. and H.A.N. components of nerve activity. At the same time, urea decreases the firing activity of nerve (Fig. 1). This fact emphasised the specificity in perception of different chemical stimuli applied to the surface of osphradium.

The chronic exposure of the animals to $HgCl_2$ (5–10 μg/l) for 1–3 weeks caused either a decrease or an increase in the high and low amplitude components of the

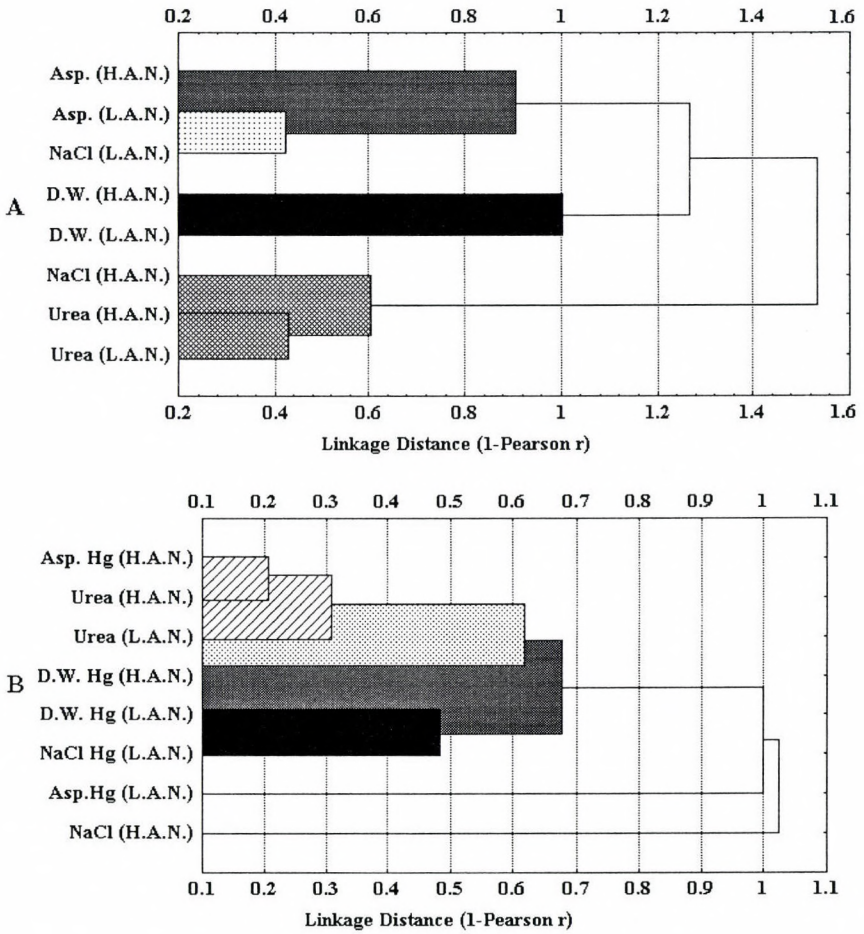

Fig. 2. Diagram of cluster analysis of two components of the right inner parietal nerve (r.i.p.n.) in control (A) and following chronic treatment by mercury (B). The chemical stimuli applied to the osphradium were: L-aspartate, NaCl, urea and the water used in molluscan culture (D.W.). H.A.N. and L.A.N. – the same as in Fig. 1

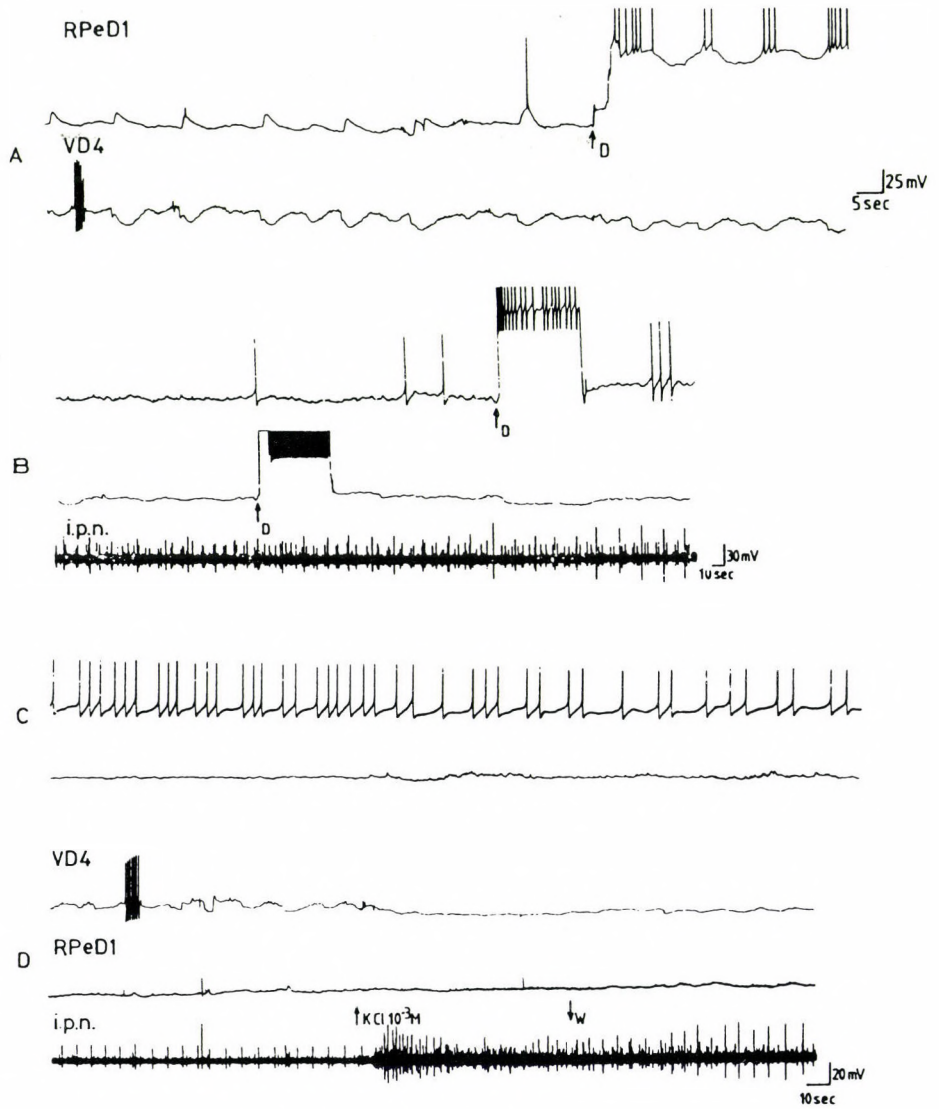

Fig. 3. Simultaneous recordings from the neurons RPeD1, VD4 and the right inner parietal nerve (r.i.p.n.). Spontaneous activity (C) and the effect of membrane depolarization (D) are seen (A, B). KCl (1 mM) applied to the osphradium caused an increase in nerve activity (D). W – wash out. IP3 – activation of the input 3 (IP3) interneuron

nerve activity depending on the chemical stimuli applied to the osphradium (Fig. 1). The cluster analysis of nerve activity revealed several clusters in the response to the chemical stimulation of osphradium which, however, were reduced to one cluster following chronical $HgCl_2$ treatment of the animal (Fig. 2).

The neurons reacting to the stimulation of osphradium were distributed to the visceral, right parietal, pedal and cerebral ganglia, including respiratory central pattern generator neurons, cardiorespiratory motoneurons and caudo-dorsal cells. Here the connection of osphradium with central neurons regulating respiration and other visceral functions is shown.

In Fig. 3 the simultaneously recorded intracellular activity of the cardiorespiratory cells is demonstrated. During slight hyperpolarization of the soma membrane of RPeD1 the axonal potentials can be registered, corresponding to the inhibitory postsynaptic potentials (IPSP) recorded from the VD4 cell with one to one correspondence (Fig. 3A). The depolarization of the cell RPeD1 to + 25 mV led to the generation of somatic AP and long-lasting hyperpolarization in neuron VD4 (Figs 3A and 3B). At the same time, depolarization of the cell VD4 to + 50 mV hyperpolarized the neuron RPeD1 (Fig. 3B).

Applying L-amino acids, urea or sucrose to the osphradium the main spike frequency of the neuron RPeD1 was decreased while NaCl caused a slight increase in its spike frequency as it was revealed using statistical analysis of firing frequency

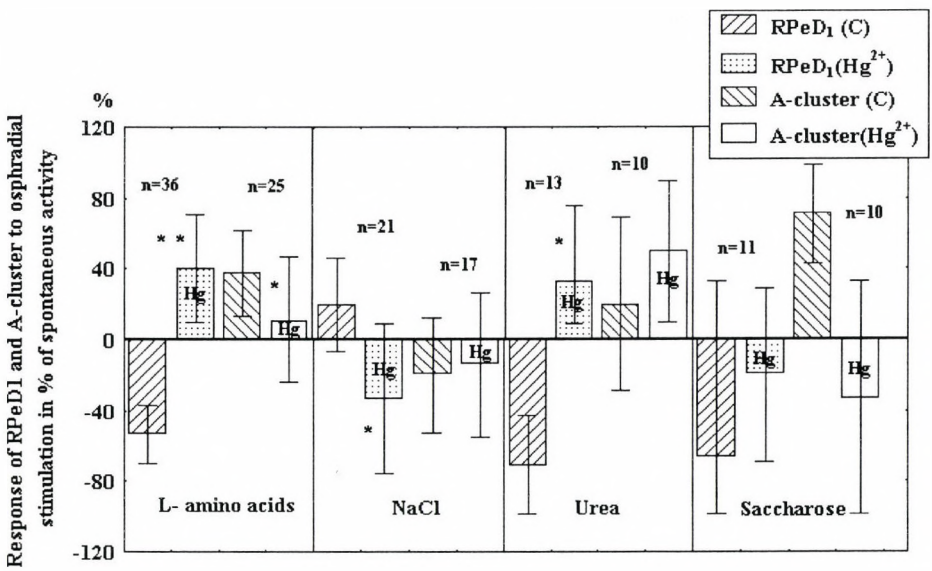

Fig. 4. Histograms demonstrating the effect of chronic exposure of the *Lymnaea* to mercury on the firing pattern of the neurons RPeD1 and A-cluster. The activity of the cells of A-cluster and RPeD1 evoked by the stimulation of osphradium before and following $HgCl_2$ (2 µg/l) treatment of the osphradium. Error bars indicate ± 1SE. Significance was $p < 0.05$ (*)

(Fig. 4). The stimulation of the osphradium modified also the synaptic connections between the neurons of respiratory circuit.

To reveal the synaptic interactions of central neurons and their alterations to the stimulation of the osphradium required more sophisticated methods. Applying L-aspartate to the osphradium caused a decrease in spike frequency of the RPeD1 if it discharged with simultaneous prolongation of the duration of silent periods between the bursts in RPaD1 evoked by VD4 (Fig. 5). Both types of reactions proved to be significantly ($p < 0.05$) different from the background mean values (Figs 6A and 6B). Thus it became evident, that the reciprocal interactions existing between the two interneurons (RPeD1 and VD4, respectively) depended from external synaptic inflow and, in particular, could be modulated by afferent impulsation originated from the osphradium. Interneuron(s) IP3 is/are known to play special role in generation of respiratory rhythm. IP3s excite RPeD1 and inhibit VD4. Some of the osphradial

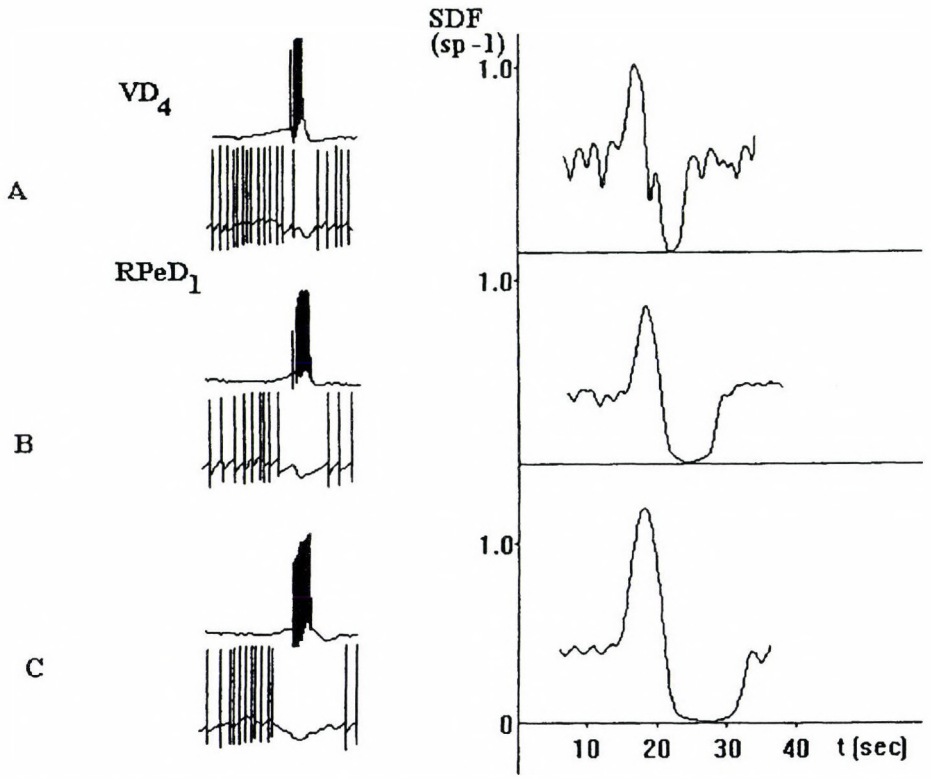

Fig. 5. Responses of the neurons VD4 and RPeD1 to the stimulation of the osphradium by L-aspartate (10^{-3} M) in control and following 15 min pretreatment of the osphradium by $HgCl_2$ (2 µg/l). A – control. B – stimulation of the osphradium by L-aspartate. C – response to the stimulation of the osphradium following treatment by $HgCl_2$

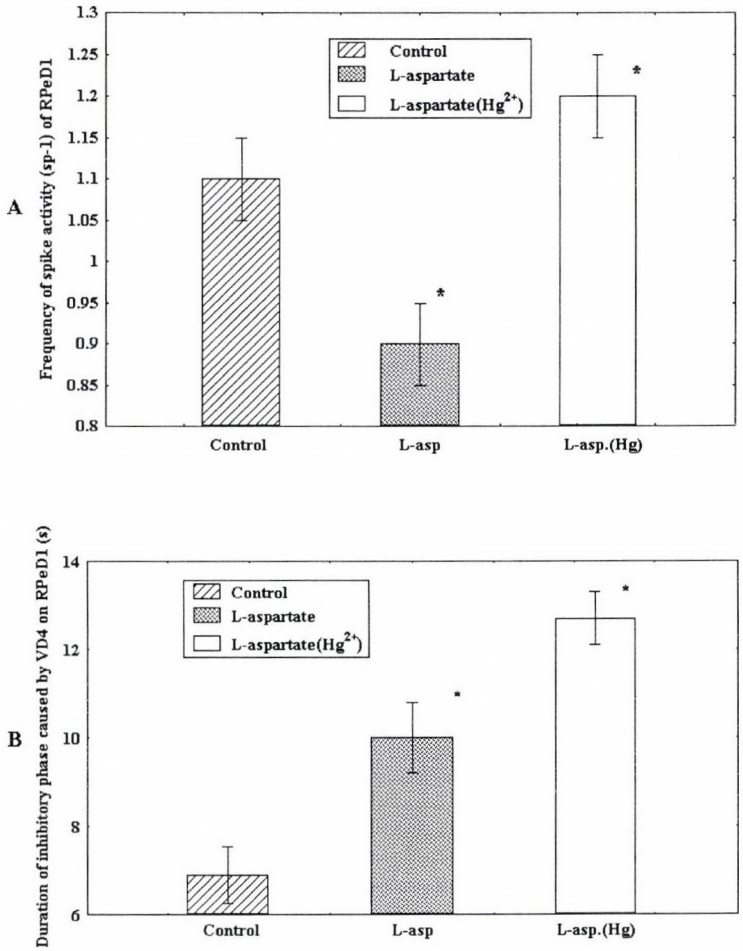

Fig. 6. Histograms demonstrating modulation of interaction between the interneurons RPeD1 and VD4 during stimulation of the osphradium in control and following treatment with $HgCl_2$. Variation in the number of spikes in the cell RPeD1 (A), and the response of the neuron VD4 (B) evoked by the stimulation of osphradium using L-aspartate before and following $HgCl_2$ (2 µg/l) treatment of the osphradium. Error bars indicate ± 1SE. Significance was $p < 0.05$

neuronal axons form synapses on the axonal branches of interneurons IP3, which are responsible for the changes in activity rhythm of RPeD1, but this latter neuron (RPeD1) can backwards hyperpolarize the neuron VD4. In these two neurons sporadically synchronous discharge of AP_s and $EPSP_s$ can be registered. The increase in AP frequency and series of EPSP were found to correlate with the burst of impulses of the right inner parietal nerve (Fig. 7A). The burst of activity in the nerve was elicited by stimulation of osphradium (Fig. 7B) appearing in spontaneous activity pattern,

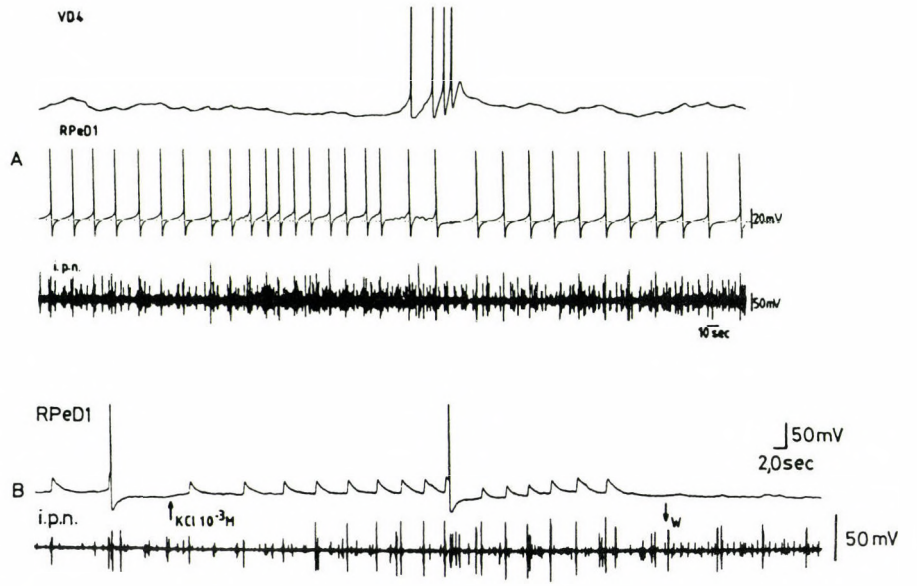

Fig. 7. Simultaneous recording from the neurons VD4, RPeD1 and the right inner parietal nerve (r.i.p.n.). Spontaneous activity (A). KCl (1 mM) applied to the osphradium caused an increase in nerve activity followed by the synchronous AP generation in the neurons (B). W – wash out. Activation of the input 3 interneuron (IP3)

too (Fig. 7A). Thus the increase in extracellular activity of the nerve precedes the occurrence of synaptic potentials in neuron RPeD1. It was obvious also in cases when the activity pattern originated from a limited number of active neurons (Fig. 7B) was extracellularly registered.

In some preparations the inverse to the above synaptical relations was seen. The neuron VD4 received periodic biphasic postsynaptic potentials, but at the same time on the cell RPeD1, no changes in rhythmic activity could be traced. This means that some of the osphradial fibres can terminate directly on interneurons RPeD1 and VD4. The character of the above synaptic inflow differs basically from that originating from IP3. The synaptic potentials having amplitude less than 1 mV with high frequency registered in the soma membrane of the neuron RPeD1 failed to cause remarkable depolarizations of membrane or AP generation. At the same time, in VD4 strong hyperpolarization was registered, corresponding to the duration of the osphradial stimulation (Fig. 3D). The cell VD4 was found as well to have contact directly with the osphradial nerve fibres, but the impulsation from osphradium caused inhibition of its firing.

The response of the cells RPeD1 was stimulus specific corresponding to the nature of substances applied to the osphradium. Solutions of urea and amino acids in concentration of 10 mM applied to the osphradium decreased the firing frequency of the

neuron RPeD1 (Figs 4A and 4C). Applying sucrose in concentration 100 mM to the osphradium, the activity of RPeD1 was inhibited (Fig. 4D), whereas NaCl solution in the same osmotic concentration caused excitation in this giant pedal cell (Fig. 4B). Various interneurons having rich synaptic connections in CNS can as well be effected by application of various salines of different osmotic pressure and amino acids to the osphradium. This confirms the data obtained on periphery and with recordings from nerve cells of the osphradial ganglion.

Neurons of the A-cluster located to the dorsal surface of the right parietal ganglion, were shown to react to the stimulation of osphradium, too. The stimulation of osphradium by the solution of L-aspartate more often switched the regular spike activity of A-cluster neurons to the bursting one. Thus, on A-neurons the silent periods between spikes are the result of the activity in neuron VD4, while burst activity originates from interneuron IP3. The firing pattern of neurons of A-cluster is connected also to the activity of neuron RPeD1. The statistical analysis of alterations in firing frequency of these cells during osphradial stimulation showed, that the activation of the neuron RPeD1 inhibits the reaction of A-cells, and on the other hand, the activity of A cells causes a pausing in RPeD1 activity (Fig. 4). Thus, information about environmental changes running from the osphradial ganglion can be transferred directly to the central neurons.

The reactions of the respiratory neurons as well as their synaptic connections to the osphradium were modified following chronic $HgCl_2$ treatment of the animals (Fig. 4) or acute treatment of the osphradium by $HgCl_2$. The results proved that the osphradium can be used for monitoring the presence of various pollutants.

The ion-currents of osphradial neurons and their modulation by $HgCl_2$

The patch-clamp method in current clamp mode was used to record membrane and action potentials in osphradial neurons during sensory processing. It was found that the action potential generation of ganglionic and sensory cells of osphradium was maintained in sodium free solution and their amplitude increased by 25% on ganglionic cells (GC1-3) and by 13% on sensory neurons (SC) if the Ca^{2+} concentration in physiological solution was doubled. After recording the spike activity of osphradial neurons the same neurons were used in voltage clamp conditions for recording ion currents. The inward Ca^{2+}-currents, corresponding to AP_s were recorded in conditions, when sodium and potassium channels were blocked by TEA (44 mM) with simultaneous omission of Na^+ from the extracellular solution and filling the patch-pipettes with CsCl. In some experiments the Na- and K-generated ion currents were blocked by tetrodotoxin (TTX 10^{-5} mM) and TEA (44 mM/l), respectively, using patch-pipette filled with CsCl.

Voltage dependent Ca-current was recorded at various holding potentials (from –40 to –120 mV) using test-impulse of 200 msec duration and increasing amplitude from –70 –60 mV to +50 +90 mV. Test impulses were applied with an interval of 3 s to avoid inactivation.

The Ca-current registered in osphradial neurons belongs to the high voltage activated Ca-current (HVA) [13]. In control saline containing 4 mM/l Ca^{2+} the threshold of activation was between –20 and 0 mV with maximal value at +30 +40 mV. Mean amplitude of HVA Ca-current was -13.6 ± 3.0 nA (n = 7) in ganglionic cells and -10.3 ± 5 nA (n = 9) in sensory cells of osphradium. The inactivation phase of Ca-currents in sensory cells was faster then that of the ganglionic cells of the osphradium. The concentration dependence of Ca-current was shown by variation of Ca-ions in bathing saline (Figs 8 and 9).

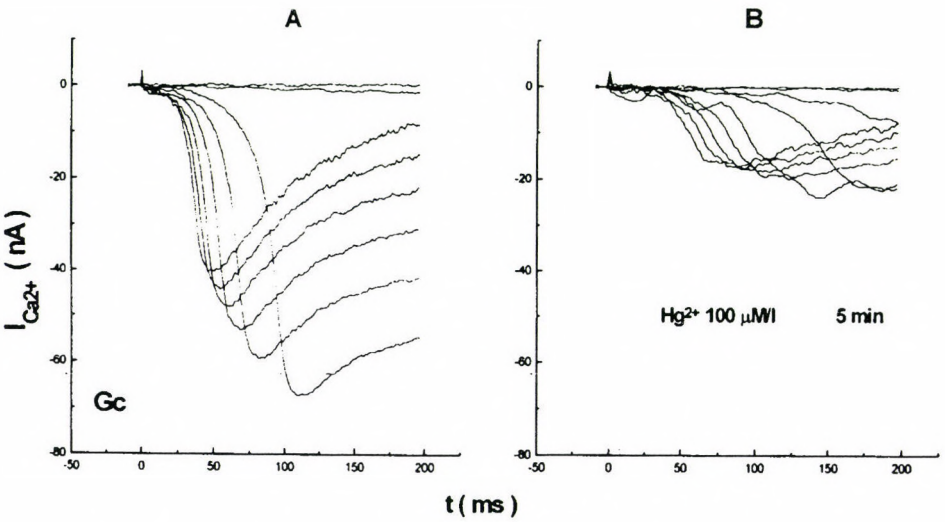

Fig. 8. High voltage-activated inward Ca-currents in the ganglionic cell (GC) of osphradium in control and following 5 min treatment with $HgCl_2$. Holding potential was –30 mV. A – control. B – treated samples

High voltage activated Ca-currents are classified into various subgroups depending on their pharmacological properties, e.g. their responses to dihydropyridines. Nifedipine (1–2 mM/l) blocked entirely the HVA Ca-inward current of sensory cells in 1–3 min and its blocking effect maintained for 30 min after returning to control saline. The Ca-current of osphradial neurons was insensitive to 10^{-2} mM/l w-conotoxin. At the same time, it was blocked completely or partially with Cd^{2+} (5×10^{-2} mM/l) or $CoCl_2$ (1 mM/l), respectively. In both types of osphradial neurons Co^{2+} decreased the amplitude of Ca-current immediately but it was restored rapidly (in 3–5 min) following wash out (Figs 8, 9 and 10). The above results proved that Ca-current of osphradial neurons belongs to the L-type of HVA Ca-currents. The same type of Ca-current was found on the neurons of *Aplysia* [15] and caudo-dorsal cells (CDC) of *Lymnaea* [13].

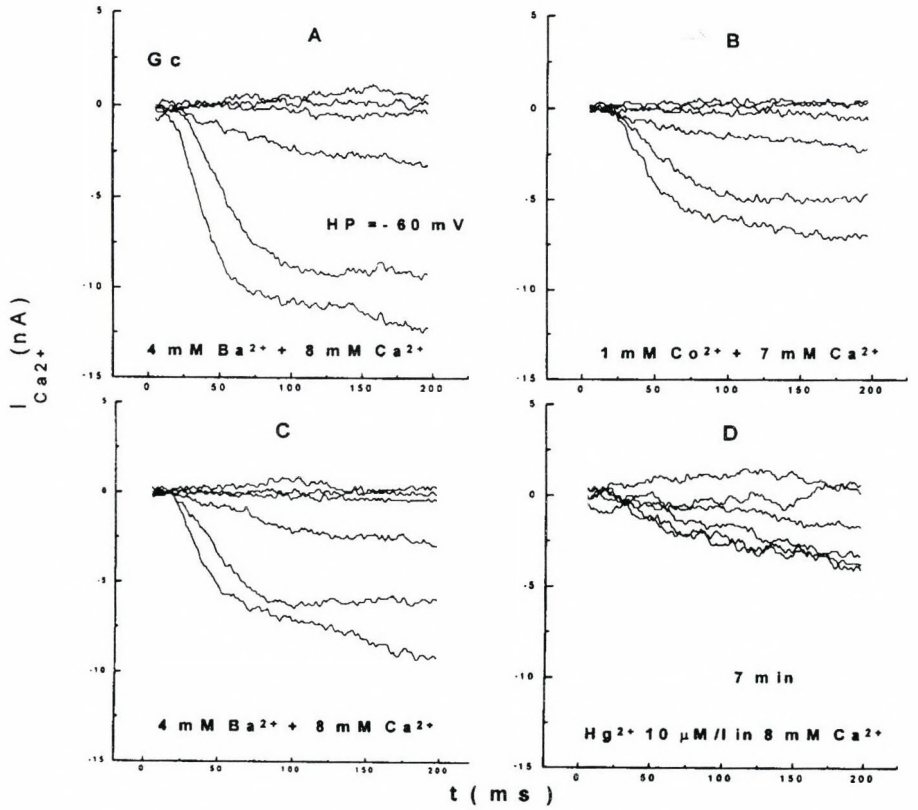

Fig. 9. Modifications of the high voltage-activated Ca-currents in the ganglionic cells (GC) of the osphradium following partial substitution of Ca-ions by Ba-ions (A and C). Effects of Co^{2+} (B) and Hg^{2+} (D) on Ca-current

We tested the effect of Hg^{2+} on Ca-current. It was shown that 10–20 µM/l Hg^{2+} reduced Ca-current by more than 80% (Fig. 9D). The blocking effect of Hg^{2+} was only partially reversible. In Figure 9 an unidentified membrane currents activated by the application of higher concentration of Hg^{2+} can as well be seen (Fig. 9D). The results of statistical treatment of the effects of Hg^{2+} are presented in Fig. 10.

Outward K-dependent currents were as well recorded from osphradial neurons. The K-currents were not blocked by 4-aminopiridine,4-AP (10^{-2} mM/l) and apaine (10^{-1} mM/l). However, TEA effected both the Ca-dependent and voltage dependent K-currents similarly to that found on other molluscan neurons [15]. Nevertheless voltage dependent K-current was blocked only partially even by 40 mM of TEA. The voltage activated K-current was completely blocked by intracellular injection of CsCl (4 mM/l) in 3 min. Ca-dependent K-current was blocked by intracellular injection of EGTA (4 mM/l) in 1 min.

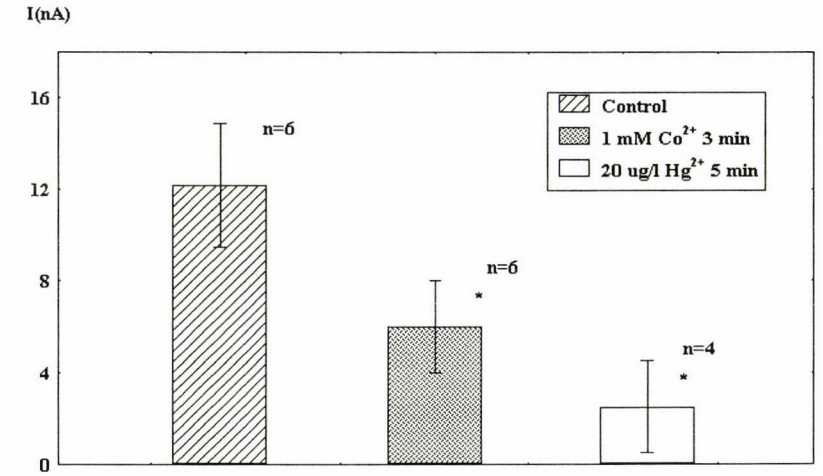

Fig. 10. Histogram demonstrating the effect of Co^{2+} and Hg^{2+} on high voltage-activated Ca-current of the osphradial sensory neurons (SC). Error bars indicate ± 1SE. Significance was $p < 0.05$ (*)

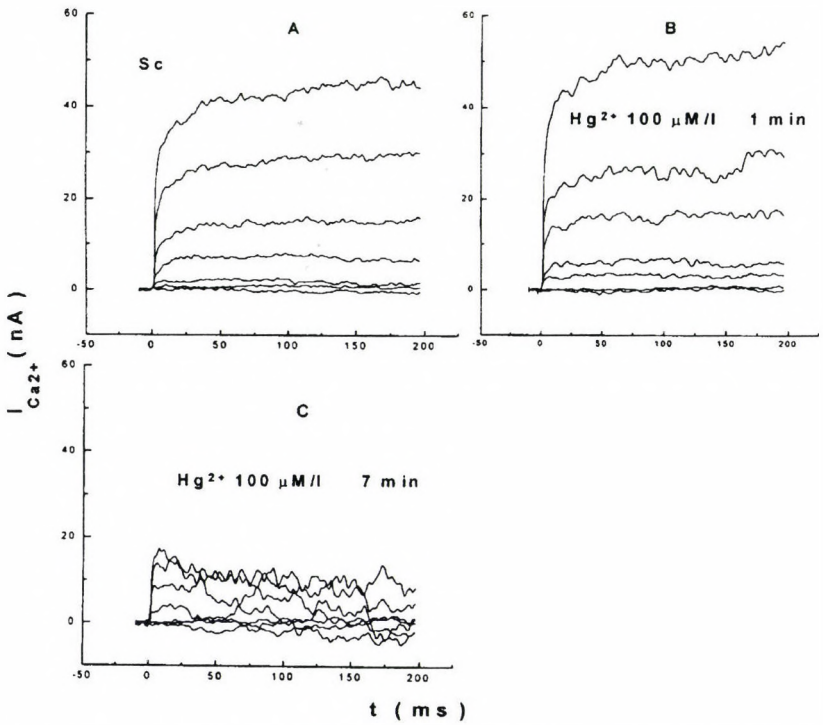

Fig. 11. Effect of Hg^{2+} on voltage-dependent outward K^+-currents of the sensory cells (SC) of osphradium. Holding potential was –60 mV. A – control. B and C – treated samples

The repolarization of AP depends on K^+-outward current and Hg^{2+} can block it. To study this question we used sodium-free extracellular solution, where the Na^+ was replaced with N-methyle D-glukamine in equiosmolar concentration and the patch-pipette was filled with KCl and EGTA. For activation of K-currents the sequence of short pre-impulses necessary for complete correction of leakage current was used and rectangular 200 ms duration test pulses were applied in 3–5 second intervals. The K^+-current recorded had threshold about –20 mV appearing to each step of testing pulse (Fig. 11). For studying the nature of K^+-currents pharmacological agents were used to block various components of K^+-current. Application of 4-aminopyridine (to 10^{-2} mM), apaine (10^{-1} mM) do not cause remarkable effects on the K^+-current. At the same time, TEA blocks both Ca^{2+}-dependent, and voltage-dependent K^+-currents being the Ca^{2+}-dependent K-current more sensitive to it. Voltage-dependent K^+-current was not completely blocked even with 40 mM TEA solution. The analysis of K^+-currents revealed its insignificant inactivation. During depolarization (40–60 mV) the phase of inactivation was absent. It turned out that Ca^{2+}-dependent K^+-current of osphradial cells has high dependence on intracellular concentration of EGTA. If patch-pipettes were filled with 4 mM solution of EGTA complete block of Ca-dependent K^+-outward current was obtained in one minute after setting up the whole cell configuration.

Following 1-minute treatment of osphradial neurons with 10–20 µM/l $HgCl_2$ slight increase in K^+-outward current was seen, but longer treatment depressed transient potassium current as well (Fig. 11). These findings were statistically verified (Fig. 12).

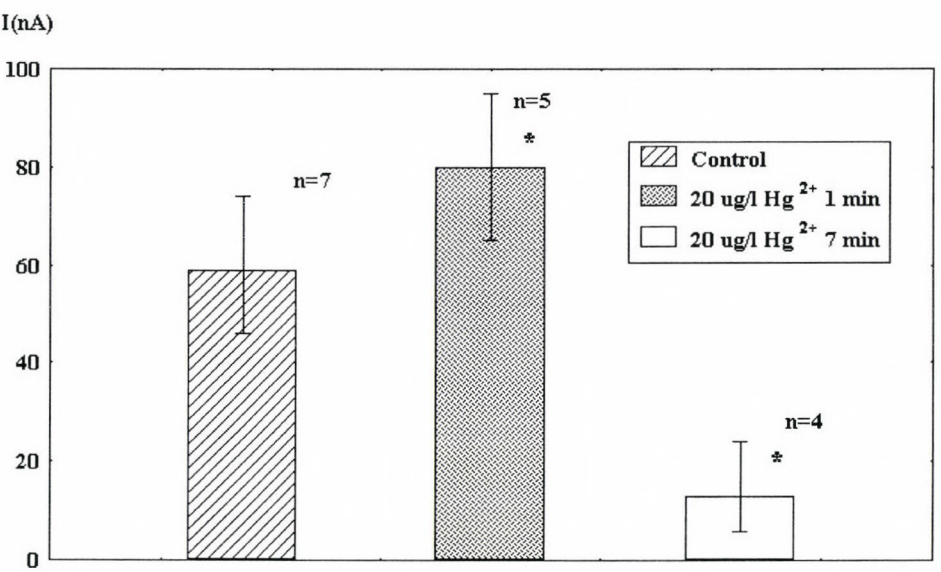

Fig. 12. Histogram demonstrating modifications of the voltage-activated K^+-currents of sensory cells (SC) of the osphradium by Hg^{2+}. Error bars indicate ± 1SE. Significance was $p < 0.05$ (*)

DISCUSSION

The osphradial sensory system was studied on the *Lymnaea stagnalis* L. (Pulmonata, Basommatophora) and the modulation of signalization running from sensory receptors of osphradium to the CNS was demonstrated following heavy metal treatment.

The chemosensory function of osphradium was shown earlier by its reaction to L-amino acids, urea and algae homogenates [7, 17, 27]. Three neurosecretory cells were found to display of AP_s with regular rhythm in sodium-free solution and to increase their amplitude depending on concentration of calcium in the perfusing solution. Inward calcium current was blocked with nifedipine, Cd^{2+} and Co^{2+}, but not with w-conotoxin. The Ca^{2+}-current in osphradial neurons was found to belong to a L-type of HVA Ca-current described earlier in the neurosecretory cells *Aplysia* and caudo-dorsal cells of *Lymnaea* [9, 13, 14, 15, 18, 21]. Increase of spike duration is the common phenomenon for neurosecretory cells, facilitating releasing of neurosecretory material from synaptical vesicles.

Both the acute treatment of the osphradium and chronic treatment of the animals with $HgCl_2$ damaged the sensory function of the osphradium. Hg^{2+} has been shown to reduce voltage activated Ca^{2+} and K^+ currents [6, 12, 26]. In this case, heavy metal can be involved in a regulation of chemosensory process in receptors and ganglionic neurons and to participate in reorganization of local neuronal osphradial networks [19]. The neurons reacting to the stimulation of osphradium were scattered throughout the visceral, right parietal, pedal and cerebral ganglia including respiratory central pattern generators, cardiorespiratory motoneurons and caudo-dorsal cells. In nervous system of pond snail more then 150 large neurons were identified [3, 8, 22, 23] forming the neuronal networks connected to the regulation of respiration, locomotion and other visceral functions with synaptical inputs [2, 3, 4, 5].

The giant pedal neuron (RPeD1) has synaptic connections with visceral interneuron (VD4) and right parietal interneuron (IP3), and generates spontaneous rhythmic firing modulated by a number of synaptic inputs. RPeD1 takes part in a neural network regulating respiratory movements in *Lymnaea* [24].

In the interneuron RPeD1 the burst of short-term spike activity is evoked by EPSPs originating from IP3 while the VD4 cell causes an inhibition on RPeD1 [22, 23] sending IPSPs to it. Our results supported these data.

The synaptic connections of these neurons with the osphradium was demonstrated in our experiments, too. Interaction between RPeD1 and VD4 interneurons was demonstrated during stimulation of osphradium. Application of L-aspartate to the osphradium caused on the cell RPeD1 two types of reaction depending on its firing pattern: it led to decrease in the number of spikes if the cell RPeD1 was active when the duration of its inhibitory phase evoked by IPSPs of VD4 was prolonged. It means that the mutual inhibitory interaction between these two interneurons (RPeD1 and VD4) can be modified by afferent signalization originating from the osphradium.

In generation of the respiratory rhythm interneuron IP3 plays a special role. Interneuron IP3 excites the neuron RPeD1 and inhibits the neuron VD4 although this connection cannot be demonstrated in each experiment. It was shown in our experi-

ments that some of the axons of osphradial ganglion cells form synapses on the dendrites of IP3, and the same fibres are responsible both for modulation of firing frequency of RPeD1 and for hyperpolarization of neuron VD4. Sometimes it was possible to record firing pattern of the osphradial nerve correlating with the AP_s or EPSPs of the cell RPeD1.

Extracellularly registered activity of r.i.p.n. preceded the EPSPs or APs generated in cell RPeD1 and their interaction was 1 : 1.

On VD4 neuron stimulation of osphradium caused an inhibition. The interneuron VD4 also was found to have direct synaptic contact with osphradium.

On the axons of A-cells the interneurons IP3, RPeD1, VD4 and further neurons regulating respiration were shown to form synapses. During stimulation of osphradium by L-aspartate the tonic firing of A-cells turned to phasic one. In this phasic pattern the burst of activity correlated with the activity of IP3, while the interburst interval corresponded to the activity of neuron VD4. In the pattern formation of A-cells RPeD1 neuron takes also part: the response of A-cells to the stimulation of the osphradium was modified by the firing of RPeD1. When RPeD1 neuron was firing the response of A-cells to the osphradium stimulation was inhibited, however, A-cells were firing if the cell RPeD1 was paused.

The giant pedal cell (RPeD1) has rhythmic spontaneous activity, change of which depends on synaptical inflow coming from interneurons in the visceral (VD4) and right parietal (IP3) ganglia. This cell is known to form a steady neuronal network generating a rhythm of respiratory movements in pond snail [24, 28]. It was shown that the osphradial stimulation results in changes in rhythmic spontaneous activity of this cell. Both the acute treatment of the osphradium and chronic treatment of the animals with $HgCl_2$ modified the synaptic connections of the respiratory regulatory neurons.

These results proved that the receptors of the osphradium are continuously perceiving the environmental changes and can be used for monitoring the pollution of the water. Osphradial multisensory system takes part in the regulation of vital vegetative and reproductive functions of *Lymnaea stagnalis* L. Heavy metal pollution led to the alterations in their regulatory mechanisms.

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CELLULAR ANALYSIS OF APPETITIVE LEARNING IN INVERTEBRATES*

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In recent years significant progress has been made in the analysis of the cellular mechanisms underlying appetitive learning in two invertebrate species, the pond snail *Lymnaea stagnalis* and the honeybee *Apis mellifera.*

In *Lymnaea,* both chemical (taste) and tactile appetitive conditioning paradigms were used and cellular traces of behavioural classical conditioning were recorded at several specific sites in the nervous system. These sites included sensory pathways, central pattern generator and modulatory interneurones as well as motoneurones of the feeding network. In the honeybee, a chemical (odour) appetitive conditioning paradigm resulted in cellular changes at different sites in the nervous system. In both the pond snail and the honeybee the activation of identified modulatory interneurones could substitute for the use of the chemical unconditioned stimulus, making these paradigms even more amenable to more detailed cellular and molecular analysis.

Keywords: Classical conditioning – appetitive learning – cellular mechanisms – *Lymnaea stagnalis* – *Apis mellifera*

INTRODUCTION

Invertebrate animals have provided useful physiological models in which significant progress has been made in the elucidation of neuronal and molecular mechanisms underlying learning [10, 40]. Some of these mechanisms, including recently described molecular modifications of gene expression, seem to be common to some forms of learning in higher animals as well (reviewed in [9]). This lends further support to the notion that a simple systems approach using invertebrate preparations is useful in creating models that are of general significance for understanding the basic mechanisms of learning.

To date, some of the most important insights into the cellular and molecular mechanisms of learning have come from work using aversive classical conditioning para-

* Dedicated to Professor János Salánki for his 70th birthday.

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digms in gastropod model systems [1, 19, 26]. However, another important form of associative behavioural modification, appetitive learning, also has been demonstrated in gastropods and other invertebrates [3, 12, 13, 18, 25, 32–34, 38, 49, 52, 55, 62]. This type of learning is based on pairing a neutral conditioned stimulus (CS) with food as an unconditioned stimulus (US). So far appetitive learning has been analysed in cellular detail in only two invertebrate organisms, the honey bee *Apis mellifera* [22–25, 44] and the snail *Lymnaea stagnalis* [37, 59, 60, 64]. The purpose of this paper is to review the results of work on these two important invertebrate models for the cellular analyses of nonaversive associative memory formation.

Cellular analysis of conditioned feeding behaviour in the pond snail Lymnaea stagnalis

One of the invertebrate models where appetitive learning has been demonstrated is the pond snail *Lymnaea stagnalis* [3, 32, 38]. A cellular analysis of appetitive learning in *Lymnaea* seemed feasible because the neuronal activity underlying feeding, the unconditioned behaviour, was already known in detail. This behaviour is generated by a central pattern generator (CPG) system [4], similar to those also commonly found in higher organisms (reviewed in [31]). The feeding CPG is synaptically connected to a variety of modulatory interneurones that can gate, initiate and help maintain CPG-driven activity [15, 45, 54, 68, 70]. The final motor output of the feeding network is produced by ten different types of motoneurones, each becoming active in one of the 3 phases of the feeding cycle, protraction, rasp and swallow [53], as a result of CPG-driven synaptic inputs [5].

In the most thoroughly investigated appetitive paradigm used in *Lymnaea* the conditioning stimulus, a tactile input applied to the lips, is paired with the unconditioned stimulus, sucrose [32–34, 59, 60]. These experiments demonstrated that *Lymnaea* can be classically conditioned by repeatedly pairing touch to the lip with food and that appetitive learning in *Lymnaea* shares important characteristics with conditioning in vertebrates [32–34].

The first type of approach to the cellular analysis of appetitive learning in *Lymnaea* was based on an *in vitro* analogue of the appetitive conditioning paradigm using a tactile CS and activation of a modulatory interneurone, called the Slow Oscillator (SO), as the US. Touch to the lip is a neutral stimulus in the intact animal and in semi-intact preparations in the sense that unlike sucrose, it cannot evoke prolonged behavioural or fictive feeding. However, it can evoke a few fictive feeding cycles in intact animals [34] and it may produce weak, brief activation of fictive feeding in some semi-intact preparations [35]. It excites modulatory neurones and has access to the feeding central pattern generator (CPG) as well [35]. Current injection into the SO reliably drives a fictive feeding pattern in the same semi-intact preparation where touch can be applied to the lips and this rhythmic response resembles unconditioned fictive feeding evoked by sucrose applied to lip receptors [35, 71]. Moreover, when during strong sucrose-evoked activation of the feeding CPG the SO

is activated together with CPG interneurones, it helps maintain a fast, regular rhythm [71]. This indicates that this modulatory interneurone may be part of the network that underlies the maintained unconditioned feeding response to food in intact animals. A major advantage of using the SO as the source of US was that its synaptic connections with other neurones of the *Lymnaea* feeding network were well understood [4].

For the *in vitro* analysis of appetitive learning [37] we used a preparation that was similar to the type of preparation first developed for *in vitro* studies of aversive learning in *Limax* [11, 20] and *Pleurobranchaea* [39]. This so-called split lip – CNS preparation was also previously successfully used to analyse chemical and tactile inputs to the feeding system in other molluscs, including *Helix* [29, 36] and *Lymnaea* [17, 35, 71]. In this type of preparation, simultaneous electrophysiological recordings were made from a number of feeding motoneurones and interneurones. The latter included the modulatory interneurones SO and cerebral giant cells (CGCs) and the central pattern generator (CPG) interneurone N1M. The *in vitro* training consisted of up to 10 pairings of a touch stimulus applied to the lips with activation of fictive feeding by depolarising the SO above threshold. In three different control groups either only touch was applied (CS alone control) or only the SO was depolarised (US alone control), or the two stimuli were applied on a random schedule (Random control). Cellular responses to lip touch were tested in the same preparations before the first trial and after the last trial.

These experiments demonstrated, for the first time, that it is possible to carry out *in vitro* appetitive classical conditioning of the molluscan feeding system. Experimental preparations that gave weak fictive feeding responses to touch before conditioning showed strong activation of fictive feeding after training (Fig. 1). Statistical analyses showed both that the increase from pre- to post-training response levels was only significant in the experimental group and that the difference between the level of unconditioned and conditioned fictive feeding response to touch was significantly greater in the experimental vs. the control groups (Fig. 2). By analysing changes in spike activity in the CGCs, we showed that these serotonergic modulatory interneurones were unlikely to play an important role in the plastic changes occurring during *in vitro* conditioning in *Lymnaea.* We also eliminated the possibility that the SO interneurone might show a facilitated touch response after conditioning. This was important because both of these modulatory cell types are known to have direct connections with the N1M type CPG interneurones [14, 67] and could have been responsible for the facilitated CPG and consequent motoneurone response to touch following conditioning.

During *in vitro* appetitive learning, possible changes could also occur at synaptic connections between the CPG interneurones themselves or between the CPG interneurones and the motoneurones, although these are unlikely to lead to a sufficiently stimulus-specific conditioning. It appears that the main remaining likely sites of plasticity are the synaptic connections between putative mechanosensory cells of the CS pathway [57] and the CPG interneurones. These may be modified during conditioning via heterosynaptic facilitation by inputs from the US pathway or even by homo-

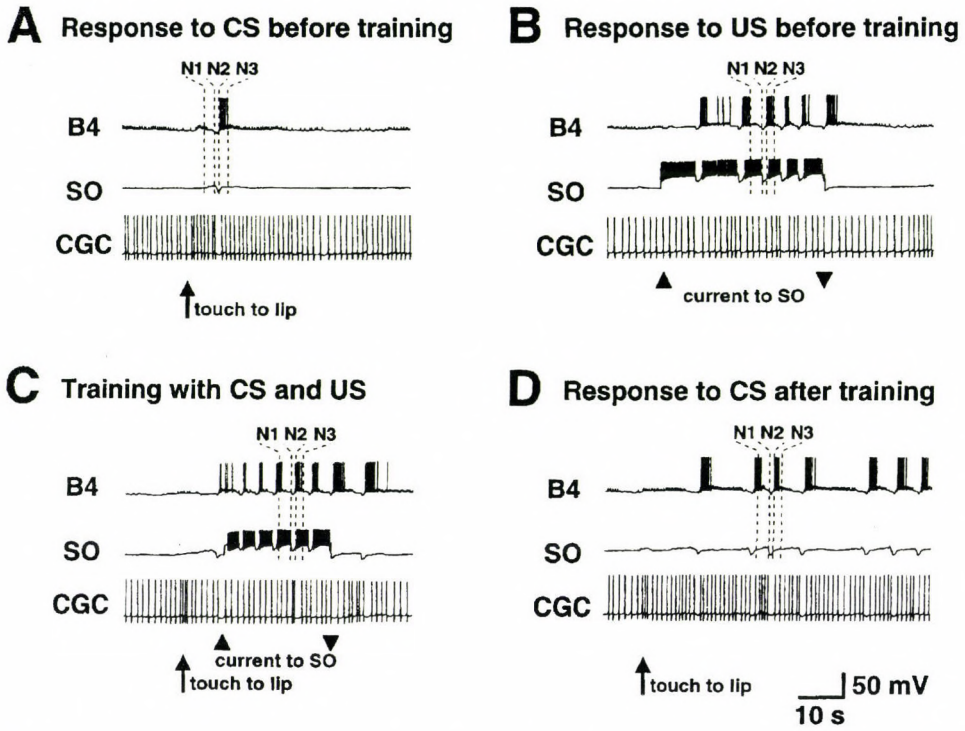

Fig. 1. An *in vitro* appetitive conditioning experiment in *Lymnaea.* (A) Before training, touch (the CS) applied to the lips causes a single cycle of fictive feeding activity in the B4 motoneurone and a small increase in tonic firing in the modulatory neurone CGC. Central pattern generator (CPG)-driven, subthreshold N1 → N2 → N3 phase inputs are also present in the modulatory interneurone SO. (B) Injection of depolarizing current into SO initiates and maintaines a strong unconditioned fictive feeding rhythm. Both the SO and the B4 cell receive typical excitatory and/or inhibitory inputs (N1 → N2 → N3) indicating activity in the feeding CPG. (C) A conditioning trial in the semi-intact preparation consists of a touch stimulus (the CS) applied to the lip, followed by activation of the feeding rhythm by current injection into the SO (the US). (D) After repeated (7) pairings of touch with stimulation of the SO the touch alone is able to evoke a conditioned fictive feeding response with clearly recognisable sequences of N1, N2 and N3-phase inputs on both B4 and SO. This response is more prolonged than the response to touch before training shown in (A). Note that the SO does not show an enhanced response to touch after conditioning and is therefore not driving the facilitated touch response in the motoneurone. Neither does the CGC show an enhanced spontaneous firing rate or an increased response to touch after conditioning (compare to A). (From Kemenes et al. [37], with permission from the American Physiological Society)

or heterosynaptic feedback from the CPG network itself. Recent work has demonstrated that certain types of motoneurones actively contribute to the generation of the feeding rhythm through electrotonic connections to CPG interneurones [59]. Plastic changes in these motoneurones may, in theory, also contribute to the increased activation of CPG neurones by the tactile CS after *in vitro* conditioning.

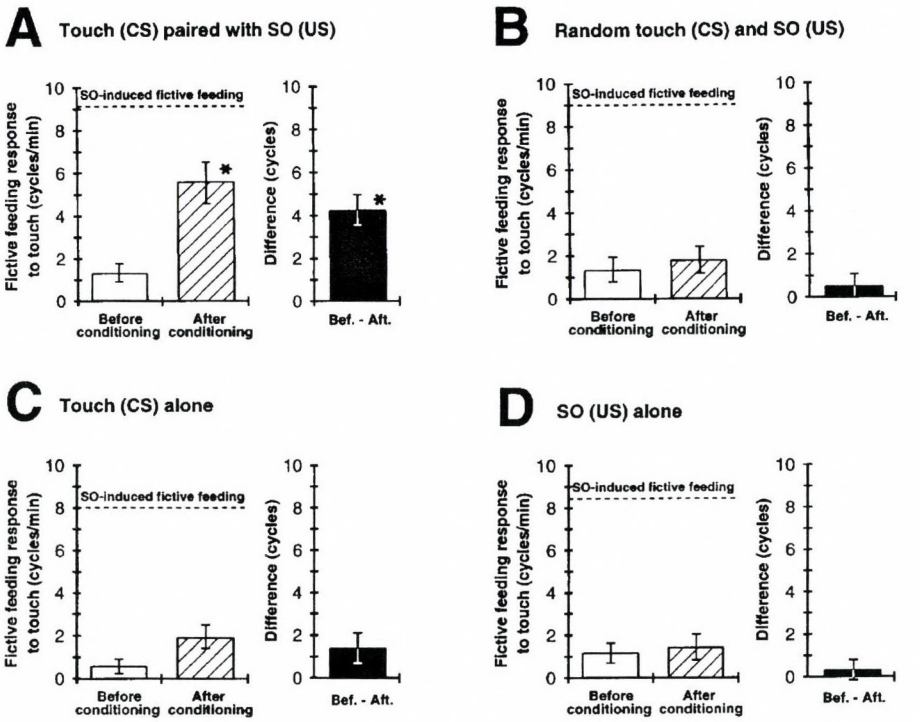

Fig. 2. Summary of the results from the *in vitro* conditioning (A) and control (B–D) experiments. A, In the experimental group, after 6–10 paired applications of the CS and US, the mean difference (black bar) between the pre- and post-training responses is significantly greater than in the combined controls. Moreover, the mean fictive feeding response to CS after conditioning (hatched bar), is significantly stronger than before conditioning (open bar). The dashed lines here and in B–D show the mean level of the SO-evoked unconditioned fictive feeding response in the group. B–D, Preparations subjected to 3 different control protocols (Random, CS alone, US alone) do not show increased fictive feeding responses. (From Kemenes et al. [37], with permission from the American Physiological Society)

The second type of approach to the cellular analysis of tactile appetitive learning in *Lymnaea* was based on appetitive conditioning of intact animals followed by a search for cellular traces of memory in semi-intact preparations [59, 60]. This second experimental approach, also successfully used in a variety of invertebrate and vertebrate preparations (reviewed in [8]), allows the potential direct links between cellular events and behaviour of intact animals to be established more easily than the *in vitro* approach.

In the first, behavioural, part of this experiment one group of animals were subjected to appetitive conditioning with 15 pairings of touch with food (sucrose) and another group were subjected to a random control protocol. In the second, electro-

physiological, part of the experiment animals from both groups were dissected into semi-intact preparations. For these experiments we used a whole-lip preparation in which the lip sensory-structures were left completely intact. The preparations made from experimental and control animals were tested for fictive feeding responses to the same touch CS that was used during the training trials. Fictive feeding activity was monitored by intracellularly recording from motoneurones of the feeding network.

The first important finding of this work was that a neurophysiological expression of the conditioned feeding response was found in semi-intact preparations made from behaviourally trained animals [59]. This was identified as patterned fictive feeding

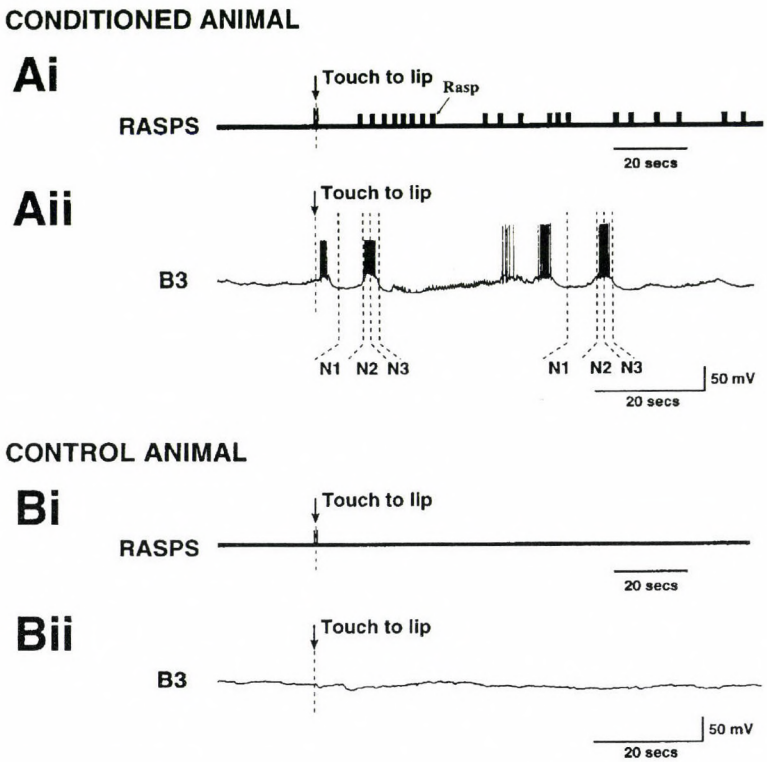

Fig. 3. Behavioural and neurophysiological analysis of the responses to the tactile stimulus (touch to lip) in behaviorally trained experimental (A) and random control (B) whole animals (Ai, Bi) and then semi-intact preparations (Aii, Bii). Ai, Feeding response to the tactile stimulus in an appetitively conditioned intact animal. The tactile stimulus elicits a continuous series of rasps (vertical lines). Aii, Fictive feeding response, recorded on a feeding motoneurone (B3), to the tactile stimulus in a semi-intact preparation made from the animal in Ai. Bi, Lack of a feeding response to the tactile stimulus in a random control animal. Bii, Lack of a fictive feeding response to the tactile stimulus in a semi-intact preparation made from the control animal in Bi. (From Staras et al. [59], with permission from the American Physiological Society)

activity in motoneurones of the feeding system (Fig. 3), triggered by the presentation of the lip touch CS, which evoked a significantly stronger response in experimental animals than in controls [59]. This finding established that behaviourally produced appetitive learning is amenable to cellular analysis in preparations made from the same animals.

More detailed analysis established that behavioural conditioning of the feeding response in *Lymnaea* leads to plastic changes that can be recorded electrophysiologically at several different locations in the neuronal network underlying rhythmic feeding [60]. In addition to the conditioned activation of the feeding CPG, an increase in the amplitude of a motoneuronal (B3) EPSP response to touch was also observed. These were preceded by changes recorded in the CS pathway, which was demonstrated by recording a conditioning-induced increase in the number of touch-evoked spikes in the cerebro-buccal connective, which forms part of the CS pathway. This indicated that plastic changes might occur at the CPG and motoneuronal level as well as upstream to both, at the level of the CS pathway mediating the mechanosensory inputs to the feeding network.

An *in vitro* correlate of an appetitive conditioned response was also found after chemical appetitive conditioning of intact animals with amyl acetate as the CS and sucrose as the US [64]. The electrophysiological tests in this experiment concentrated on the $CV1_a$ cells, a pair of modulatory interneurones in the cerebral ganglia [45]. In most semi-intact preparations (~80%) taken from experimental snails, application of amyl acetate to lip tissue led to depolarisation of the $CV1_a$ cell membrane and in ~30% of them, to strong rhythmical bursting activity. Interestingly, in some preparations where the buccal feeding central pattern generator system was removed, $CV1_a$ continued to show an excitatory response to amyl acetate after conditioning. This indicates that, as in tactile appetitive conditioning [60], one site of plasticity may be located upstream to the buccal feeding system, perhaps even in the $CV1_a$ neurones themselves.

Despite the progress that has been made in identifying potential sites of plasticity after appetitive conditioning in *Lymnaea,* still little is known about the tactile and/or chemical CS and US pathways mediating the unconditioned and conditioned responses. Neurones of the buccal and cerebral feeding network have been shown to receive both mechano- and chemosensory inputs [35, 61] so they can serve as potential sites for convergence of CS and US pathways. Recently, it also has been shown that both mechano- and chemosensory inputs from the lips are widely distributed to inter- and motoneurones of the feeding system [61, Kemenes et al., in preparation]. However, progress with the analysis of the contribution of identified neurones of the CS and US pathways to appetitive learning so far has been hampered by the technical difficulties involved in finding primary mechano- and chemosensory neurones that provide inputs to neurones of the feeding system in *Lymnaea.* Also very little is known about the biochemical/molecular mechanisms underlying appetitive conditioning in *Lymnaea.* Although some of the transmitters used by inter- and motoneurones of the feeding system have been identified [7, 16, 50, 51, 69], and it also has been demonstrated that the US pathway uses NO as one of its main transmitters [17],

the targets for these transmitters have not yet been characterised in molecular detail. It is not known either what transmitter(s) the neurones of the CS pathway use in their synaptic connections with the feeding system.

Cellular analysis of the conditioned proboscis-extension response (PER) in the honeybee (Apis mellifera)

Bees display a strong unconditioned response (UR) when their antennae or proboscis are stimulated by food, such as sucrose. This is the proboscis extension response (PER), which can serve as the UR in associative conditioning paradigms. The PER can be conditioned by pairing an odour CS with a sucrose US applied to the antennae and proboscis. After only one trial the initially neutral CS becomes associated with the US and subsequently elicits a conditioned PER [6].

The olfactory CS pathway projects to a number of structures in the bee brain, such as the antennal lobes, the mushroom bodies *(mb)* and the lateral protocerebral lobe [2, 21, 47]. So far very little has been known about changes in identified neurones of the CS pathway contributing to the formation of the conditioned PER. This is probably due to technical difficulties involved in trying to record very small cells during olfactory PER conditioning. However, a single identified *mb*-extrinsic neurone, PE1, has been found to change its response to the CS after conditioning [44]. A single forward pairing of an odour with sucrose reduced the response to the odour for a short time after pairing. By contrast, multiple differential conditioning trials lead to a short-lived increase of the PE1 response to CS+ and no change of the response to the CS– [44]. It is not yet clear what role these transient electrical changes in a CS pathway neurone play in olfactory learning in the bee. However, these findings indicate that a possible site of plasticity lies within or upstream of PE1, that is in the CS pathway itself [25].

Significantly, an important modulatory neurone of the US pathway used in the bee olfactory learning paradigm also has been identified [21]. The VUMmx1 neurone responds to sucrose stimulation of the antennae and the proboscis, and its axonal arborisations converge with the olfactory CS pathway in the antennal lobes, the lateral protocerebral lobe and the mb-s. Moreover, in *in vivo* preparations supra-threshold depolarisation of the VUMmx1 neurone has been shown to substitute for the US in a single conditioning trial [21]. This neurone therefore provided the first direct access to the cellular mechanisms underlying the reinforcing properties of the US pathway in the bee olfactory conditioning paradigm [21]. Further experiments revealed that VUM neurones show octopamine (OA) immunoreactivity [41] and that pairing an odour CS with local injection of OA to the convergence sites between the VUMmx1 neurone and the CS pathway produces a conditioned PER, which is similar to the one achieved by pairing the CS with the activation of VUMmx1 [22, 24].

DISCUSSION

Although the cellular and molecular analyses of appetitive learning in invertebrates have not yet progressed as far as those of aversive learning, both the *Lymnaea* and bee models have already yielded important data that indicate that there may be important similarities as well as differences between some of the mechanisms underlying these two important forms of associative memory formation. Both the *Lymnaea* and bee findings showed that, at least in invertebrates, stimulation of single identified central interneurones could substitute for the US for *in vitro* conditioning. In *Aplysia* aversive learning, direct activation of central facilitator interneurones, important in modulating synaptic transmission between mechanosensory neurones of the CS pathway and motoneurones of the US pathway [8], produced the same presynaptic facilitation in the sensory neurones that an electric shock applied to the tail US site could produce [27, 28, 43].This suggests that in both appetitive and aversive conditioning in invertebrates intracellular stimulation of identified central neurones can substitute for a sensory US. However, it is not yet known if during appetitive learning such neurones play a presynaptic facilitating role as well as evoke and/or modulate the unconditioned response. It is interesting to note that neither the *Aplysia* facilitator neurones nor the bee VUMmx1 neurones are capable of evoking the unconditioned response. Therefore, the associations formed after pairing the tactile or odour CS with the activation of these cells is more likely to be of a stimulus-stimulus (S-S) rather than of a stimulus-response (S-R) nature [30]. By contrast, the SO interneurone in *Lymnaea* can actually drive fictive feeding, the cellular correlate of unconditioned feeding behaviour. Therefore, it seems more likely that *in vitro* appetitive conditioning using stimulation of the SO neurone as the US [37] leads to an S-R association between the CS and some unit (probably the feeding CPG) controlling feeding motor activity.

As with aversive classical conditioning paradigms in *Aplysia* (for reviews see [8, 10]), appetitive learning in *Lymnaea* (but not in the bee) was based on the amplification of a weak response (weak feeding motor output in response to touch), rather than on the formation of a completely new one. This was indicated by the observation that it was a prerequisite for successful *in vitro* conditioning that preparations should show a clear (albeit weak) fictive feeding response to touch prior to training (alpha-conditioning, [63]).

One question that remains open in the aversive conditioning work on *Aplysia* as well as in the appetitive conditioning work in both *Lymnaea* and the bee is whether there are multiple sites of plasticity or all changes could be ultimately derived from plastic changes at a single site. The original cellular model for associative conditioning in *Aplysia* was essentially a single-site, non-Hebbian model in which the most important plastic changes took place in the pre-synaptic terminals of the sensory neurones of the CS pathway [26]. Subsequent work raised the possibility that peripheral as well as central sites of plasticity exist [42] and that Hebbian as well as non-Hebbian mechanisms might contribute to classical conditioning in *Aplysia* [20]. Recent work in *Lymnaea* has demonstrated that cellular traces of behavioural condi-

tioning could be recorded at several specific sites in the feeding network [60]. This is consistent with a multistage or multisite cellular model of appetitive learning but does not rule out a single common site of plasticity, say in the sensory periphery, either. In the bee olfactory paradigm, the rich axonal arborisation of the VUMmx1 neurone in three different sites of the brain that receive olfactory inputs points to the possibility that neural substrates of associative learning may be located at these multiple sites of CS-US convergence [25]. However, no electrophysiological evidence has yet been presented to show that plastic changes actually take place in these structures and thus a single common site for plastic changes, say in the mushroom bodies, cannot be ruled out in the bee model either.

Perhaps the most important difference between the findings of work on appetitive and aversive conditioning in invertebrates concerned the effect of protein-synthesis inhibitors on the formation of long-term memory (LTM). When it was first investigated in the bee, the formation of long-term olfactory memory did not seem to require protein synthesis [46, 65]. This was in contrast with observations using aversive paradigms in *Aplysia,* where, as in many vertebrate models, a clear role for protein synthesis in the formation of long term memory had been demonstrated [48, 56]. However, recent work has demonstrated that long-term retention of olfactory memories in bees does depend on protein-synthesis and suggested that the type of memory subjected to analysis in previous work was a medium- rather than a long-term memory [66]. An analysis of the role of protein synthesis during appetitive LTM formation in *Lymnaea* has not been carried out yet but preliminary evidence suggests that the expression of certain types of cAMP responsive element binding (CREB) proteins may be upregulated after classical conditioning of the feeding response (unpublished observations). This is significant because work in both invertebrates and vertebrates have already shown that transcription during long-lasting plasticity is mediated by CREB [9].

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EFFECTS OF VERATRIDINE AND ITS DERIVATIVES ON THE Na-CONDUCTING CHANNELS IN *HELIX* NEURONS*

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Effects of veratrum alkaloids were studied on the Na-channels of the land snail *Helix pomatia.* It was found that veratridine and its analogues depolarize the membrane due to the increased Na-permeability. The inactivation was shifted right along the voltage axis and the recovery from the inactivation was faster after veratridine treatment. After alkaloid treatment the selectivity of the Na-channel decreased, however, the selectivity sequence was not altered. The activation curve was not shifted. Veratridine derivatives, which appeared to be more effective on insects, had almost no effect on the Na-current.

Keywords: Veratridine – channel selectivity – Na-channel – molluscan neuron – *Hellix pomatia*

INTRODUCTION

The veratrum alkaloids are steroids that occur in liliaceous plants, which were used for a long time as insecticides [3]. Among veratrum preparations the greatest biological significance is attributed to cevaratrum esters, which are characterized by a high degree of hydroxylation and a hemiacetal bridge between the A and B rings [21].

Recently new analogues of the veratrum alkaloids were synthetized and their toxic effects on insects and mice were determined. 3-pivaloyl veracevine was as toxic as the natural alkaloids to the housefly and the milkweed bug. 3-(3,5-dimethyoxybenzoyl)veracevine was several times more toxic than veratridine (VTD) on insects. Some species specificity was established related to the stereochemistry of the derivatives of veracevine [21]. Aim of the experiments described below was to study the mechanism of action of these newly synthetized analogues at the cellular level, and to compare the effectiveness of the VTD derivatives on *Helix* Na-channels.

On vertebrates, VTD causes a membrane depolarization as a result of persistent opening of sodium channels or slowing its normal inactivation [15]. The mean open time of sodium channels is prolonged by VTD [4], and the single channel conduc-

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tance is decreased [1, 25]. Two modes of binding by VTD have been described, a rapid binding to open channels, and a slow binding, perhaps to inactivated channels [18, 20, 22]. Whichever pathway modification is activated, the characteristics of the modified channels appear to be identical [18, 19]. Once a channel has been modified by VTD, its properties resemble of batrachotoxin (BTX)-modified channels, with some quantitative differences. Electrophysiological and single-channel studies have shown that BTX shifts the voltage-dependent activation curve of the Na-channel toward more hyperpolarized potential, and totally prevents inactivation [6, 7, 9, 13, 16]. The voltage dependence of activation of VTD modified Na-channels is shifted by (–50)–(–90) mV in both muscle and nerve [10, 17]. The ionic selectivity is less than that of unmodified channels, yet differs from BTX modified channels [14], furthermore all alkali metal cations are permeant through VTD-modified channels [17].

In *Helix* neurons VTD depolarized the membrane and caused slow oscillations. In voltage-clamped neurons VTD led to a slowly developing maintained inward current. This maintained inward current disappeared in Na-free Tris-substituted saline, demonstrating, that this current was carried by Na-ions [11, 12]. The VTD effect on snail neurons, however, differs from that on myelinated nerve fiber in two aspects: *(a)* on *Helix* neurons VTD caused oscillations and *(b)* TTX blocked the VTD effect on frog nerve, but was ineffective on *Helix* neurons.

In the experiments described here, the effects of veratrum alkaloids were studied on the kinetics and selectivity of voltage-gated Na-channels of *Helix* neurons.

MATERIALS AND METHODS

Preparation

Experiments were performed on neurons RPa3 and LPa3 located in the subesophageal ganglion of *Helix pomatia* L. Although most of the neurons in *Helix* brain are characterized with Na- and Ca-dependent mechanisms of spike generation, these are known as cells possessing with Na-spikes in nominally Ca-free saline.

Solutions

The normal extracellular solution contained (mM/l): NaCl 80 mM, KCl 4 mM, $CaCl_2$ 10 mM, $MgCl_2$ 5 mM, sucrose 10 mM and Tris Cl 10 mM. Solutions had a pH of 7.4. Na currents were recorded in NaTEA-saline with composition of: NaCl 80 mM, KCl 4 mM, $CaCl_2$ 1.2 mM, $MgCl_2$ 5 mM, TEACl 30 mM, 4 AP (4-aminopyridine) 4 mM and TrisCl 5 mM. Na substituted solutions contained appropriate substitutes in equimolar amounts. The drugs used in this study were all applied externally. In all experiments with veratridine derivatives, the solution was freshly prepared from a 10^{-3} M stock solution made up on DMSO. The DMSO final concentration, which was <1%, did not have any effect on the Na-currents. All experiments were performed at room temperature (~20 °C). The sources of chemicals were as follows.

Veratridine and its derivatives were synthesized by Ujváry (Plant Protection Institute, Budapest). Derivatives used in the study are listed and their chemical structure shown in Fig. 1. Aconitine, 4-aminopyridine (4AP), tetraethylammonium-chloride (TEACl) and TTX were purchased from Sigma.

Current-recording

The experiments were performed using the two-microelectrode voltage-clamp technique. For monitoring voltage and current, electrodes were filled with 2.5 M KCl and had resistance of 1–2 Mohm. Current electrodes were covered with silver paint and grounded in order to minimize the coupling capacitance between the two electrodes. The bath was connected via a 2.5 M KCl-agar and Ag-$AgCl_2$ electrode to operational amplifier.

The voltage-clamp steps were generated from a computer and membrane currents were sampled at 2 KHz with Interface (Axon Instruments). Currents were measured and analysed using the pClamp program and results were traced on an 7475A Hewlett Packard plotter. All currents were leakage compensated using the P/N protocol provided by the computer program. Inactivation curves were fitted with a Boltzmann function, $1 + \exp [(E - E_{0.5})/k]^{-1}$, where E is the membrane potential, $E_{0.5}$ the midpoint potential and k is the slope factor. The recovery from inactivation was fitted by single exponential function.

Ion-selectivity: The selectivity of ionic channels was compared determining the relative permeability of the membrane in Na and the test solutions. Since the internal concentrations of ions were not known, however their concentrations were constant the relative permeability was computed as [5]:

$$P_s/P_{Na} = \exp [zF(V_{rev,\,S} - V_{rev,\,Na})/RT]$$

The equation is derived from the Goldman–Hodgkin–Katz constant-field equation, where $V_{rev,\,Na}$, and $V_{rev,\,S}$, are the reversal potentials in Na containing and Na substitute solutions, respectively, P_S and P_{Na} are permeabilities of the Na-channel for Na and S ions. $[S]_0$ and $[Na]_0$ are extracellular concentrations. R, T, and F have their usual thermodynamic meanings, and z the valence of the current carrying ion.

RESULTS

In normal physiological solution veratridine and cevadine (10^{-5} M) depolarized the membrane of snail neurons. The action potential (AP) amplitude became smaller and often membrane oscillations were observed. When membrane oscillations reached the threshold of the AP, burst of spikes occurred. Depolarization caused by alkaloids was resulted from the increased resting sodium permeability, since in Na-free saline VTD had no effect on the membrane potential [11].

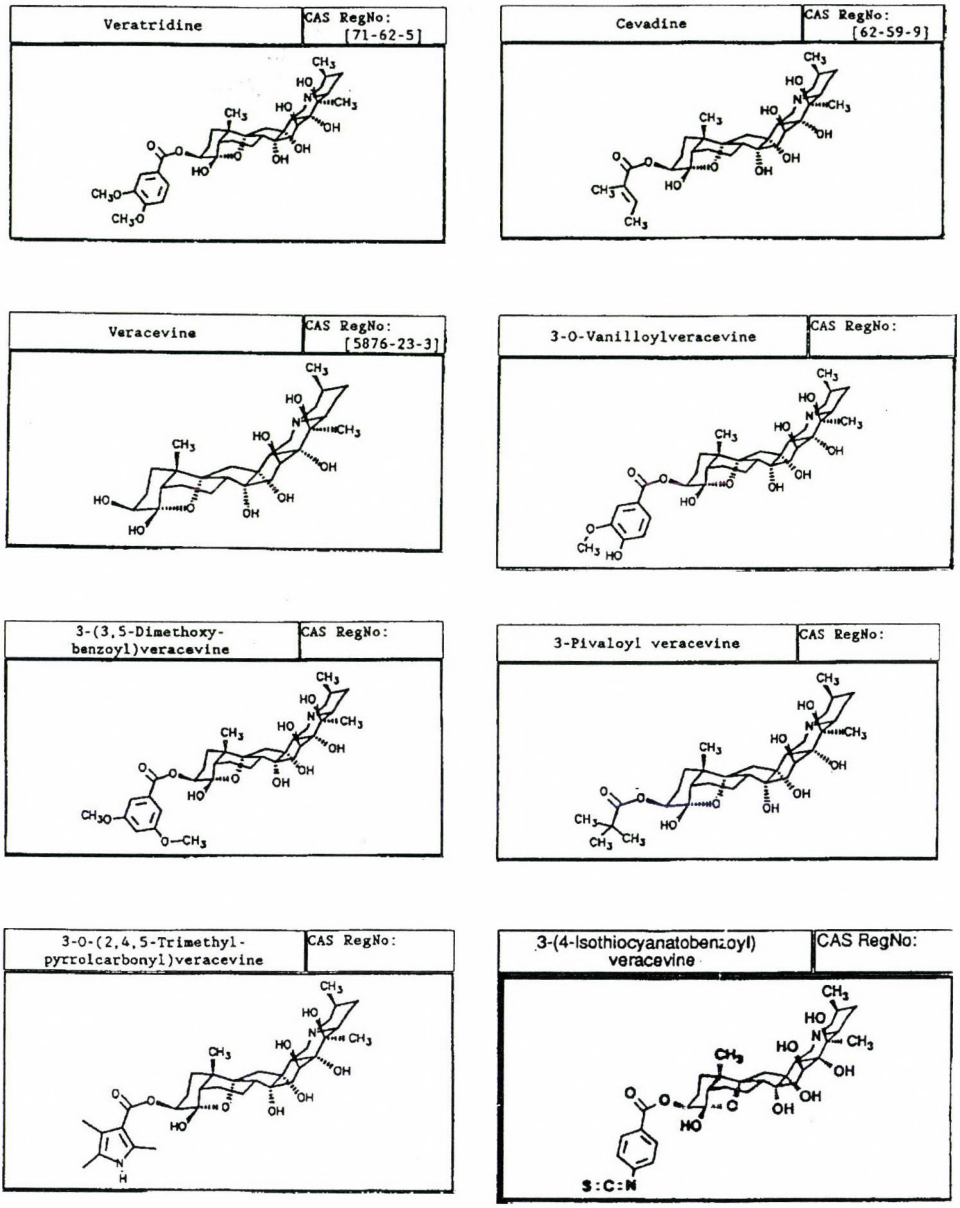

Fig. 1. Chemical structure of lipid soluble alkaloids

Effect of veratridine (VTD)

The effect of VTD and its analogues at concentrations of 10^{-4}–10^{-5} M was studied in voltage-clamped neurons. Figure 2 shows Na currents from the snail neurons during a depolarizing pulse before (a) and after (b) external application of 10^{-5} M VTD. In the untreated fiber, a depolarization causes Na-channels to activate and a normal transient Na current appears, rising to a maximum and then declining as Na-channels inactivate. Characteristic feature of the untreated *Helix* neuronal Na-channels, that they do not inactivate completely [8]. By the end of the 40 ms pulse, inactivation is therefore incomplete and Na current has a steady-state value (Fig. 2). Treatment with VTD had two obvious effects on the I_{Na}: in most cases an increase of the peak amplitude and slowing of the inactivation of the sodium inward current (I_{Na}) was obtained. Current traces clearly show all features caused by VTD binding to the Na-channel. In the presence of VTD, the amplitude of the inward current was larger and the activation occurred earlier. At large depolarizing test potentials, a slowly developing and slowly inactivating inward current component was recorded (b records in Fig. 2). This can be interpreted as a progressive conversion of the normal Na-channels into the alkaloid modified ones. Subtraction of the control current curve from the VTD modified one revealed that the fraction of the modified channels is small. After VTD

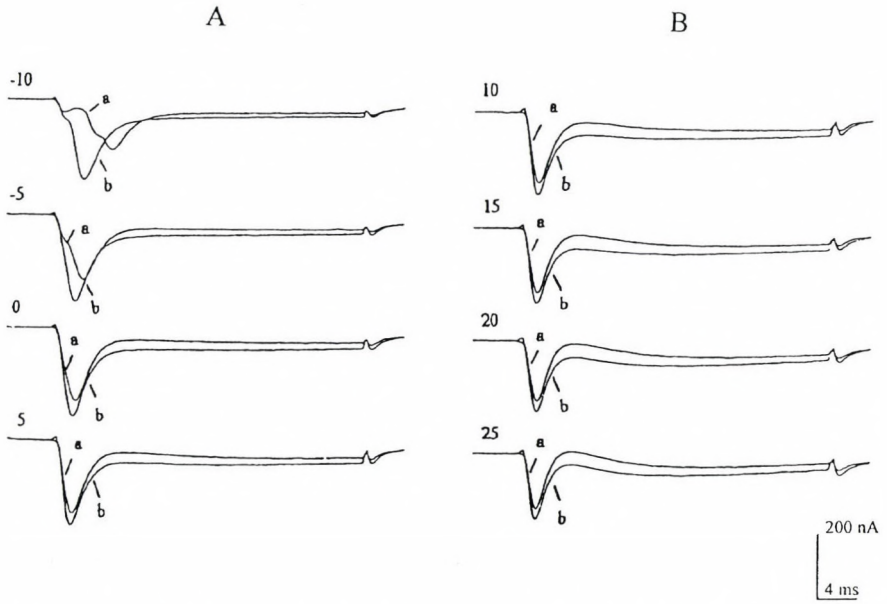

Fig. 2. Sodium inward currents recorded from a neuron bathed in NaTEA-saline (a) and in the presence of 10^{-5} M of VTD. HP = –50 mV. Numbers on the left side of the current traces show potentials up to the membrane was depolarized. Both the increase of the peak I_{Na} amplitude and the steady-state component are seen. At large depolarizations (over 5 mV) the second inward component can be seen, as well

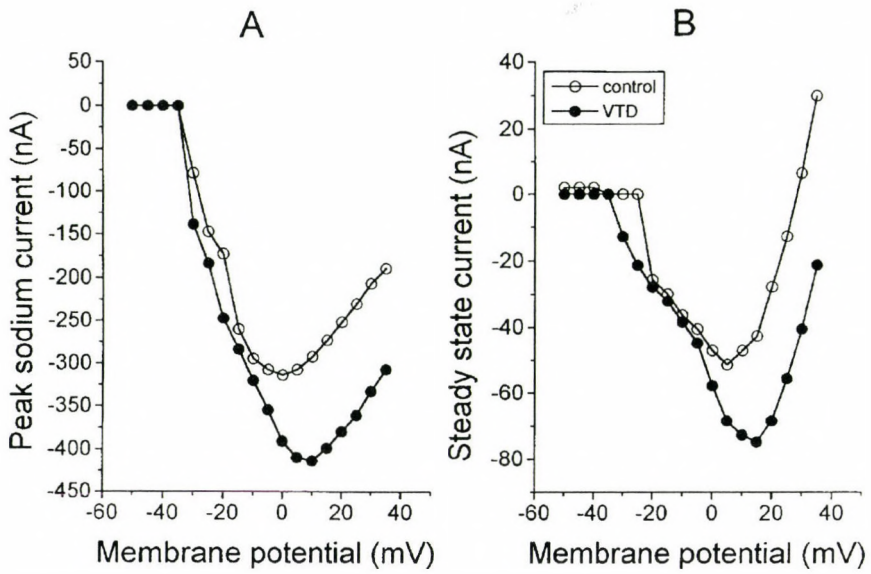

Fig. 3. Current-voltage relationship of I_{Na} in control and VTD treated neurons. Peak amplitudes (A) and the steady-state amplitudes (B) of the I_{Na} measured at the end of 40 ms test pulse are plotted as a function of the potential, before and during VTD application. HP = –50 mV

treatment an augmentation of the I_{Na} amplitude was obtained (Fig. 3A). In 20% of experiments no change, or even a decrease of the I_{Na} amplitude was observed. In both cases, however the steady-state current-voltage (I–V) relationship was shifted in inward direction in the presence of the VTD (Fig. 3B). The effect of the VTD could be observed 3 min following the application, which may suggest that the alkaloid binds to the Na-channel in its inactivated or closed state, too. No use-dependency of the VTD-effect was observed. The VTD-effect is partly reversible on the snail neuronal Na-channels after intensive perfusion with control physiological solution at least for 30 min. Complete restoration was not obtained.

Kinetic analysis of the VTD effect

The activation gating of the VTD-treated neurons was practically similar to the control ones. The steady-state value of the sodium channel activation parameter was calculated from the peak amplitudes of the INa and was plotted as function of the membrane potential (Fig. 4A).The time-to-peak plot showed no difference between the control and VTD-treated neurons. The inactivation parameter was measured applying a double-pulse protocol. Test pulses of constant amplitude to –10 mV and durations (40 ms) followed the 40 ms prepulses which varied in amplitude between –50

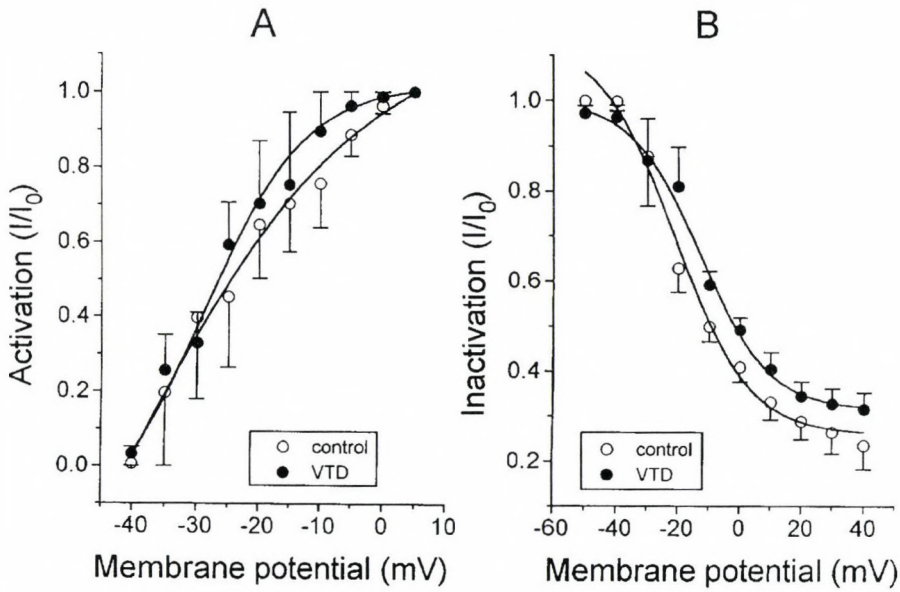

Fig. 4. Potential dependence of activation (A) and inactivation (B) variables of I_{Na}. Data marked with open circles are from the control and full circles are from the VTD treated neurons (n = 6–8). Vertical bars denote the standard error of mean

and +40 mV's. The slope factor (k = 23.7 mV) was larger compared with the control (k = 17.3 mV).

The inactivation was shifted in depolarizing direction. The midpoint of the inactivation curve occurred at –10 mV in control, and +2 mV in the presence of the VTD (Fig. 4B). The difference between these two values proved to be statistically significant (student-test) in average 8.5 mV.

Recovery from the inactivation was determined using a double-pulse method with increasing gap between two identical pulses. Figure 5A shows original current traces recorded before (A) and after adding VTD to the bath (B). On the current traces all of the characteristic changes caused by the alkaloid tratment are clearly seen: increase of the peak amplitude, increase of the steady-state current. The amplitude of the depolarizing pulses was selected to induce maximum response. Fit of the peak values of I_{Na} elicited by second pulse at increasing time interval followed the first pulse, showed a simple exponential function (Fig. 6). Recovery from inactivation was faster in alkaloid treated neuron than in control.

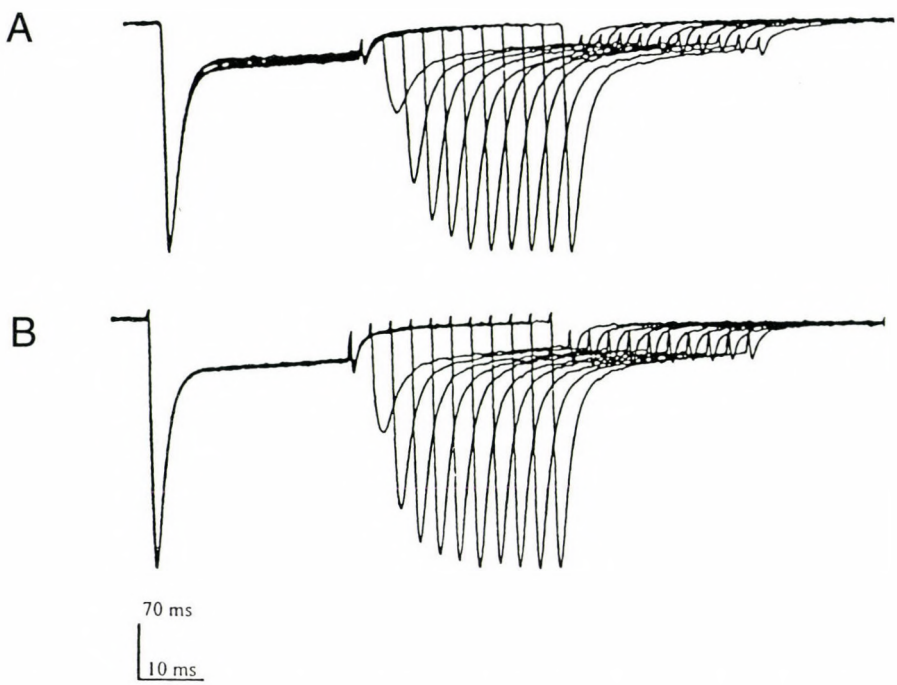

Fig. 5. Original current traces from the recovery experiment. A – control, B – VTD-treated neuron

Other analogues

Similar kinetic analysis was made in the presence of derivatives of the VTD. The results are presented in Table 1.

The kinetic properties following the VTD derivative, treatment were qualitatively similar of that observed in the case of the VTD.

In all neurons investigated, cevadine, veracevine, 3-0-(2,5-trimethylpyrrocarbonyl) veracevine, 3-0-vanilloveracevine, 3-pivaloil-veracevine, 3-(3,5-dimethoxybenzoil)-veracevine and 3-(4-isothiocyanatobenzoyl)veracevine changed the peak I_{Na} in a non-significant manner. In some cases 5% increase or decrease of the I_{Na} amplitude was observed. In other instances they had no effect either on the peak amplitude or on the current amplitude measured at the end of the 40 ms test pulse. In *Helix* neurons sodium channels are not highly selective for cations. In control saline the selectivity order is: Li (1.25) > Na (1.0) > TEA (0.62) > Rb (0.61) > Cs (0.4). The selectivity was determined from the maximum values of the I–V curves (lower-row in Table 1), or from the difference of the reversal potentials of the Ina obtained in Na containing or substituted saline (upper row in Table 1). It can be concluded that all of the VTD analogues have almost equally slightly decreased the selectivity of the

Table 1
Kinetic parameters and ion selectivity of the snail Na-channels

Compound	Erev (mV ± S.D.)	$\Delta h_{0.5}$ (mV)	k (mV)	P_{Li}/P_{Na}	P_{Rb}/P_{Na}	P_{Cs}/P_{Na}	P_{TEA}/P_{Na}	Recover $\Delta\tau$ (ms)
Control	57.5 ± 8.7 (n = 39)	–	17.3 ± 2.7	1.25 0.88	0.37 0.61	0.37 0.40	0.61 0.62	–
VTD	64.6 ± 20.1 (n = 15)	+8.53	23.7 ± 3.1	1.08 1.15	– 0.73	0.60 0.80	0.70 0.72	1.82
VCN	66.5 ± 19.5 (n = 6)	+6.86	9.7 ± 1.3	0.96 0.52	0.34 0.50	0.57 0.50	0.59 1.20	1.98
CEV	65.5 ± 14.7 (n = 6)	+6.9	14.5 ± 1.4	0.93 0.78	0.62 0.52	0.25 0.59	0.31 0.47	3.93
PIV	56.9 ± 9.6 (n = 7)	+5.4	18.8 ± 2.8	–	–	–	–	1.25
VANILLO	57.1 ± 12.8 (n = 8)	2.8	14.6 ± 1.5	–	–	–	–	0.1
DIMETH	63.0 ± 11.6 (n = 6)	0.17	16.8 ± 2.0	– 0.81	–	– 0.53	– 0.85	1.96
TRIMETH	53.6 ± 9.3 (n = 10)	10.8	18.1 ± 1.3	–	–	–	–	–1.2
ISOTH	45.6 ± 13.6 (n = 8)	–5.3	12.8 ± 1.7	–	–	–	–	0.3

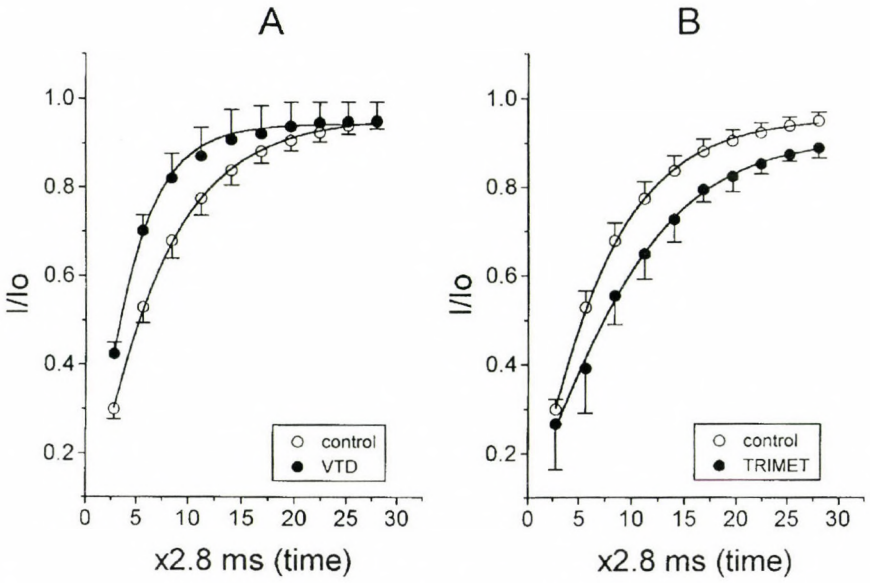

Fig. 6. Recovery from inactivation fitted by single exponential. In the presence of veratridine the recovery from inactivation was faster than in control saline (A). Trimethylveracevine had an opposite effect on the recovery, it slowed down the kinetic of the Na-channel (B)

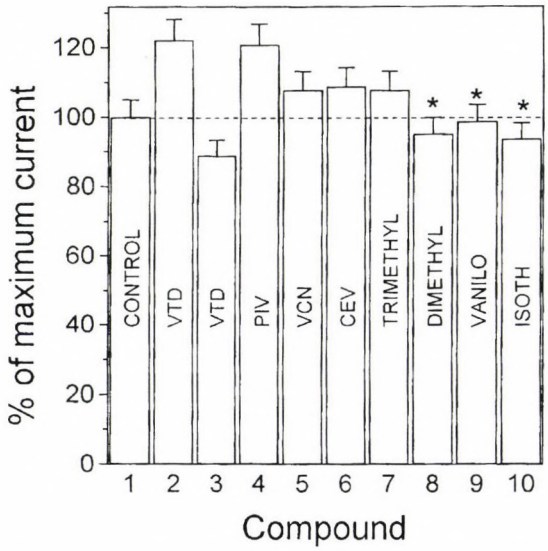

Fig. 7. Summary of the effects of veratridine analogues on the I_{Na}. The decreasing and increasing effect of VTD were separated while both were statistically significant. Stars above bars denote statistically not significant differences at 95%

channel, however, the selectivity sequence was not altered. All VTD analogues have modified the properties of the sodium channel similarly to that of VTD or cevadine. The VTD derivatives did not influence the activation neither of the time to peak curves. Only quantitative differences in the action were observed, except trymethylveracevine, which shifted the recovery from inactivation curve opposite compared to VTD (Fig. 6B). VTD, VCN and cevadine proved to be the most effective compounds among VTD derivatives. Figure 7 summarizes the effect of VTD analogues on the I_{Na} amplitude. All analogues increased the peak values of the I_{Na} except vanillo-, dimethyl- and isothiocyanatobenzoyl-veracevine, which consistently decreased the amplitude of the I_{Na}. This decrease was not significant. Except for vanillo- and dimethoxy-veracevine, a positive shift of the inactivation curve was observed. Vanilo- and isothiocyanatobenzoyl-veracevine had no influence on the time constant of the recovery from inactivation (Table 1).

DISCUSSION

The major goals of this work were (i) to compare the actions of veratridine analogues on the snail neuronal Na-channels, and (ii) to seek for differences in the effects, that might explain difference in their toxicity [21]. We conclude that (i) VTD, and its analogues depolarize the membrane which depolarizations are caused by increased permeability of Na-channels; (ii) The effect was observed almost immediately (2–3 min) upon drug application; (iii) VTD and its analogues applied extracellularly to snail neurons make inactivation of INa incomplete during a depolarizing voltage-clamp pulse; (iv) Neither of the investigated alkaloids shifted the activation curves significantly; (v) The inactivation parameter and recovery from inactivation was affected significantly; (vi) VTD and its analogues decreased the selectivity of the Na-channels towards cations.

The lipid-soluble alkaloid VTD and its derivatives are reversible activators, or partial agonists of the voltage-dependent Na-channels of several excitable cells. These toxins, binding to the receptor site 2 of the Na-channel, cause persistent activation of Na-channels at the resting membrane potential by blocking channel inactivation and shifting the activation and inactivation curves towards more negative potentials [2]. The VTD actions on the Na-channels are very similar on the frog muscle and nerve cells [10, 18, 20, 23, 24], rat myocytes [26] and neuroblastoma cells [1]. It is widely accepted that VTD readily binds to open Na-channels, but poorly, if at all to the channels being in closed or inactivated states.

The effects obtained on *Helix* neurons, are therefore somewhat different from those obtained on vertebrate preparations. One of the similarities is the depolarizing effect. The order of effectiveness causing depolarization is equated with the effectiveness changing of the I_{Na} amplitude (Fig. 7). Difference was obtained in shifting both of the activation and inactivation curves along voltage axis compared to the data obtained on vertebrate Na-channels.The activation parameter was not shifted, while the inactivation parameter was shifted towards positive potentials. A major result of

this study is the demonstration that the selectivity of the alkaloid modified channels have changed. No VTD derivative proved to be more effective on the *Helix* Na-channels than VTD. This is not the case in insects where in the toxicological experiments 3-pivaloilveracevine was as toxic as VTD, and 3-(3,5-dimethyoxybenzoyl)veracevine proved to be several times more toxic than VTD or cevadine. This difference in the effectiveness of VTD derivatives could be explained by species specificity of the alkaloids tested [21], or by differences observed in the molecular structure of the Na-channels between vertebrates and insects.

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BIRDS AS OBJECTS IN BIOINDICATION OF RADIOACTIVE POLLUTION*

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This article is a review the recent results of research in the accumulation of natural and artificial radionuclides in birds from Russia (Adigea, Krasnodar, Rostov, Astrahan and Moscow regions, Novaya Zemlya isles), Ukraine, Vietnam, Poland, Ethiophia and Mongolia after global precipitation and local pollution, such as in the East-Urals radioactive region and radioactive zones after the Chernobyl accident. Resident birds reflect local level of radionuclide contamination. The ^{90}Sr concentration in the food of the Pied Flycatcher had a tendency to increase in dependent of age. The Common Jay and the Mallard were the most contaminated with ^{137}Cs in the Bryansk region. The total content of various radio-isotopes of plutonium in bird bones from Southwest Russia were hundred and thousand times more, than in Mongolia. Activity levels in specimens from Ethiopia bear record to Ethiopia can notbe a "pure" control site in radioecological research and radioactive background since it does not significantly differ from Turkmenia and Mongolia.

Keywords: Birds – radionuclides – bioindicators – local contamination – global contamination

INTRODUCTION

The problem of radioactive pollution is extremely urgent in Russia in connection with presence of territories polluted by radionuclides on places of nuclear tests, in zones around the enterprises on production, processing and storage of radioactive materials, and also in areas of emergency pollution in the Southern Urals and Chernobyl. But also attempt of creative use of underground nuclear explosions for a lining drainpipes and the creations of reservoirs at Semipalatinsk nuclear range and in Perm area have shown, that this problem was not previously solved, as again created lakes, or rather – grounds, surrounding them, are polluted by radionuclides over limits admitting their economic use. All the same wild plants and animals constantly inhabit on these polluted grounds, as well as on grounds of technological radioactive pollution at the mining and smelting and chemico-metallurgical, radiochemical

*Dedicated to Professor János Salánki for his 70th birthday.

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enterprises for manufacture of nuclear meaterials, which include 14 areas, territories and republics of Russia; the original ecosystems, which develop in conditions of strong influence of ionizing radiation form up there. The clearing of such soils from radionuclides, first of all ^{90}Sr, ^{137}Cs, ^{239}Pu, manages more than in 2 mlrd. rubles for one hectare at the price of 1995. Therefore such grounds will not be disactivated in the foreseeable future, as the area only of technological radioactive pollution averages 61,000 hectares in Russia, and there are even more zones of emergency pollution. Soviet and Russian ecologists have monitored dynamic of such ecosystems for a long time, including the change of a biodiversity on the polluted grounds [6, 7, 15].

The Chernobyl disaster, that acquired the significance as worldwide ecological catastrophe, aroused interest in investigating the processes of radionuclide migration in ecosystems. After the accident, large wetlands, that are the living and reproductive habitats of many species of birds were contaminated with radionuclides. Birds which occupy middle and last levels of trophic chains are an important components of terrestrial and water ecosystems. The problem of radionuclide accumulation in birds has been already in the spotlight, since the danger of the impact of artificial radioactive isotopes in ecosystems after accidents, nuclear tests and waste products on living organisms had been recognized.

Birds as objects of bioindication of radioactive contamination present undoubted interest. This animal group has an intensive metabolism and consumes large quantities of food per unit of body mass. Birds are calcium concentrators, that is very important in control ecosystems polluted with ^{90}Sr, and vertebrates, which having large musculature, are accumulators of ^{137}Cs. Polluted biotopes are often attractive for birds in areas without human settlements where they enter a process of biogenic migration of ecotoxicants. Thanks to these factors it is possible to release a monitoring program using birds as bioindicators of radioactive contamination [8, 9, 11].

The main aim of this article is to review the recent results of our research in the accumulation of natural and artificial radionuclides in birds after global precipitation and local pollution, such as in the East-Urals radioactive region and territories, surveying the radioactive zones after the Chernobyl accident (Ukraine and the Bryansk region of Russia).

Ethiopia and Mongolia attracted our attention as a likely are for "pure" controls for the study of accumulation and migration of artificial radionuclides in food chains of natural ecosystems. Non-transformed natural steppe landscapes were conserved in Mongolia. There are no any nuclear power plants in Ethiopia and the adjoining countries, nuclear tests did not affect Ethiopia, and it was supposed that Ethiopia was not polluted with radionuclides after the Chernobyl accident in April 1986.

MATERIALS AND METHODS

The study was carried out in 1989–1992 in the vicinity of the Chernobyl nuclear power plant (NPP) in the Prepyat Polesye region, NW Ukraine, Bryansk region and in the East-Ural radioactive region. This places of research were two areas with sim-

ilar habitats but with different levels of radioactive contamination on site with similar biotopes and different levels of radioactive contamination. In the Urals, three sites of birch forest were selected: "Lejnevka" (level of contamination by ^{90}Sr on ground was 800 kBq/kg), "Berdenish" (140 kBq/kg) and the control (5 kBq/kg); in the Chernobyl area – three sites of mixed forest: "Schepelevichi" (6 km of the NPP, before 885 Ci/km^2), "Izumrudny" (7 km SE of the NPP, before 91 Ci/km^2) and "Oranoye" (40 km of the NPP, less 5 Ci/km^2). The level of soil contamination with radioactive substances was up to ^{90}Sr Ci/km^2 (according to "The map scheme of radiation damage of coniferous forest in the region of accident on the Chernobyl Nuclear Power Station, Syktyvkar, 1991"). A method of gamma non-destructive sectrometer measurement and radiochemistry analysis for determination of ^{137}Cs, ^{134}Cs and ^{90}Sr in eggs, bodies and in the digestive tract contents of the Great Tits *Parus major* and the Pied Flycatchers *Ficedula hypoleuca* (Chernobyl, Urals), ^{137}Cs in muscles of the Mallard *Anas platyrhynchos,* the Northern Shoveler *A. Clypeata,* the Common Partridge *Perdix perdix,* the Corncrake *Crex crex,* the Common Snipe *Gallinago gallinago,* the Common Cuckoo *Cuculus canorus* and the Common Jay *Garrulis glandarius* (Bryansk region) was used.

Radionuclide (cesium ^{137}Cs, strontium ^{90}Sr, plutonium $^{238,239,240}Pu$, potassium ^{40}K, thorium ^{232}Th and radium ^{226}Ra) concentrations were measured in bones and bodies of birds from the Rostov region (the Common Quail *Coturnix coturnix,* the Common Buzzard *Buteo buteo*), Mongolia (the Dalmatian Pelican *Pelicanus crispus,* the Roody Sheld *Tadorna ferruginea,* the Black Kite *Milvus migrans,* the Tawny Eagle *Aquila rapax,* the Greater Spotted Eagle *A. clanga,* the Upland Buzzard *Buteo hemilasius,* the Saker Falcon *Falco cherrug,* The Chukar Partridge *Alectoris chukar,* the Herring Gull *Larus argentatus*), Ethiopia (the Marabou *Leptoptilos crumeniferus* and the African race of the Indian White-backed Vulture *Gyps bengalensis,* domestic hen). This was first determination of plutonium in birds from Mongolia. The radionuclide content was determined by a method of non-destructive spectrometer measurement and radiochemistry analysis. Radionuclide contents are in Bq/g of dry weight. The analysis was carried out in the Laboratory of Radioecology at the Institute of Biology, Komi Branch of the Ural Department from the Russian Academy of Sciences.

Animals were collected in Mongolia and Ethiopia for radio-chemistry analysis according to the scientific programs of Russian-Mongolian and Russian-Ethiopian Expeditions, and we collected bones of dead animals on roads and near electrical lines. Skeletons were prepared and combined in one specimen for each species equal to 1 kg.

Part of this study was carried out 1995–1998 in different regions. Materials were presented by birds from Russia (Adigea, Krasnodar, Rostov, Astrahan and Moscow regions, Novaya Zemlya isles), Ukraine, Vietnam, Poland. Part of materials was collected in 1998 with Russian-Ethiopian biological Expeditions in tropical forest (South-Western Ethiopia, Jima). Bird bodies were dried and homogenized. Multi-element analysis was realized for 43 individuals of 17 species of birds: the Blue Monarch *Hypothymis azurea,* the Kingfisher *Alcedo atthis* (Vietnam), the Pied Wagtail *Motacilla alba* (Novaya Zemlya), the Reed Warbler *Acrocephalus scir-*

paceus (Krasnodar region), the Chiffchaff *Phylloscopus collybita* (Adigea), the Pied Flycatcher *Ficedula hypoleuca* (Moscow region), the Great Tit *Parus major* (n = 5, Adigea; n = 1, Eastern cost of Azov sea, Krasnodar region; n = 1, Rostov region, and n = 1, Vietnam), the Swift *Apus apus,* the Tree Sparrow *Passer montanus* (n = 2, Rostov region; n = 1, Moscow region, n = 2, Ukraine, Odessa; n = 2, Adigea; n = 2, Eastern cost of Azov sea, Krasnodar region; n = 1, Poland, Warsaw) the House Sparrow *P. domesticus* (n = 1, Astrahan region; n = 2, Rostov region; n = 1, Adigea; n = 2, Rostov-on-Don), the Goldfinch *Carduelis carduelis* (n = 1, Rostov region, n = 1, Adigea), the Serin *Serinus serinus* (Rostov region), the Common Rosefinch *Caprodacus erythrinus* (Adigea), the Olive Sunbird *Nectarinia olivacea* (n = 4), the Variable Sunbird *Nectarinia venusta* (n = 1), the Yellow-fronted Canary *Serinus mozambicus* (n = 2), he Tawny Pinia *Prinia subflava* (n = 1) (Ethiopia).

A method of ionic plasma spectrometry (ICPMS) with a Plasma Quad "V G Instrument", which led to obtaining quick authoritative data of element concentration until the 10^{-12}th digit, was used for the first time on animals in this study. Determination was done in the Institute of Geology of mineral deposits, petrography, mineralogy and geochemistry RAS (Moscow).

RESULTS

Chernobyl radioactive zone

Radionuclide activities in the food and body of the Pied Flycatcher at different ages are presented in Table 1. Nestlings of the Pied Flycatcher raised in strongly contaminated sites accumulated more radionuclides (about ten times), then those raised at the control site. This was a common trend for all radionuclides: activities of radionuclides in birds decreased along gradient from the strongly contaminated area to the control site (Fig. 1). Fledglings of this species had a similar trend. Levels of contamination of sites influenced the levels of ^{137}Cs, ^{134}Cs and ^{90}Sr accumulated in the Pied Flycatcher bodies (for ^{137}Cs ANOVA: df = 2 + 73, F = 28.73, P = 0.0001; for ^{134}Cs: ANOVA: df = 2 + 73, F = 9.68, P = 0.0002; for ^{90}Sr ANOVA: df = 2 + 73, F = 33.86, P = 0.0001). Age-depended variation of ^{90}Sr accumulation were founded in the Pied Flycatcher (ANOVA: df = 2 + 73, F = 8.72, P = 0.0004). Fledglings more accumulated more radio-strontium than nestlings and adult birds (Table 1, Fig. 2). There was not a similar trend in cesium accumulation. Age-factor did not have a significant influence on the strontium content.

The East-Urals radioactive region

The consistent pattern of radionuclide accumulation, that had been found near Chernobyl, was not accidental. It was affirmed by the pattern of radionuclide accumulation of the Pied Flycatcher and the Great Tit in the East-Urals radioactive region.

Table 1

^{137}C, ^{134}Cs and ^{90}Sr activities (Bq/g d.w.) in food and body of Pied Flycathcer at different ages from Chernobyl area

Specimens	Radioactive site "Schepelevichi"							Radioactive site "Isumrudny"							Control site "Oranoye"						
	^{137}Cs			^{134}Cs		^{90}Sr		^{137}Cs			^{134}Cs		^{90}Sr		^{137}Cs			^{134}Cs		^{90}Sr	
	N	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	N	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	N	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD
FOOD																					
Nestlings	1	1.1	0.09	0.1	0.05	1.2	0.2	–	–	–	–	–	–	–	2	0		0		1.5	0.56
Fledglings	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1	0.8	0.17	0		3.9	0.96
BODY																					
Nestlings	9	1.8	1.13	0.26	0.13	6.29	4.01	20	0.55	0.69	0.1	0.21	2.03	0.93	18	0.09	0.14	0.02	0.07	0.82	0.63
Fledglings	–	–	–	–	–	–	–	17	0.27	0.15	0.05	0.07	5.28	1.89	7	0.13	0.03	0.02	0.06	0.68	0.69
Adults	–	–	–	–	–	–	–	3	0.23	0.06	0.09	0.01	2.63	2.10	–	–	–	–	–	–	–

(N – sample size, $\bar{x}$ – mean, SD – standard deviation)

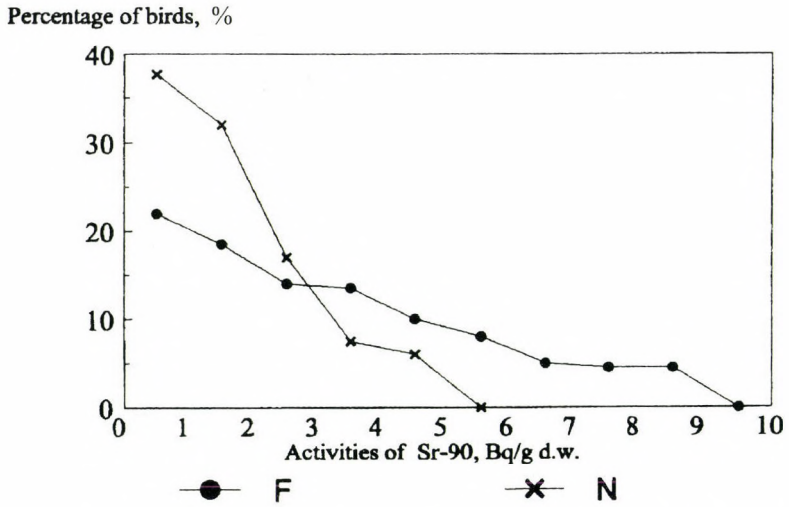

Fig. 1. Average activities of radionuclides (Bq/g of dry tissue) in bodies of the Pied Flycatcher fledglings in gradient of radioactive contamination in Chernobyl

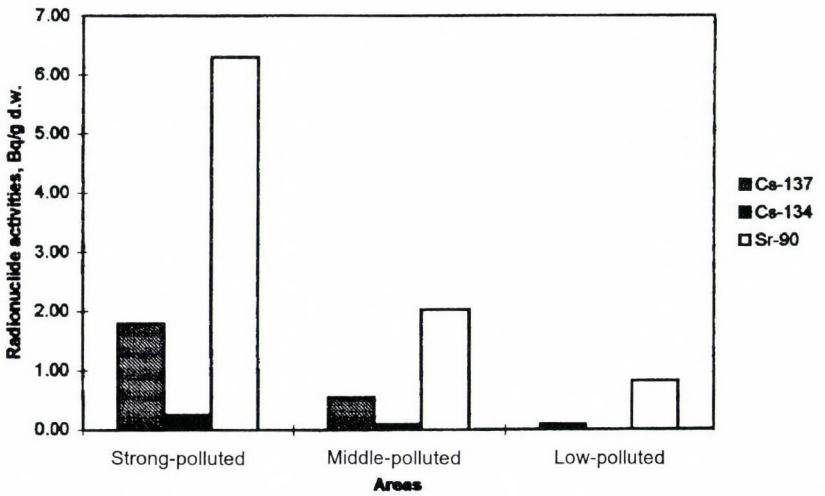

Fig. 2. Average activities of radionuclides (Bq/g of dry tissue) in bodies of fledglings and nestlings of the Pied Flycatcher and the Great Tit, "Izumrudny" area in Chernobyl

The Great Tits, inhabiting a more contaminated area ("Lejnevka"), accumulated more significantly radionuclides, than those living in low radioactive biotope ("Berdenisch") (Fig. 3). However, the Pied Flycatchers from the low polluted site accumulated more cesium and strontium. But, samples of the two species, that we analyzed, were not homogeneous, because adult birds came in less contact with radioactive biotope as compared with nestlings and fledglings in the Great Tit sample.

Bryansk region

Contamination of birds with ^{137}Cs from Bryansk region were unequal for different species. The pattern of the average activity of this radionuclide in seven species of birds is shown in Fig. 4. The Common Jay and the Mallard were the most contaminated. The Mallard is a commonly hunted species, which are usually killed by hunters in great numbers. All of the ducks collected in August for research were polluted with strontium. Young ducks born locally had a higher concentration of radionuclides (on the average, 39,805 kBq/kg, n = 19), than adult birds (on the average, 37,526 kBq/kg, n=4).

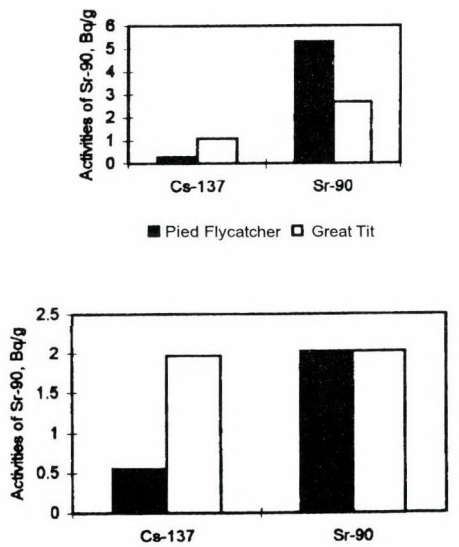

Fig. 3. Average activities of ^{90}Sr and ^{137}Cs (kB/g of dry tissue) in bodies of the Pied Flycatcher and the Great Tit in the East-Urals radioactive trace

Table 2
Concentration of thorium and uranium isotopes in bird bodies from different areas, ppm of d. w.

Species of birds	^{232}Th 10^{-2}	^{238}U 10^{-2}	Area
Alcedo atthis	27.9	18.9	V
Hypothymis azurea	5.8	12.6	V
Motacilla alba	19.1	46.7	NZ
Apus apus	2.9	8.6	R
Acrocephalus scirpaceus	0.3	1.1	K
Phylloscopus collybitus	0.3	0	A
Ficedula hypoleuca	2.7	2.7	M
Parus major	2.6	9	K
	0.2–30.7*	0–34.3	A
	0.1	0	R
	7.0	17.6	V
Passer montanus	2.5–5.9	0	K
	0.6–2.0	0	R
	78.6	39.3	M
	59–126	146–155	U
	2.5–5.9	0–6.3	A
	4.4–4.9	0	K
	7.0	0	P
P. domesticus	0.9–5.3	0	R
	3–11.1	0–95.1	R
	9.6	0	As
	3.6	0	K
Carduelis carduelis	1.8	15.4	R
	4.1	0.7	A
Serinus serinus	2.7	19.1	R
Caprodacus erythrinus	5.9	0	A
Nectarinia olivacea	0–14	0	E
N. venusta	0	0	E
Serinus mozambicus	3–19	0	E
Prinia subflava	0	0	E

*Limits of concentrations

Areas abbreviations: V – Vietnam, NZ – Novaya Zemlya, R – Rostov region, K – Krasnodar region, A – Adigea, M – Moscow region, U – Ukraine, Odessa, P – Poland, As – Astrahan region, E – Ethiopia

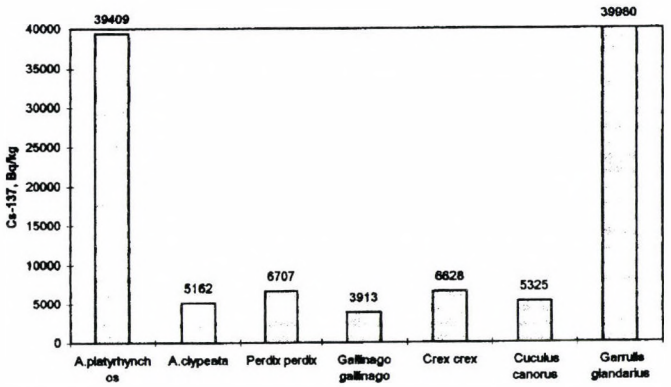

Fig. 4. Average activities of ^{137}Cs (Bq/g of dry tissue) in birds from the Bryansk region

Global level accumulation of radionuclides in birds

We estimated a content of natural radionuclides of thorium and uranium in small passerine birds from eight different areas (Table 2, Fig. 5). Thorium content varied on areas (ANOVA: $df = 8 + 32$, $F = 10.304$, $P < 0.001$), as well as uranium content (ANOVA: $df = 8 + 32$, $F = 17.345$, $P < 0.001$). The lowest content levels of natural radionuclides in birds were in Ethiopia. Data describing the activities of some artificial and natural radionuclides in bodies or bones of birds in the 1980s and 1990s is presented in Table 3.

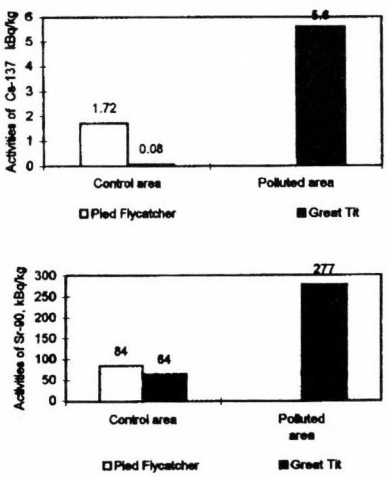

Fig. 5. Average concentration of thorium and uranium (ppm) in bodies of small passerine birds from different areas

Table 3

Concentration of radionuclides in bodies of birds from different ecosystems.
The data are given in Bq/kg d.w. Data marked with star were obtained 1960–1970s

Species	Ecosystem	Localities	Tissue	^{137}Cs	^{90}Sr	$^{238,239,240}Pu$	^{40}K	^{232}Th	^{226}Ra
Sterna paradisaea	Arctic	Franz-Joseph Land	Bones	19	8.34	1.2			
Aethya cristatella	Tundra	Great Dyamid Island	Bones	23	56.5				
Lagopus lagopus	Taigra	Pechera-Ilichsky	Bones	36					
Lagopus lagopus		Reservate	Musculus	70	23				
Tetrao urogallus			Bones		38				
Bonasa bonasia			Bones	60	18				
Parus major	Mixed forest	Beresinsky Reservate	Body	40	28				
Emberiza citrinella		Dalny Vostok	Body	6	15				
Dendrocopos leucotos			Body	6	8				
Buteo buteo	Steppe	Rostov region	Body	16	117.8	0.21			
Coturnix coturnix			Body	147.1	344.1	2.6			
Corvus frugilegus			Body		1.587	0.1			
Aquila rapax		Central Mongolia	Bones	16	27	0.057	380	46	68
*Aquila clanga**			Bones	0					
*Milvus migrans**			Bones	0					
Milvus migrans			Bones	0.1					
Milvus migrans			Bones	1.4	1.86	1.5			
Tadorna ferruginea			Bones	24	38	0.098	300	56	54
*Pelicanus crispus**			Bones	21	27	0.006	280	49	61

*Alectoris chukar**			Bones	0.1					
Corvus corax			Bones	1.2	34.1	0.6			
Larus argentatus			Bones	4.6	25.4	0.6			
Larus argentatus			Bones	18.6	53	0.4			
Buteo hemialasius			Bones	5.1	7.3	0.6			
Falco cherrug			Bones	16	270		270	31	39
Domestic hen	Savanna	Ethiopia	Bones	0.41	60.8				
Leptoptilos crumeniferus			Bones	1.9	6.6	2			
Gyps bengalensis			Bones	2.82	368.9	0.7			
Aquila heliaca	Deserted	Kalmykia, Reservate	Body	16	33				
Aquila rapax	Steppe	Daghestan	Bones	19.4					
Aquila rapax			Bones	3.0	250				
Aquila heliaca			Bones		250				
Buteo rufinus	Deserts	Kara-Kum, Body	16						
Melanocorypha calandra		Repetek Reservate	Body	11	10				
Galerida cristata			Body	7					
Podoces hendersoni		Gobi, Mongolia	Body	34					
Milvus migrans			Bones	2.8	20.6	0.5			

Table 4
Radionuclide content in birds and mammals from arid ecosystems in 1990s, Bq/kg d.w.

Area	^{137}Cs		^{90}Sr		$^{238,239,240}Pu$	
	sample	mean ± SE	sample	mean ± SE	sample	mean ± SE
Rostov region	2	81.55 ± 65.55	3	154.4 ± 100.6	3	1.35 ± 1.77
Dagestan	3	8.40 ± 5.50	3	173.5 ± 76.5	–	–
Mongolia	13	8.31 ± 2.57	9	26.74 ± 15.3	13	0.57 ± 0.47
Ethiopia	11	3.51 ± 0.84	11	67.95 ± 34.47	14	0.94 ± 0.51
Australia	–	–	8	43.88 ± 7.79	8	<2 ± 0

We compated data of radionuclide content in birds and mammals from Ethiophia with data we collected in other arid areas [10] and with data collected by D. A. Krivolutski and V. E. Sokolov in arid ecosystems of Australia (Table 4). ^{137}Cs content significantly varied in birds and mammals independent of areas (ANOVA: df = 3 + 25, F = 9.006, P = 0.003), but the effect of "area" for ^{90}Sr was not significant (ANOVA: df = 4 + 29, F = 1.479, P = 0.2341). Animals from the Rostov region contained ^{137}Cs in quantities about 23 times greater than those from Australia, and 10 times more, then those from Ethiopia. Thus, Eastern Africa is an area registering a higher global radionuclide precipitation than Australia.

DISCUSSION

Local contamination

A trophic-dynamic view in researching a consistent pattern of pollutant transformation in ecosystems, is very important for selection of objects as bioindicators of environment contamination. It is well known that the concentration of several toxic substances increase in the last elements of food chain. According to this, all insectivorous and raptor bird species are interesting as objects of bioindication. The Great Tit and the Pied Flycatcher occupy different ecological niches of the ecosystem. One of the important factors of life in a contaminated environment is the contact of adult birds and nestlings with sources of ionizing radiation. Differences in species' biology (habits of feeding, nest building, and position in the food chains) can determine how vulnerable a species is to ionizing radiation.

A comparison of radionuclide accumulation of these species showed, that fledglings of the Pied Flycatcher from the polluted area contained about twice more strontium, but cesium content in its bodies were twice less as compared fledglings of the Great Tit (Fig. 2). Nestlings of this species did not differ by strontium content, while cesium activity of the Pied Flycatcher nestlings was twice less than that of the

Great Tit (Fig. 2). Such differences could be explained by the different diets of the species. It is known that the Great Tit fledged nestlings prefer larves of *Lepidoptera* and collect prey in middle forest layer, whereas the Pied Flycatcher chooses more hitinized preys of the forest, in bottom layer. More cesium content in nestling and fledgling bodies of the Great Tits may be explained by the domination of fleshy caterpillars in diet of this species. The differences in cesium accumulation in the boies of the two species form gradually and become more significant as the fledgling age, when the diet of the two species differ to a larger degree than at nestling age.

The degree of concentration of radioactive substances in an animal's body depends on many factors. There are three ways of radionuclide entrance in organism: cross respiration organs, alimentary tract and integument [3]. The Great Tit and the Pied Flycatcher bring to nestlings imago and larvae of *Lepidoptera,* that are consuments of the first order in food chain. The Pied Flycatcher bring often spiders, consuments of the second order, had a lower concentration of ^{90}Sr than the *Lepidoptera.* Radionuclide concentration in skeletons of the Great Tits from the contaminated area in the East-Ural radioactive region was 14.5 times more, than that in the food of birds [3]. Radionuclide concentration in bodies of forestry birds, feeding in the tree canopy, was significantly lower than that in birds feeding on prey near the ground [3].

The main dose-forming radionuclides 3 years after of the Chernobyl NPP accident are ^{137}Cs and ^{90}Sr [2]. The Great Tit has strong contact with the contaminated area during breeding. It also uses the different parts of radioactive habitat for feeding, nest building and nesting. During that time the Great Tits visit nesting area often, collecting from the ground and from different parts of the tree crown nesting material and foraging for nestlings [9]. The size of the foraging grounds of Great Tits that nest in similar conditions are practically similar in size, constituting 10,400 m^2 [4]. Thus, adult birds which nest in radioactive area probably come in close contact with radioactive sources during the breeding season.

Radionuclides enter the body of Great Tit via the food chains of the radioactive ecosystem and, being incorporated in metabolism instead of Ca (^{90}Sr) and K (^{137}Cs), they generate an endogenous β- and γ-radiation. It is known that the diversity of food items consumed expands in the sequence "nestlings – fledglings – adults" [4]. According to our study the concentration of ^{90}Sr in the food of the Great Tit [12] and the Pied Flycatcher (Table 1) at these ontogenetic stages also had a tendency to increase. This can be explained by the fact that arthropods appeared in the food of fledglings and adults. Arthropods dwell in the lower layer of radioactive ecosystem, and radionuclide accumulation accrue more quickly than they are excreted from these organisms. The average concentration of ^{90}Sr in the food consumed by Great Tits in the Polluted area was roughly 3–5 times higher than that in the diet of tits from a less contaminated area [12].

One of the problems of bird radioacology is associated with their ability to move great distances during migration [1]. Because of this, radionuclides accumulated in bird bodies, were carried over thousands of kilometers from the radioactive areas.

Territorial links of the Ural's birds are wide and cover almost all of southern Eurasia and Africa. Wintering areas for waterfowl from the Urals are regions of the Caspian, Black and Mediterranean seas [13]. Migrant and wild birds entered into a process of biogenic radionuclide migration and most importantly included those in humans' diet (ducks, geese, gallinaceous, rallen and charadriiformes). It is very important to monitor toxicant content in the bodies of game birds. Resident birds reflect local level of radionuclide contamination, however, migrating species are able to move toxicants across large distances. We examined resting and feeding flocks of waterfowl that were during multing but before migration on radioactive lakes polluted with ^{137}Cs (from 2.5×10^{-5} to 3.9×10^{-3} muCi/l) and ^{90}Sr (from 2.3×10^{-3} to 1.6×10^{-1} muCi/l) in the East-Ural radioactive region. According to our estimation about 8000 geese congregated from August to September on these reservoirs, that attract birds through their protected status. According to the data from Novaya Zemlya [5], the King Eider *Somateria spectabilis* accumulated in its liver up to 1000 Bq/kg of D. W., young individuals of the Snowy Owl *Nyctea scandica* – 580 Bq/kg, the Bean Goose *Anser fabalis* – up to 890 Bq/kg and the Long-tailed Duck *Clangula hyemalis* – up to 700 Bq/kg ^{137}Cs. An activity of β-emitters in waterfowl bodies, objects of sport hunting, taken from near Chernobyl, was on average in 1986: 13,172 Bq/kg for Teals *Anas crecca* and *A. querquedula,* 9657 Bq/kg for the Mallard and 4329 Bq/kg for the Common Coot *Fulica atra,* and 8 years after accident activity of γ-emitters in muscles of the Mallard was 1.7 kBq/kg [16].

Monitoring of radionuclide accumulation in a population of resident birds, inhabiting in radioactive ecosystems, is important for the research of long-term effects of chronic radioactive contact on a population, but monitoring of radionuclide accumulation in migrating birds is significant for the estimation of the safety of territories, which include the processes redistribution of radionuclides.

Global level accumulation of radionuclides in birds

Local pollution allowed us to find a pattern of toxicant accumulation with a significant effect only on a limited area; however, we can register traces of several radioactive elements over wide territories. Micro-quantities of some elements can be biogeochemical markers of a geographic population. Concentrations of natural radionuclides, for example thorium and uranium contents, may be used as geographic markers.

While it is not enough to prove negative trends of accumulation radionuclides in Mongolian birds, it is possible to note following: radio-strontium in bones of some species of birds was not found in 1982, or its values were ten and hundred times less than in 1996. We analyzed our and earlier published data on the radionuclide contents in arid and sub-arid ecosystems of Eurasia (Table 3). The comparison of the radionuclide contents of birds in 1996 from the steppe zone in Russia and Mongolia showed that the total content of various radioisotopes of plutonium in bird bones from Southwest Russia were hundred and thousand times more, than in Mongolia.

This can be explained by radionuclide accumulation as a result of global precipitation after the Chernobyl accident on the European steppe. Activity levels in specimens from Ethiopia bear record to Ethiopia cannot be a "pure" control site in radioecological research and radioactive background since it does not significantly differ from Turkmenia [14] and Mongolia (Tables 3, 4).

In conclusion, it may be shown the concentrations of artificial radionuclides in Ethiopia's animals were similar to the level of contamination of Palearctic biota. Thorium traces were detected, but there was not any trace of uranium. It is necessary to use as bioindicators a bird species, which habitats both contaminated and pure biotopes. However, it is difficult to find a pure area that could correspond to all characteristics of the control sites. The pattern of radionuclides in birds can be used as biomarkers of local populations. According data from the Urals [3, 13] birds breeding on reservoirs contaminated with ^{90}Sr obtained a radioactive "label" by natural means. Thus, individuals of a local population have their own chemical markers thanks to the local geochemical make-up of habitat. Birds as objects of bioindication and monitoring of radioactive contamination are very usable due to their ecological diversity. Material for analysis may be collect without special killing, we may use only Museum collection, dead birds and bag of hunters.

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THE CONTRIBUTION OF A PYRETHROID INSECTICIDE TO THE MASSIVE EEL *(ANGUILLA ANGUILLA)* DEVASTATION, IN LAKE BALATON, IN 1995*

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In the summer of 1995, 30 tonnes of eel *(Anguilla anguilla)* died in Lake Balaton, Hungary. An investigation was carried out to find the causes of this ecocatastrophe. During this investigation, certain biochemical parameters, i.e. the blood sugar level, the acetylcholinesterase (AChE, EC 3.1.1.7), lactate dehydrogenase (LDH, EC 1.1.2.3), glutamic-oxaloacetic transaminase (GOT, EC 2.6.1.1), and glutamic-pyruvic transaminase (GPT, EC 2.6.1.2) activities in the blood serum of the collected surviving and dying eels were examined. Deltamethrin, the active ingredient of the insecticide K-OTHRIN 1 ULV, used against mosquitoes was detected in different animal species, i.e. eel, bream *(Abramis brama),* pike perch *(Stizostedion lucioperca),* and the common gull *(Larus canus)* and in sediment samples from the lake. Additionally, laboratory experiments were carried out to study the effects of deltamethrin on eels.

During the investigation in the field it appeared that the AChE activity was significantly lower in the blood serum of the dying eels as compared to that in living animals ($P < 0.05$, Student's *t*-test). The blood glucose content exhibited a difference, too: it was 2.5 times higher in the dying eels than in the surviving ones. A huge increase in the LDH level was measured in the dying eels.

The GOT activities of the serum were twice as high in the dying eels as in the living fish, while the GPT was not significantly changed.

Deltamethrin was detected in different tissue samples of the dying eels: 2.70–18.1 μg/kg in the liver, 9.0–31.1 μg/kg in the gill and 3.0 μg/kg wet tissue in the muscle. Deltamethrin residues were found in tissue samples from other animals, in the following concentrations: 0.44 μg/kg in bream, 2.14 μg/kg in pike perch and 1.06 μg/kg wet tissue in dead gulls. The sediment samples collected from the sites of the devastation contained deltamethrin in a concentration of 5.50–30.00 μg/kg wet sediment at the time of the eel deaths, and in a concentration 7.00–8.75 μg/kg wet sediment a month later.

Laboratory experiments with the insecticide K-OTHRIN 1 ULV revealed that 1.00 μg/l of its active ingredient, deltamethrin, caused the death of 50% of the eels after an exposure time of 96 h. During this experiments similar trends could be observed in changes of enzyme activities of the treated eels to those that were detected in filed study during the eel devastation in Lake Balaton.

At the end of a one-week treatment with the insecticide at the concentration of 0.5 μg/l of its active ingredient the gills of the treated eels contained deltamethrin at 12.6–44.8 μg/kg wet tissue concentration, while at the 24th hour after the treatment (11.2–42.7 μg/kg wet tissue) deltamethrin concentration in the liver of treated eels could be detected.

* Dedicated to Professor János Salánki for his 70th birthday.

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All the above-mentioned changes and the detected deltamethrin residue in the eels appear to demonstrate the contribution of deltamethrin to the severe eel devastation. This information on the ecological risk of pyrethroid insecticides might be useful in their further application.

Keywords: Acetylcholinesterase – blood sugar level – deltamethrin residue – eel devastation – lactate dehydrogenase – transaminases

INTRODUCTION

Deltamethrin (or decamethrin, DM; a type II pyrethroid; (S)-α-cyano-3-phenoxybenzyl-(1R)-*cis*-3-(2,2-dibromovinyl)-2,2-dimethylcyclopropane-carboxylate, $C_{22}H_{19}Br_2NO_3$) is the active ingredient of several pesticides used in Hungary: Decis 2.5 EC (25 mg/l), K-OTHRIN 1 ULV (1 mg/l), etc. These pesticides are considered to be among the safest classes of insecticides available. However, under clean water laboratory conditions, exposure of fish to this pyrethroid demonstrates its toxicity. The 96-h LC_{50} values range between 0.4 and 2.0 μg/l [13]. Extensive field studies in experimental ponds have shown that this high toxicity potential is not realised [41]. However, a few cases of fish devastation have been reported in Hungary when pyrethroid poisoning was blamed [33]. An eel catastrophe similar to that which occurred in the summer of 1995 was recorded in 1991. At that time, brain and liver samples contained DM residues at 20 μg/kg wet tissue [12]. Gönczy suspected that one of the causes of the massive eel loss was the presence of DM in the fish.

There is no significant difference between the acute toxicities in cold and warm water fish species [36]. It seems that DM affects fish at both high and low temperatures, although a negative temperature coefficient has been claimed for certain pyrethroids [18]. Furthermore, the toxicity of pyrethroids (including DM) for fish is not influenced by the hardness or pH of the water [22].

DM has been observed to cause neuromuscular dysfunctions in organisms [32]. Its primary effect has been attributed to a slowing of the sodium channel of function nerve cells. Additionally, there are many secondary effects on the whole organism. Rose and Dewar state that [32], DM has at least two distinct actions: a short-term pharmacological effect at near-lethal dose levels and a long-term neurotoxic effect that results in sparse nerve damage.

The literature contains many extensive references as to the secondary effects of DM on various parameters such as AChE, ATPases [34], noradrenaline and adrenaline contents in rats [6], choline transport [21], induction of chromosome aberrations in mice [5], and the subpopulations of muscarinic and nicotinic ACh receptors [11]. DM has also been proven to have negative impacts on hatching and the mortality of fish embryos [39]. However, few investigations have been made on the mechanism of acute toxicity in fish [13, 43].

The present study involves a biochemical characterization of certain enzymes in eel. The blood serum activities of AChE, LDH, GOT and GPT and blood glucose levels were determined in living and dying animals. The changes in these enzyme activities have been used to demonstrate tissue damage in fish [3, 17, 26]. Similarly, an

increase in blood glucose level has been used to demonstrate metabolic stress [26, 40]. Changes in the AChE activity of the serum were also studied. The DM residues from tissues and sediment samples were detected by HPLC. This study was performed to evaluate the contribution of DM to this catastrophe.

MATERIALS AND METHODS

Enzymes and blood sugar

In the field experiments, eels *(Anguilla anguilla)* were caught and blood and tissue samples were taken from them. Blood samples were centrifuged to remove the blood cells. Glycerine was added to the sera in equal volume. Blood samples were transported to the laboratories at 4 °C, whilst the tissue samples were carried in liquid nitrogen.

Eels of both sexes, weighing 400–600 g, were used in the laboratory experiments. Fish were kept individually in air-saturated water at 22 ± 1 °C in a 100 l aquarium. The animals were not fed during the experiments. The DM concentration in the aquarium was 0.5, 1.0 or 2.0 μg/l. The duration of exposure was 96 and 168 h. After 168 h exposure the eels survived the treatment were taken in clear water in order to study their recovery from the poisoning.

AChE, GOT, GPT and LDH activities and the blood glucose level were measured in a haemolysis-free serum. The values are expressed as the averages of the results on 9–10 individuals (± S.D.). The differences between the data on surviving and dying animals were analysed by the Student's *t* (paired) test.

AChE was determined with acetylthiocholine as substrate by the method of Ellman et al. [10]. The GOT and GPT activities were assayed according to Reitman and Frankel [31], and the LDH activity according to Annon [2] with Reanal kits (Hungary).

Enzyme activities are given in U/l (1 U = 1 μmol substrate min^{-1}). Blood sugar level was determined by the glucose oxidase-peroxidase method. The reaction mixture was 2.5 ml glucose reagent [2 ml peroxidase (Reanal, Hungary) + 12.5 mg glucose oxidase (Reanal, Hungary) diluted in 50 ml 0.1 M phosphate buffer (pH 7.0) + 3.3 mg *o*-dianisidine (Reanal, Hungary) diluted in 1.0 ml distilled water] + 0.1 ml blood serum. After a 35-min incubation at room temperature, the absorbance was measured at 450 nm.

Determination of DM residues

The sediment samples (10 g of each) were measured and mixed with 10 ml water and 50 ml acetone. After a 5-minutes shaking, the samples were incubated for 1 h, and the DM was extracted with 10 ml hexane. The hydrophobic phase containing DM was dried under vacuum. After drying the samples were dissolved in 100 μl of the HPLC eluent used.

Samples of animal tissues for DM detection were prepared according to Akhtar [1]. The procedure described for GC was modified to HPLC.

All chromatographic experiments were performed with a Gynkotek HPLC system consisting of a high precision pump (Model 480), a degassing unit and a Peltier column thermostat for HPLC. The detector used was a Gynkotek variable UV detector operated at 230 and 210 nm. The injector was a Rheodyne 8125 (California, USA) with a volume of 50 µl. The stationary phase used was a Bondapack C18 (3.9 × 300 mm) column. The eluent was acetonitrile: 0.1% H_2SO_4 in deionized HPLC grade distilled water (70 : 30). The flow rate was 1 ml/min, and the column temperature was 50 °C.

The data were collected and evaluated with the GynkoSoft Chromatography Datasystem. Eluents, i.e. acetonitrile, hexane and acetone were obtained from Sigma Chemical Company. The HPLC grade water was deionized and distilled in a Milli Q system (Millipore, Bedford. USA). Decis insecticide was used, as a standard which contains 25 g/l DM.

Since the DM concentration of the samples was just above the detection limit, standard amounts of DM were added to them in order to make the method more sensitive.

RESULTS

Results of the field experiments

AChE

The AChE activity in the serum of dying eels was significantly lower (60% decrease), while in the surviving individuals the level of this enzyme was normal (Fig. 1). One of our former experiments on carp *(Cyprinus carpio)* revealed that DM can reduce the AChE activity in the serum even at low concentration [19, 38]. Earlier experience indicated that the LC_{50} of DM for eels is 0.47 µg/l (96 h). It is well known that α-cyanopyrethroids such as DM cause a long-lasting prolongation of the normally transient increase in sodium permeability of the nerve membrane during excitation, resulting in long-lasting trains of repetitive impulses in the sense organs and a frequency-dependent depression of the nerve impulses in the nerve fibres [41]. Since the mechanisms responsible for nerve impulse generation and conduction are basically the same throughout the entire nervous system, pyrethroids may well act in a similar way in various parts of the central nervous system [41].

The clinical signs of DM toxicity are tremor and salivation in animals [16, 30]. In fish, signs of toxicity include irregular movement of the operculum and disturbances in the coordination of swimming. During our field and laboratory research, we observed similar syndromes in eels.

A near to the lethal dose of DM produces biochemical changes in the peripheral nerves, consistent with sparse axonal degeneration [41]. Swelling and disintegration

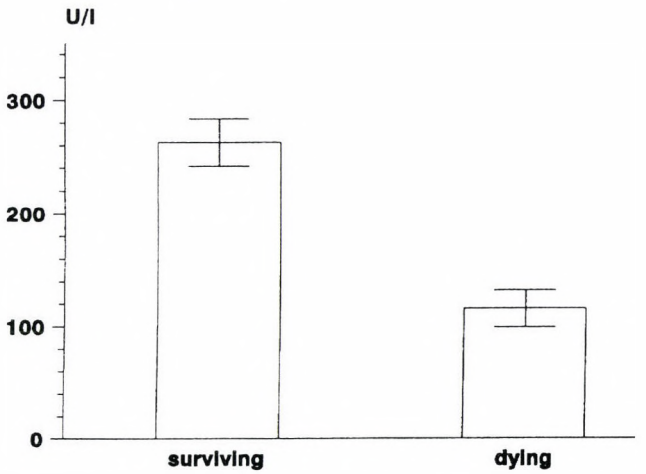

Fig. 1. AChE activities of surviving and dying eels. Activities, given in U/l, are the means ±S.D. for 9 to 10 specimens. *Note.* Values are significantly different according to the Student's *t* (paired) test ($P < 10^{-7}$)

of the axons of the sciatic nerve may be observed [41]. This destruction could be the cause of, among others, the decrease in AChE activity of the heart and intestine [4, 37, 38]. AChE inhibition is particularly dangerous in the heart, since the cholinergic system has a decisive role in the innervation of the heart in fish [28]. AChE inhibition potentiates the vagal tone, which may cause adverse effects in the metabolic processes related to circulation. In this case, inhibition of the heart function interferes with the uptake of O_2 and the release of CO_2 at the gills, which may result in hypoxia at the tissue level [14].

GOT and GPT

The GOT activities in the blood serum were significantly higher in the dying eels than in the surviving animals (Fig. 2). GPT did not display significant changes in the dying fish as compared to the surviving animals (Fig. 3). These trends are similar to those observed earlier in carp treated with DM [19, 38].

The damage of tissue containing considerable amounts of GOT and GPT is well-detectable through the increasing activities of these enzymes in the blood serum [15]. The distributions of GOT and GPT vary in the different organs [27], and the ratio of the activities of the two enzymes permits reasonably exact conclusions on the injured tissues.

The elevated GOT and the practically unchanged GPT activities observed in our studies were presumably due to liver damage. However, other organs may have also been damaged (e.g. the kidney or/and the gill).

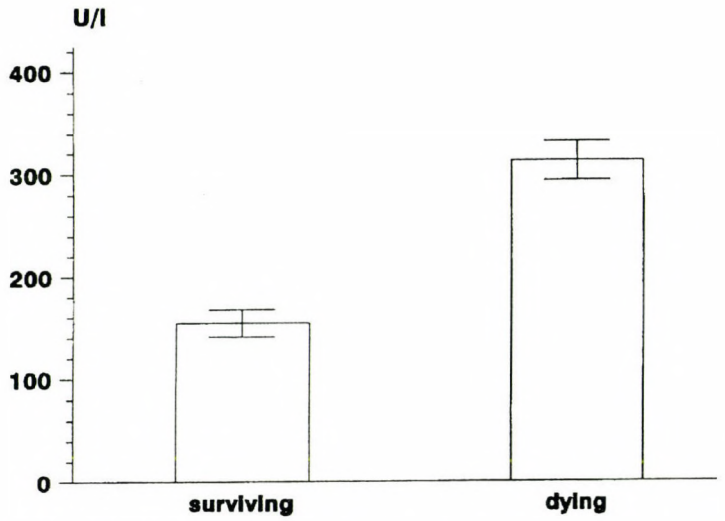

Fig. 2. OT activities of surviving and dying eels. Activities, given in U/l, are the means ±S.D. for 9 to 10 specimens. *Note.* Values are significantly different according to the Student's *t* (paired) test ($P < 2 \times 10^{-7}$). For experimental conditions see Materials and Methods

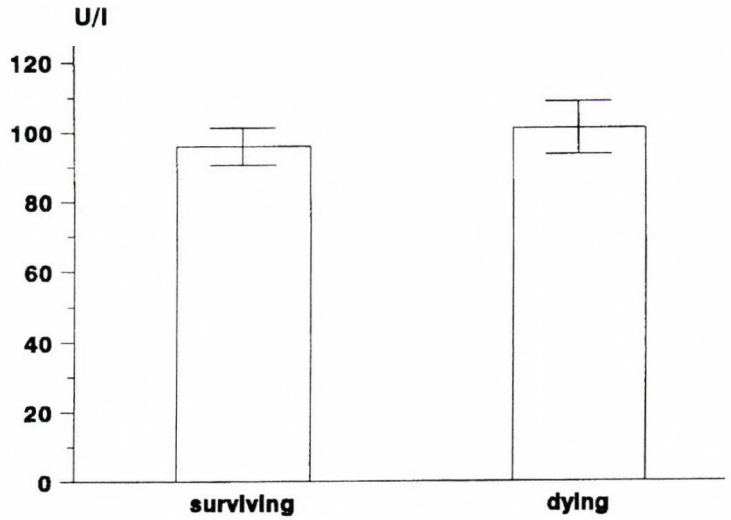

Fig. 3. GPT activities of surviving and dying eels. Activities, given in U/l, are the means ± S.D. for 9 to 10 specimens. For experimental conditions see Materials and Methods

Blood sugar level

Pyrethroid poisoning in fish produces a syndrome characterized by tremors, abnormal operculum movement, ataxia and hyperactivity. This stressed stage was marked by a higher blood sugar level: 2.5 times higher in the dying animals than in the surviving ones (Fig. 4).

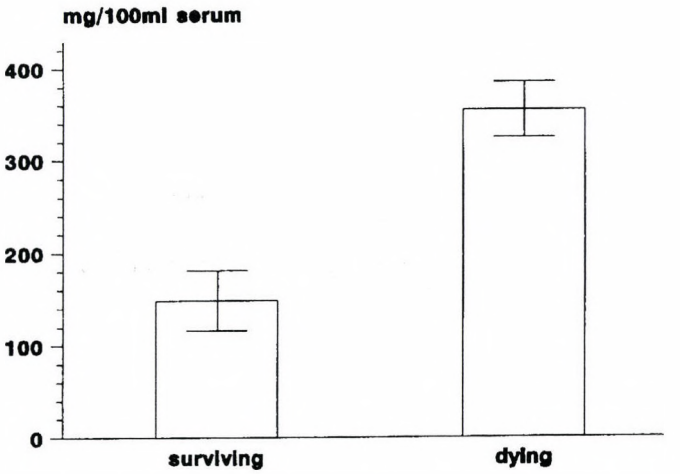

Fig. 4. Blood glucose levels of surviving and dying eels. Concentrations, given in mg glucose/100 ml blood serum, are the means ± S.D. for 9 to 10 specimens. *Note.* Values are significantly according to the Student's *t* (paired) test ($P < 6 \times 10^{-7}$). For experimental conditions see Materials and Methods

The DM-induced "non-motor" symptoms, i.e. the increases in brain blood flow and blood glucose level, may result from a supraspinal component of DM activity [7]. Permanent stress includes impaired γ-globulin formation and depressed interferon production, which play important roles in the resistance of fish to various bacterial, viral [27, 40] and nematode infections [23].

LDH

There was a significant increase in LDH activity of an extent we have never observed before (Fig. 5). It has already been noted that DM treatment can cause an LDH activity increase in the serum [38].

The increased LDH activity could indicate metabolic changes in the stressed fish, primarily in the muscle cells: the catabolism of glycogen and glucose is shifted towards the formation of lactate, which can be lethal for fish [24, 35].

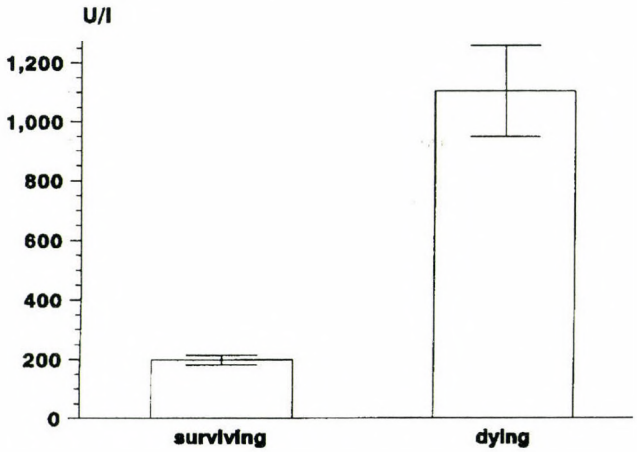

Fig. 5. LDH activities of surviving and dying eels. Activities, given in U/l, are the means ± S.D. for 9 to 10 animals. *Note.* Values are significantly according to the Student's *t* (paired) test ($P < 2 \times 10^{-7}$). For experimental conditions see Materials and Methods

DM residues in the sediment and in different species of fish

DM could be detected in the different organs of dying eels collected during the devastation (Table 1). The DM concentration observed in the liver was 2.7–18.5 μg/kg wet tissue. The highest DM residue was measured in the gill, at 9.0–31.1 μg/kg wet tissue, while the muscle contained DM at 3 μg/kg wet tissue. It must be mentioned that the eel was not the only species in wich DM was detected. Four weeks after the latest K-OTHRIN 1 ULV spraying, the presence of DM was proved in the bream, the pike perch and even the common gulls consuming the carcasses of eels. Such an accumulation of deltamethrin in the food chain is suprising since the pyrethroids are known not to bioaccumulate.

The presence of DM in the sediment samples collected from six different places was also detected at the time of the devastation, at 5.5–30 μg/kg wet sediment. Repeated measurements a month later demonstrated DM at a quarter of the concentration observed previously. The pyrethroids are said to be rapidly degradable pesticides. Their half-life in natural water at summer temperature is 72–96 h [41]. The main reason for it is the considerable ability to bind floating particles [41]. In the sediment, the degradation of DM depends mainly on the features of the sediment, i.e. its temperature, the size of the grain and the aerobity. This procedure in the sediment takes a longer time: approximately 11–72 days [41]. The data of the Hungarian Meteorological Service can explain the presence of DM in the sediment of the lake. They reported that immediately after the spraying there was a huge storm with 45.8 mm of precipitation (pers. com. Vissy, K.). This huge rainfall, which was exactly 75% of the monthly precipitation, washed soil from the shore into the lake, together with the pesticides bound to the soil particles.

Table 1
DM contents of tissues originating from different fish and of sediment samples

Samples	Date of collection	Deltamethrin residue*
Liver of dying eels	24–28 July, 1995	2.7–18.5
Gill of dying eels	24–28 July, 1995	9.0–31.1
Muscle of dying eels	24–28 July, 1995	3.0
Eels	7 August, 1995	0.1–0.3
Bream	7 August, 1995	0.4
Pike perch	7 August, 1995	2.1
Common gull	3 August, 1995	1.1
Sediment from site No. 4	26 July, 1995	13.0–16.2
Sediment from site No. 5	26 July, 1995	12.4–30.0
Sediment from site No. 9	26 July, 1995	19.0–26.6
Sediment from site No. 21	26 July, 1995	5.6–8.0
Sediment from site No. 4	24 August, 1995	7.0
Sediment from site No. 5	24 August, 1995	8.8
Liver of the poisoned eel	24th hour of the treatment	11.2–42.7
Liver of the poisoned eel	168th hour of the treatment	6.6–28.3
Liver of the poisoned eel	168th hour of the recovery phase	4.8–21.7
Gill of the poisoned eel	24th hour of the treatment	0–2.1
Gill of the poisoned eel	168th hour of the treatment	12.6–44.8
Gill of the poisoned eel	168th hour of the recovery phase	3.2–17.5

*The concentrations are expressed as µg/kg wet tissue or µg/kg wet sediment. *Note.* The mentioned places are the areas where the eel devastaion occurred. The date of the most recent spraying with K-OTHRINE 1 ULV was 13–14 July, 1995.

Results of laboratory experiments

During the laboratory experiments with the insecticide K-OTHRIN 1 ULV, it appeared that the formulated insecticide concentration of 1 µg/l relative to the active ingredient caused the death of 50% of the eels after exposure for 96 h. None of the untreated fish died. Tremor, salivation and disturbed in swimming coordination were observed in treated fish.

The GOT level of the treated eels at the 48th hour after the treatment was more than 1.5 times higher (340.6 ± 39.8 U/l) than the control level (193.6 ± 86.6 U/l), while at the 96th hour of the treatment the GOT level increased to 478.7 ± 61.8 U/l. At the 24th hours of the recovery phase GOT activity decreased to 257.3 ± 51.4 U/l

level, but only at the 188th hours of the recovery phase returned back to the control level. The GPT level did not show significant changes in the laboratory experiments as well as the field experiment.

The blood sugar level of the treated eels was approximately twice higher (158.8 ± 18 mg/100 ml) at the 96th hours after the treatment than the control level (81.4 ± 23.6 mg/100 ml) and even at the 188th hour of the recovery phase it was higher (100.4 ± 35 mg/100 ml) than the control level. The LDH activity of the treated eels reached the 5473.3 ± 178.6 U/l level at the 96th hours after the treatment which showed an almost 3.5 times elevation compares to the control level (1570.6 ± 459.2 U/l). This enzyme activity returned to the control level only at 188th hours of the recovery phase.

The bioconcentration of DM was investigated in eels treated by K-OTHRIN 1 ULV at 0.5 µg/l of its active ingredient. (At this concentration 25% of the poisoned eel died within 96 hours). DM could be detected in the gills at the 24th hours after the treatment. At the end of the 1-week treatment the DM concentration increased and even at the end of the 1-week recovery phase DM could be detected from the gill (Table 1). The largest DM concentration in the liver of treated eels could be detected at the 24 hours after the treatment. From this time the DM concentration decreased, but even at the end of 1-week recovery phase the liver contained DM (Table 1). Deér et al. [8] have desribed that the DM can cause induction of the cytochrome P450-dependent monooxygenases at low concentration which may lead to metabolism of the insecticide. This finding can explain the decrease of the DM residue in tissues.

During the laboratory experiments with K-OTHRIN 1 ULV treated eels, trends could be observed in changes of enzyme activities similar to those which were detected in the study during the eel devastation in Lake Balaton and the DM accumulation was at the same range as detected in the tissues of dying eel during the field investigation.

DISCUSSION

Besides the investigation described above, several research institutes have carried out research to determine the factors that could have contributed to this large-scale devastation. The VITUKI institute found permetrin (another pyrethroid the active ingredient of the insecticide Reslin Super ULV also used against mosquitoes at Lake Balaton) in the different organs of dead eels: 1.7 µg/kg in the muscle, 24 µg/kg in the liver and 96 µg/kg wet tissue in the gills [20]. That investigation showed the proportion of the cis and trans isomers of permetrin to be the same as in Reslin Super ULV. They suggested a microlayer theory, according to which the sprayed insecticide can form a very thin layer on top of the water. Eels often poke their head out of the water, especially at night and in the process they become contaminated by the insecticide. This theory is based on the fact that the highest permetrin residue was detected in the gills of dead eels [20]. Some Hungarian researchers have questioned the role of the

pyrethroids in this catastrophe. They referred to the rapid degradation of pyrethroids in natural waters [41]. According to Pénzes, the main reason for this devastation was the *Aeromonas punctata* infection, together with other stress sources such as worm infection [29]. The relatively high water temperature observed (19–24 °C) and the eutrophic water conditions led to the proliferation of these bacteria. It was his opinion that the mass deaths of the eels would have stopped if the water temperature had decreased below 20 °C [29]. It should be mentioned that the worm *(Anquillicola crassus)* infection held to be the main cause of the eel devastation in 1991 was also investigated [23]. In 1991, the swimbladders of dead and moribund eels contained 30–50 pecimens of adult nematode *A. crassus,* and the swimbladder wall of the infected eels had thickened to 3 to 4 mm [23]. The research performed in 1995 did not reveal a direct connection with the dead and infected eels. Only 63% of the dead eels contained worms in their swimbladders. At the same time numerous living but worm-infected eels were found (approximately 60% of the examined vital eels) [9].

CONCLUSIONS

From the present research it appeared that pyrethroids might have contributed to the eel devastation that occurred in Lake Balaton in 1995. This is suggested by the DM and permethrin residues detected in the tissues of the eels and in the sediment samples. The changes observed in the levels of several enzymes, i.e. the reduction of AChE activity, the elevated GOT and LDH activities in the serum of moribund eels, etc., which are similar to those observed during laboratory experiments with DM, the similar range of the DM residuse detected in the organs (especially in liver and gill) of dying eels during the field study and in the poisoned eels during the laboratory investigation might be regarded as evidence in favour of pyrethroid poisoning.

Nevertheless, several other factors have also to be taken into account. Researchers agree that the physiological condition of the eels living in Lake Balaton was very poor, due to the seriously eutrophicated water, which can be a source of bacterium and worm infection. In this case, insecticide contamination was an extremely large burden for the weak eels. This insecticide poisoning can therefore be regarded as the main reason for this devastation, although the poor physiological condition of the fish was also a very important factor.

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SEROTONIN INHIBITS CILIARY TRANSPORT IN ESOPHAGUS OF THE NUDIBRANCH MOLLUSK *TRITONIA DIOMEDEA**

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Both serotonin and the molluskan pedal neuropeptides (TPEPs) cause increased ciliary beating rate of cells of the foot epithelium of the nudibranch mollusk, *Tritonia diomedea*. Here we compared responses of the ciliated epithelium of the esophagus with that of the foot, and report fundamental differences. Serotonin reduces the ciliary transport rate of the esophagus. We find also that the serotonin driven inhibition of esophagus is blocked and the excitation of foot epithelium is reduced by the serotonin receptor blocker ketanserin. On the contrary, ergometrine completely blocked the serotonin effect in the esophagus, and does not block the serotonin effect in the foot. Neither the TPEP driven excitation of ciliated cells of the foot nor that of the esophagus is blocked by ketanserin and ergometrine. Clearly, serotonin and TPEP regulation of different ciliated epithelia involve different receptors. Thus, mechanisms of serotonin control of different ciliated epithelia in the same animal are apparently fundamentally different, and unlike responses in all previous reports, 5HT here inhibits a ciliated epihelium.

Keywords: Serotonin – inhibition – cilia – mollusk – *Tritonia*

INTRODUCTION

Ciliated cells occur widely in animals, and are often responsible for the motile force underlying diverse processes including mucus transport, locomotion, reproduction, respiration, circulation and feeding. In multicellular animals, ciliated cells are often organized in bands or laminar epithelia and can be controlled by the nervous system.

The underlying neural control mechanisms are partially understood [13, 15]. In veliger larvae and ascidians, cilia beat with an endogenous maximal frequency, and neuronal control is exerted by intermittent, abrupt arrest of beating [2, 3, 19]. In these cases, the neurotransmitter is unknown although in veligers evidence suggests involvement of a peptide [7]. Some veligers are also capable of graded control of ciliary beating [3, 9].

*Dedicated to Professor János Salánki for his 70th birthday.

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Another type of ciliated epithelium described in the sea slug *Tritonia diomedea* and freshwater snail *Planorbis corneus,* is controlled by pedal ganglion neurons which provide tonic excitation of otherwise motionless cilia of the foot [4, 5, 10]. Finally the ciliary beating frequency of lateral cilia of *Mytilus* gill and larvae of several marine invertebrates can be modulated by different CNS inputs [1, 8, 22, 23].

Serotonin (5-hydroxytryptamine, 5HT) generally has a cilio-excitatory effect [18]. For instance, in the freshwater snail *Lymnaea stagnalis,* 5HT facilitated activity of the ciliated epithelium of the foot and doubled the crawling velocity [24, 26]. 5HT has a direct excitatory influence on ciliary beat frequency in veliger larvae of some species of marine mollusks and in embryos of *Helisoma trivolvis* [7, 12]. In Tritonia, the identified pedal neurons mentioned above which excited motionless cilia, contained 5HT [4, 5]. Injection of 5HT into intact *Tritonia* caused prolonged pedal ciliary beating [6]. 5HT increased the motor activity of isolated ciliated epithelia of *Tritonia* foot, and ciliary beat frequency of isolated ciliated cells [27].

Recently discovered neuropeptides in *Tritonia,* the Pedal Peptides (TPEPs) are also involved as mediators of ciliary locomotion. Similar to 5HT, TPEPs excite activity of isolated epithelium of *Tritonia* foot and directly facilitate ciliary beat frequency of isolated cells [27]. Thus, a novel, double control of the ciliated cells of the pedal epithelium by two different mediators is apparently involved. We do not know if one of the mediators duplicates mechanisms of the other, or if there are different mechanisms that supplement each other to regulate normal locomotion [28]. Another question is whether parallel control of ciliated epithelia by two transmitters is exceptional or a more generally occurring phenomenon? Recent investigation of 5HT and TPEPs effects on ciliated epithelium of the salivary duct in *Tritonia* showed that both mediators had excitatory influences on ciliary beat frequency [11], suggesting similar mechanisms to that controlling the foot.

In this paper, we demonstrate that 5HT and TPEP affect activity of ciliated epithelia of the esophagus and foot in *Tritonia,* and result in opposite motor responses. Mechanisms of 5HT control of ciliated epihelium of the foot and the esophagus in the same animal are apparently fundamentally different and involve different 5HT receptors. And we report the first observation of serotonergic inhibition of ciliary transport in animals.

MATERIALS AND METHODS

We used 20 nudibranch mollusks *Tritonia diomedea* in these experiments. Slugs (125–460 g) were collected by SCUBA diving in waters of Puget Sound, near Tacoma, WA, USA, and maintained in aquaria at Friday Harbor Laboratories. Tritonia were fed on sea pens *(Ptilosarcus gurneyi).* We dissected 4 patches of esophagus and the same number of foot epithelium from each slug. After the dissection in seawater, each pair of patches (one pair each from esophagus and from the foot) was placed ciliated epithelium up, stretched maximally to overcome confounding effects of slow contractions of underlying muscles, and pinned in a Sylgard-coated Petri

dish. Every dish was filled with 5 ml of filtered sea water and considered as a separate experiment. We immersed the dishes in 1 l jar with filtered sea water and storaged them in a refrigerator at 5 °C. Preparations in every dish were investigated once a day for 4 days. To avoid abrupt changes of temperature during the experiment each dish was taken out of the refrigerator 1 h prior to experiments. All experiments were carried out at room temperature.

Normal on-going beating of the ciliated epithelium evoked a visible flow of sea water over the patch. The rate of ciliary beating was determined by the rate at which a droplet of Sumi drawing ink (applied from a fine pipette) was transported along a defined path over the epithelium. The passage of ink along the patch was observed through a stereomicroscope. We studied the effects of serotonin (5HT), TPEP-NLS (abbreviated TPEP here), 5HT receptor blockers (ketanserin), dopamine (DA) and the ergot alkaloid ergometrine maleate (= ergonovine maleate) on activity of ciliated epithelia. All reagents were obtained from Sigma, except TPEP which was synthesized by the Protein Core Facility of the University of Washington. We measured ink transport in sea water and then 10–30 m after addition of reagents. Fresh solutions were made just before tests. A small aliquot was pipetted directly into the 5 ml dish to bring the seawater in the dish to the desired final concentration. In experiments to determine dose dependency, 5HT and TPEP were added in ascending series of doses. Each exposure of patches in 5HT or TPEP alternated with wash (15 min) in an aquarium with continuously flowing sea water. At the end of daily experiments all preparations were washed (2 h) in the same manner.

In all experiments, except those reported in Figures 1 and 6 as noted, reagent concentrations were 5HT and TPEP, 10^{-4} M; ketanserin, 10^{-3} M; ergometrine 10^{-4} M. Dopamine solutions ($10^{-2} - 10^{-3}$ M) were prepared with ascorbic acid (50 μM) as antioxidant.

To record beating rates of isolated ciliated cells of the esophagus, we used methods described earlier by Willows et al. [27].

RESULTS

Responses of pedal and esophageal ciliated epithelia to serotonin and receptor blockers

In prior experiments [27] we reported that serotonin and the *Tritonia* pedal ganglion neuropeptides (TPEPs) elicit increased ciliary beating both in patches and in isolated cells of the pedal epithelium (threshold $10^{-8} - 10^{-7}$ M). We confirmed that result here (Fig. 1C, for serotonin exposure of isolated patches of pedal epithelium). However exposure of patches of ciliated esophageal epithelium to serotonin (10^{-4} M) here elicited inhibition of ciliary transport rate (Fig. 1B) with threshold also between $10^{-8} - 10^{-7}$ M (Fig. 1A). The inhibition of transport rate increased as the concentration of 5HT increased in the range $10^{-8} - 10^{-4}$ M.

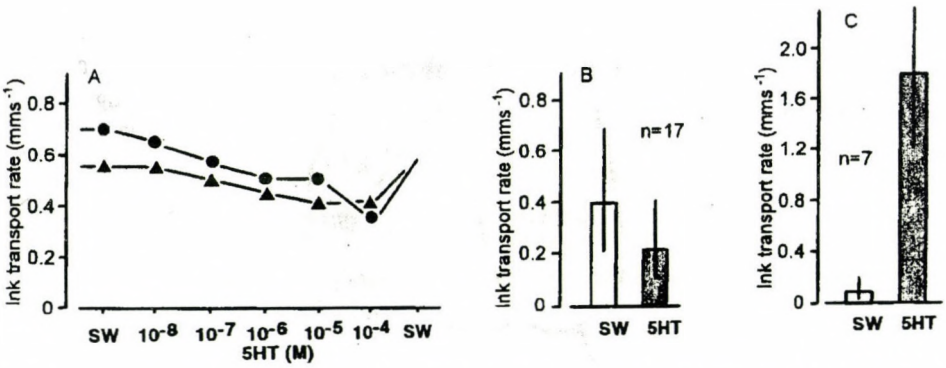

Fig. 1. Epithelial transport rate responses to 5HT in isolated patches of esophagus (A, B) and foot (C)

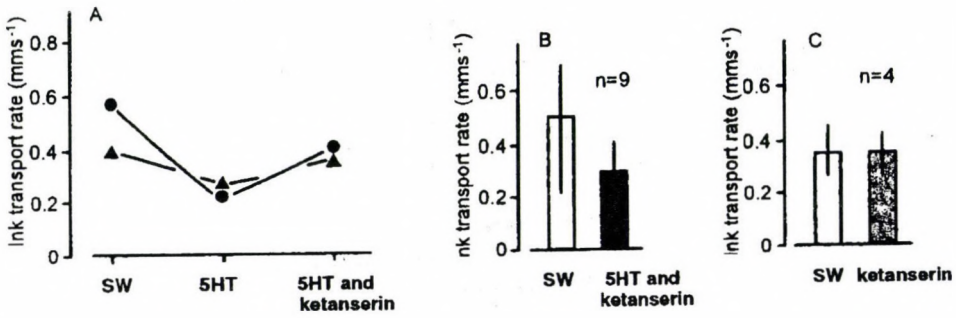

Fig. 2. Ketanserin effects on epithelial transport rate responses to 5HT in isolated patches of esophagus (A, B) and control (C)

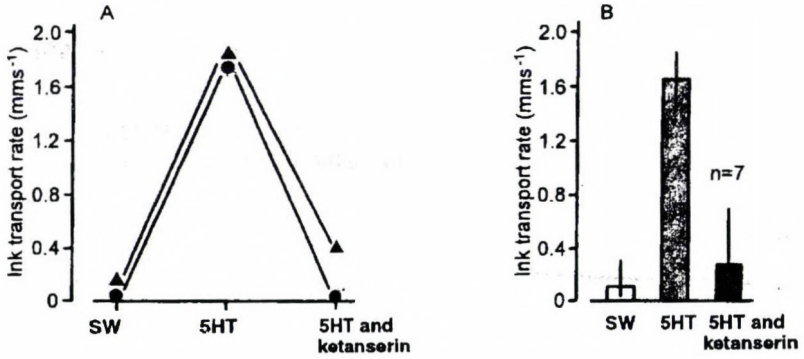

Fig. 3. Ketanserin effects on epithelial transport rate responses to 5HT in isolated patches of foot

To learn whether the serotonin receptors involved in these inhibitory and excitatory responses are pharmacologically similar to receptors known from prior work in other organisms, we measured transport rate changes in response to serotonin in the presence of the receptor blocker ketanserin (to block type $5HT_{1D}/5HT_{1c}$ receptors), and ergometrine (to block inhibitory dopamine receptors). In Fig. 2 responses of the esophagus to serotonin (concentration 10^{-4} M, in all experiments) and serotonin in combination with ketanserin (concentration 10^{-3} M, in all experiments) indicate that the inhibitory response of the esophagus to serotonin is partially blocked in the presence of ketanserin (Fig. 2A, B). Exposure to ketanserin alone had no effect on ink transport rate (Fig. 2C). In these experiments, the outcome did not depend upon whether the esophagus was furst exposed to serotonin (n = 6) or to ketanserin (n = 4).

In experiments similar to those illustrated in Fig. 2, patches of ciliated foot epithelium were exposed to 5HT. Unlike responses of the esophagus, 5HT enhanced markedly ciliary beating in foot (Fig. 3). When foot patches were exposed to 5HT in combination with ketanserin, the excitatory effects of 5HT were blocked. In 4 experiments we used ketanserin before 5HT. In both orders of presentation, ketanserin blocked 5HT excitation of ciliary beating. We noted also that ketanserin alone had no effect on epithelial transport (Fig. 2C).

To explore the nature of the unexpected inhibition of esophageal ciliary transport reported above (Fig. 2), we used the dopamine receptor blocker ergometrine in combination with 5HT (Fig. 4). We observed that the inhibition of esophageal ciliary transport was blocked by ergometrine. We presented 5HT then added ergometrine in 3 experiments, with similar results. We observed also (Fig. 4C) that ergometrine alone did not elicit a measurable change in ink transport rate.

By contrast, exposure of foot epithelial patches to ergometrine, revealed that the excitatory reponse to 5HT was not changed (Fig. 5).

To determine the response characteristics of esophagus further, we exposed patches to TPEP (Fig. 6). A dose response series indicated that as for 5HT, an excitatory threshold exists at approx. 10^{-8} M, with saturation between 10^{-5} and 10^{-4} M. A combination with ketanserin did not reduce the esophageal excitation of ink transport rate elicited by TPEP (Fig. 7). As above, the order of presentation of ketanserin and TPEP

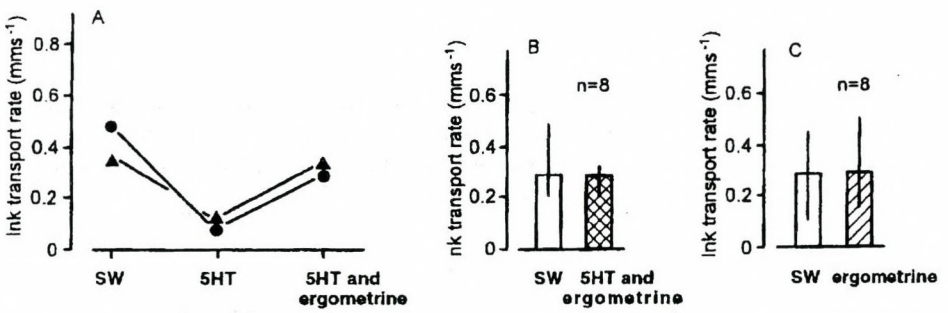

Fig. 4. Ergometrine effects on epithelial transport rate responses to 5HT in isolated patches of esophagus

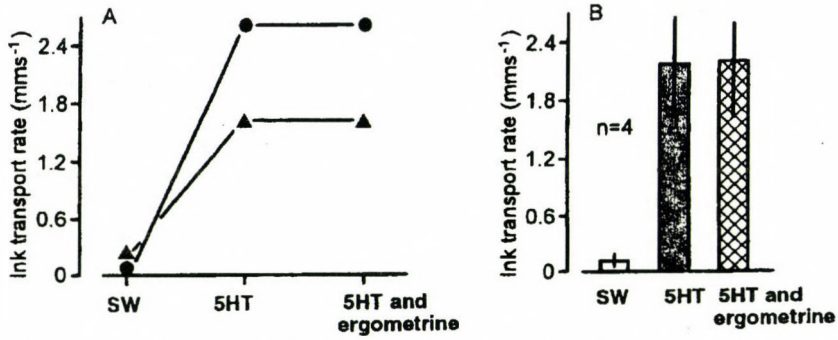

Fig. 5. Ergometrine effects on epithelial transport rate responses to 5HT in isolated patches of foot

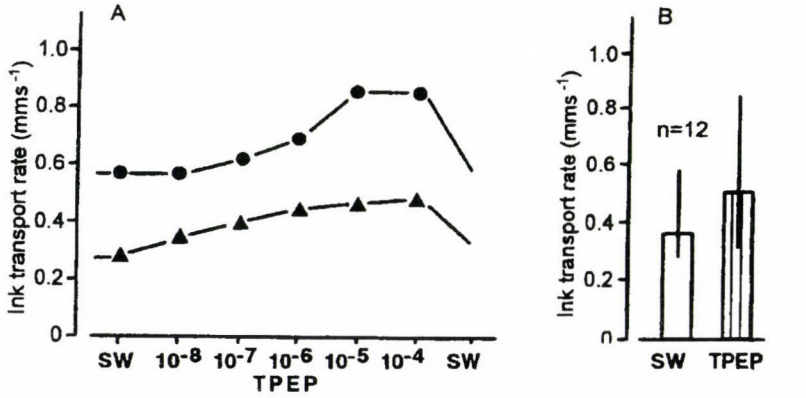

Fig. 6. Epithelial transport rate responses to TPEPs in isolated patches of esophagus

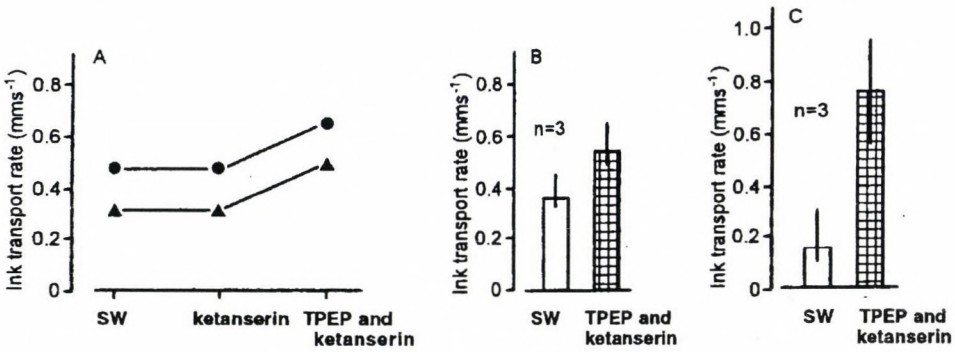

Fig. 7. Ketanserin effects on epithelial transport rate responses to TPEPs in isolated patches of esophagus (A, B) and foot (C)

was mixed in these trials. Similar results for TPEP exposure to foot epithelia (not shown) were obtained and have been reported earlier [27].

We reported above (Fig. 3B) that ketanserin alone elicits no response in foot patches. But we observed and report here that ketanserin has no effects on TPEP elicited excitation of ink transport rates in esophagus (Fig. 7A, B) or on foot epithelia (Fig. 7C).

In another set of experiments (results similar to Fig. 7, not shown), we found that ergometrine did not itself elicit transport rate changes, nor did it alter TPEP excitation in either esophagus or foot.

In order to determine whether a dopamine mediated pathway might regulate ciliary transport, we exposed esophagus and foot patches to 5HT and dopamine ($10^{-2} - 10^{-3}$ M) directly in 3 experiments. We found that although these patches responded with excitation (foot) and inhibition (esophagus) to 5HT, dopamine elicited no observable change in ciliary transport rate.

Finally, to examine the effects of 5HT and TPEP on *isolated* esophageal cells we exposed and monitored beating rates. Unlike the excitatory responses of foot cells reported earlier, by Willows et al. [27], we observed no inhibitory nor excitatory effects to direct application of either 5HT nor TPEP on esophageal ciliated cells.

DISCUSSION

The date presented here (summarized in Fig. 8) demonstrates an unexpected inhibitory control mechanism for a ciliated epithelium. Serotonin apparently markedly inhibits the ciliary transport rate of the esophageal epithelium in *Tritonia.* Jorgensen [16] indicated that ciliated cells fall into two categories, 5HT sensitive and insensitive. Isolated cells from *Tritonia* esophagus did not increase ciliary beating rate when exposed to 5HT. Like the abfrontal cilia of the mussel *Mytilus edulis* [25], they are insensitive to 5HT. In contrast to these esophageal cells however, isolated ciliated foot cells were sensitive to 5HT and increased their beating rate in the presence of 5HT [27, 28].

Nevertheless we could not exclude direct 5-HT direct influence on esophageal ciliated cells. Another mechanism for regulation of ciliary beating is to vary ciliary stroke amplitude (or angle). Thus, in mussels, the laterofrontal cirri beat in a plane vertical to the long axes of the filaments, and the recovery phase brings the cirrus into the plane of the gill surface, resulting in a beat angle of 90 °C [17]. In mussels, 5HT reduced the angle of beat, and high concentrations arrested the cirri in the vertical position. One possible mechanism of 5HT inhibitory action on *Tritonia* esophageal ciliated cells would be to decrease ciliary stroke amplitude (or angle) resulting in ciliary transport rate decrease.

Our results indicate that 5HT receptors on both esophagus and foot are sensitive to ketanserin. Ketanserin is a selective antagonist of 5HT2/5HT1c receptors. We suggest that ketanserin blocks 5HT excitatory responses in foot and reduces inhibitory action of 5HT in esophagus via different ketanserin sensitive receptors.

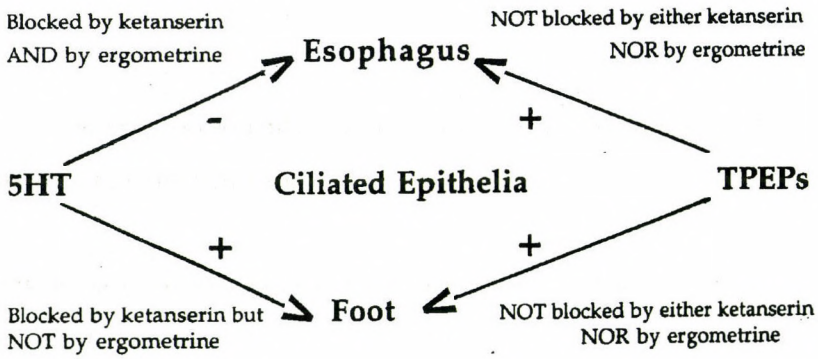

Fig. 8. Summary of responses of esophageal and foot epithelia to exposure to 5HT and TPEPs and actions of ketanserin and ergometrine

5HT inhibitory action on esophagus (but not excitation of the foot) apparently also involves an ergometrine sensitive 5HT receptor. A dopaminergic pathway is probably not involved, however. Our initial observation that ergometrine blocked the esophageal inhibitory response to 5HT, led us to consider the possibility that dopaminergic neurons might be involved. In a subsequent experiment, we found that direct exposure of esophageal epithelium to dopamine (10^{-2}–10^{-3} M) elicited no apparent change in ink transport rate. This result coupled with previous reports of ergometrine sensitive 5HT receptors [21] suggests therefore that this unusual inhibition of ciliary beating by 5HT could involve such receptors either post-junctionally on the ciliated cells or via prejunctional inhibition. Thus, the 5HT receptors may be of one or two types, sensitive to ketanserin and/or ergometrine and inhibition may occur as a result of direct or prejunctional action on ciliated esophageal cells. Our speculation would be that there are two receptors types, and that they exist post-junctionally (excitatory, ketanserine sensitive) and pre-junctionally (inhibitory, ergometrine sensitive).

Comparative investigation of different ciliated epithelia (the foot, the salivary duct and the esophagus) in *Tritonia* indicated coexistence of control of motor responses by 5HT and TPEP. Both these and previous results indicate the possibility of dual control of motor activity by peptidergic and serotonergic systems. Similar dual control was reported in *Aplysia* where 5HT stimulates swimming [20], and pedal neurons containing peptides (APEP, similar to TPEP) participate in locomotor control [14].

In the esophagus of *Tritonia,* 5HT and TPEP apparently have different receptors and cause different responses. In the foot, the mediators have different receptors but mimic one another. Future work is needed to determine if 5HT and TPEP facilitated motor activity of *Tritonia* foot, result from different receptor mechanisms.

ACKNOWLEDGEMENTS

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THE ACTIONS OF FXRFAMIDE RELATED NEUROPEPTIDES ON IDENTIFIED NEURONES FROM THE SNAIL, *HELIX ASPERSA**

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Intracellular recordings were made from identified neurones in the suboesophageal ganglia of the snail, *Helix aspersa*. The actions of the eight FxRFamide analogues were investigated on these neurones. These peptides included ones isolated from arthropods and nematodes. All the peptides excited certain neurones while inhibiting others, though their relative potencies varied. Overall on neurones inhibited by these peptides the potency order was: DNFLRFamide > FMRFamide > PDVDHVFLRFamide = KNEFIRFamide > FLRFamide ≫ SDRNFLRFamide = SDPNFLRFamide > KHEYLRFamide. However, if the responses are compared on individual cell types, then the picture becomes more complex. For example, on cell F-2, KNEFIRFamide proved to be potent with an EC-50 value of 0.54 μM. On neurones F-13/16 and E-16, PDVDHVFLRFamide was inhibitory while FMRFamide, FLRFamide, SDRNFLRFamide and SDPNFLRFamide were excitatory. In terms of overall excitatory actions, the data are less complete but an approximate order of potency is: FMRFamide > DNFLRFamide ≫ SDPNFLRFamide > PDVDHVFLRFamide ≫ KNEFIRFamide = KHEYLRFamide = SDRNFLRFamide. However this again varies between specific neurones. These results demonstrate that peptides from insects, crustacea and nematodes are active on *Helix* neurones and may activate specific receptor subtypes, indicating the possible presence of endogenous analogues of these non-molluscan peptides in the *Helix* nervous system.

Keywords: Helix aspersa – FxRFamide peptides – nematode – arthropod – neurones

INTRODUCTION

There have been many studies investigating the action of RFamides on central gastropod neurones and other tissues and this family of peptides plays an important role in the physiology of this group of molluscs [3, 9]. These peptides can excite and or inhibit neurones and probably act through a number of receptor subtypes [2, 4]. While it is likely that most RFamides act through a G protein system there is now excellent evidence that FMRFamide can excite neurones by directly gating a sodium channel [5] and this amiloride-sensitive peptide-gated channel has been cloned [15].

*Dedicated to Professor János Salánki for his 70th birthday.

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0236-5383/99/$ 5.00

This peptide-gated channel shows a clear preference for the tetrapeptide rather than the extended analogues of FMRFamide [4].

Since the identification of FMRFamide by Price and Greenberg in 1977 [20] many analogues of this peptide have been identified including heptapeptides and further N-terminally extended peptides up to decapeptides [25]. Many of these extended peptides are FLRFamides rather than FMRFamides [19, 21]. Identification of extended peptides has been demonstrated in insects, crustaceans and nematodes [1, 6, 22, 24]. Tetrapeptides, e.g. YMRFamide and YLFRamide have been identified in annelids, e.g. leeches [8].

The current investigation extends earlier studies and tries to determine whether RFamides from other groups are active on gastropod neurones and if possible the type of receptor subtype they are activating. For this study peptides have been selected from nematodes, i.e. KNEFIRFamide, KHEYLRFamide and SDPNFLRFamide, from crustacea, i.e. SDRNFLRFamide and insects, i.e. PDVDHVFLRFamide. In addition a synthetic analogue was tested which proved to be a potent inhibitory compound, i.e. DNFLRFamide and for comparison the two gastropod tetrapeptides, FMRFamide and FLRFamide were included.

MATERIALS AND METHODS

The experiments were performed on identified neurones from the suboesophageal ganglia of locally collected garden snails *Helix aspersa.* The isolated ganglia were removed from the connective tissue with a carbon blade pinned down in a Sylgard coated bath of 0.5 ml volume and continuously perfused (3 or 4 ml/min) with saline of the following composition: NaCl 100 mM, $CaCl_2$ 7 mM, KCl 4mM, $MgCl_2$ 5mM, Tris-basic 5 mM, pH 7.5. For experiments involving ion substitution: in low chloride NaCl was replaced by Na isethionate, in low sodium or sodium free, Na was replaced with Tris, in high potassium KCl was increased to 16 mM and NaCl reduced to 88 mM. Experiments were also performed in high magnesium (16 mM) and low calcium (0.5 mM) to confirm direct action of the peptide on the experimental cell. Intracellular recordings were made from neurones on the dorsal surface of the visceral and left parietal ganglia, identification was based on the map of Kerkut et al. [12]. Experiments were repeated on at least four different preparations. Electrodes were filled with 2M potassium acetate with a resistance of 8 to 12 Mohms. Signals were recorded using a single electrode Dagan 8100 with permanent records made on a Clevite brush chart recorder.

Peptides were made up in distilled water, stored frozen in 0.2 ml aliquots (5×10^{-3}M) until required then diluted by saline to the required concentration, they were bath applied for one minute in the main perfusion system.

4-Aminopyridine (4-AP), tetraethylammonium (TEA), acetylcholine, FMRFamide, FLRFamide and one sample of PDVDHVFLRFamide were obtained from Sigma. The other peptides were synthesised by Dr. R. Sharma and Dr. M. L. Chen. Statistical analysis was performed using unpaired Student's t-test. The EC-50 value

for AF-1 inhibition was calculated from mean concentration-response curves, this value causing 8 mV hyperpolarization. The values calculated for other peptides were not EC-50 values but the concentration of peptide which hyperpolarized or depolarized the neurones by 8 mV. This provides an indication of relative potency.

RESULTS

The identified neurones used in this study were located in either the visceral ganglion, cells E12-16, or the right parietal ganglion, cells F-1, 2 and 5/6. As can be seen from Table 1, all the peptides tested in this study could excite certain neurones while inhibiting others. Figure 1 provides a summary of the results, pooled from the neurones used in this investigation. The peptides were applied at 10 μM and so provide a guide as to their relative potencies at this concentration on these neurones. On neurones inhibited and hyperpolarized by the peptides the potency ratios were as follows: DNFLRFamide > FMRFamide > PDVDHVFLRFamide = KNEFIRFamide > FLRFamide ≫ SDRNFLRFamide = SDPNFLRFamide > KHEYLRFamide. However, if the responses of these peptides are analysed in terms of their actions on specific identified neurones then it is clear that the picture is more complex. Figure 2 shows the relative potencies of these peptides on identified cell groups, F-1 and E-12 areas, Figure 2A, and F-5/6 and E-14 areas, Figure 2B. From this it can be seen that for cells in the F-1 and E-12 areas, PDVDHVFLRFamide is more potent than FMRFamide ($p < 0.0001$). In contrast, for cells in the F-5/6 and E-14 areas, FMRFamide is very potent while PDVDHVFLRFamide is almost inactive ($P < 0.0001$). In both cases FLRFamide is slightly less active than FMRFamide and SDRNFLRFamide is more active than SDPNFLRFamide.

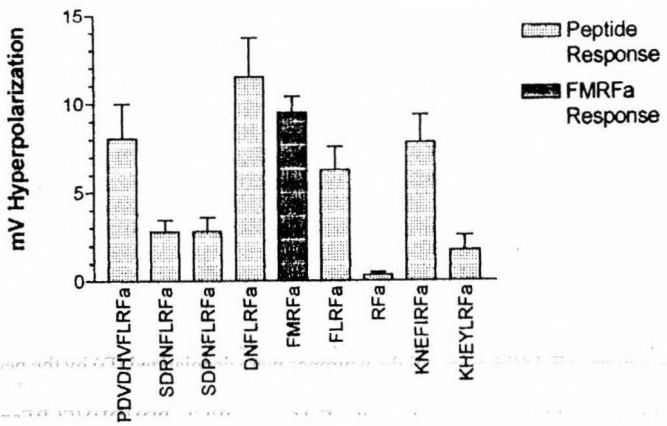

Fig. 1. Summary of the results from over 50 neurones located in the visceral and right parietal ganglia which were hyperpolarized (H cells) by FMRFamide. Each peptide was applied at least five times at a standard concentration of 10 μM. Error bars indicate S.E.M.

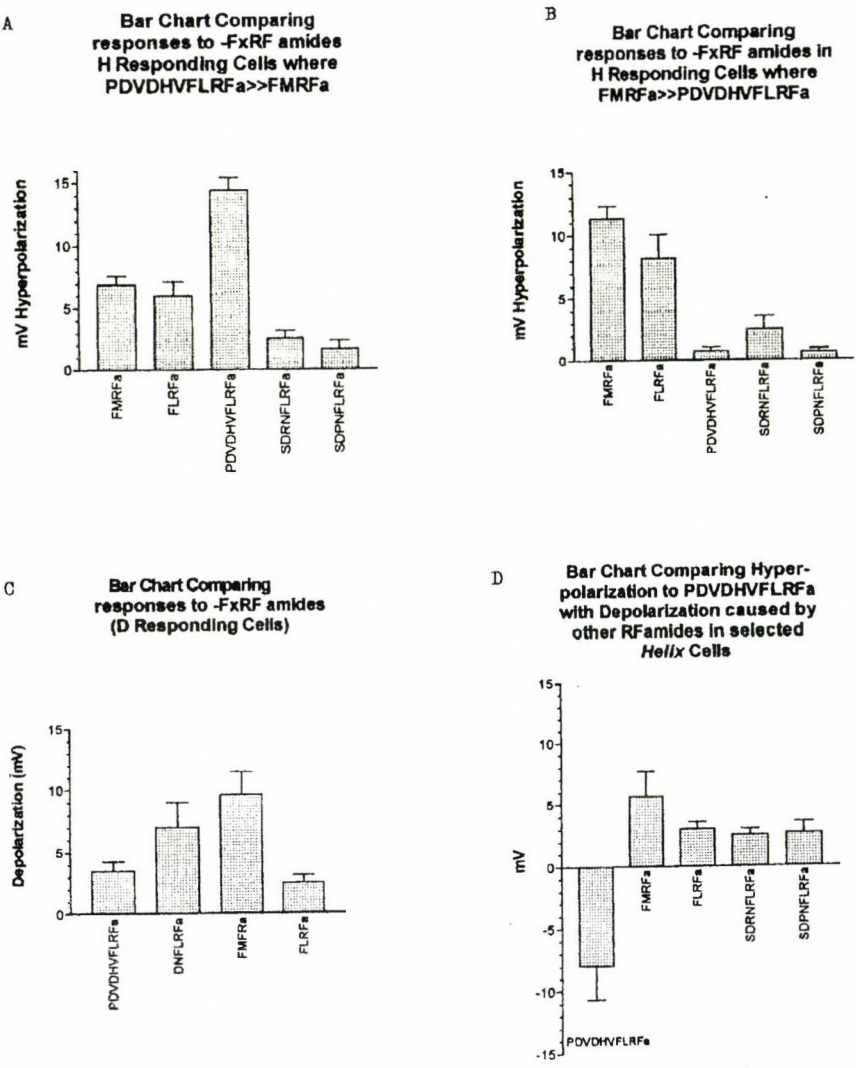

Fig. 2. A). Summary of the results from a minimum of six preparations using cells F-1 and E-12 area. All the neurones were hyperpolarized (H) by the peptides when applied at a concentration of 10 μM. Error bars indicate S.E.M. – *B)* Summary of the results from a minimum of six preparations using neurones F-5/6 and E-14 area. All the neurones were hyperpolarized (H) by the peptides when applied at a concentration of 10 μM. Error bars indicate S.E.M. – *C)* Summary of the results from a minimum of four preparations using neurones E-14/16 area. All the neurones were depolarized (D) by the peptides when applied at a concentration of 10 μM. Error bars indicate S.E.M. – *D)* Summary of the results from two neurones in the F-13/14 area and two neurones from the E-16 area. While PDVDHVFLRFamide inhibited these neurones, the other peptides depolarized them. All the peptides were applied at 10 μM. Error bars indicate S.E.M.

As seen in Table 1, not all cells are inhibited by these peptides, for example, neurones in the E-14/16 area are also excited, Figure 2C, with FMRFamide the most potent and FLRFamide the least potent. Overall PDVDHVFLRFamide is not very active on these neurones. However, this peptide has an interesting effect compared with the other peptides on certain neurones in the F-13/14 and E-16 areas, Figure 2D. In this case while PDVDHVFLRFamide is inhibitory, the other peptides tested were all excitatory. This demonstrates that PDVDHVFLRFamide can act on a receptor which is different to that activated by the tetrapeptides FMRFamide and FLRFamide and the octapeptides SDRNFLRFamide and SDPNFLRFamide. An example of the responses to FMRFamide, FLRFamide, SDRNFLRFamide, SDPNFLRFamide and PDVDHVFLRFamide is shown in Figure 3. In this figure, 10 μM FMRFamide produced a very strong depolarization and increase in cell firing rate. By comparison, FLRFamide, 10 μM, produced only a slow and modest increase in firing rate accompanied by little depolarization. Both SDRNFLRFamide and SDPNFLRFamide, 10 μM, depolarized and excited the cell with a potency between that of FMRFamide and FLRFamide. Following the addition of the other peptides, the response to FMRFamide was reduced but still produced a clear depolarization and excitation of the cell. A concentration-response curve for PDVDHVFLRFamide hyperpolarization on cell E-12 is shown in Figure 4. The EC-50 value for this peptide on E-12 would be around 3 μM. The relative potency value given in Table 1 is 4.6 μM. In comparison, the relative potency value for this peptide on cells in the F-5/6 and E-14 areas is 60 μM suggesting these cells are devoid of a specific receptor for this peptide. Table 1 provides an overall idea of the relative potencies of the peptides in terms of excitation: FMRFamide > DNFLRFamide ≫ SDPNFLRFamide > PDVDHVFLRFamide ≫ KNEFIRFamide = KHEYLRFamide = SDRNFLRFamide.

Table 1
Summary of the potencies of seven FxRFamide peptides expressed in μM

Peptide	Concentration in μM to		Cell type
	hyperpolarize	depolarize	
KNEFIRFamide (AF-1)	0.54	>100	H: F-2; D: E-14, F-77
PDVDHVFLRFamide	4.4	60	H: E-12; D: E-14
DNFLRFamide	6	11	H: E-13; D: E-14, E-11
FMRFamide	7	7	H: various; D: various
SDRNFLRamide	30	>100	H: F-1, F-5; D: F-13, E-16
SDPNFLRFamide (PF-1)	60	90	H: F-2; D: E-14
KHEYLRFamide (AF-2)	>100	>100	H: F-2; D: E-14

The value for KNEFIRFamide inhibition is a true EC-50, where this value induced a hyperpolarization of 8 mV. All the other values are not EC-50s for the compounds but values where the peptide either hyperpolarized or depolarized the neurones by 8 mV. This provides nevertheless an indication of relative potency.

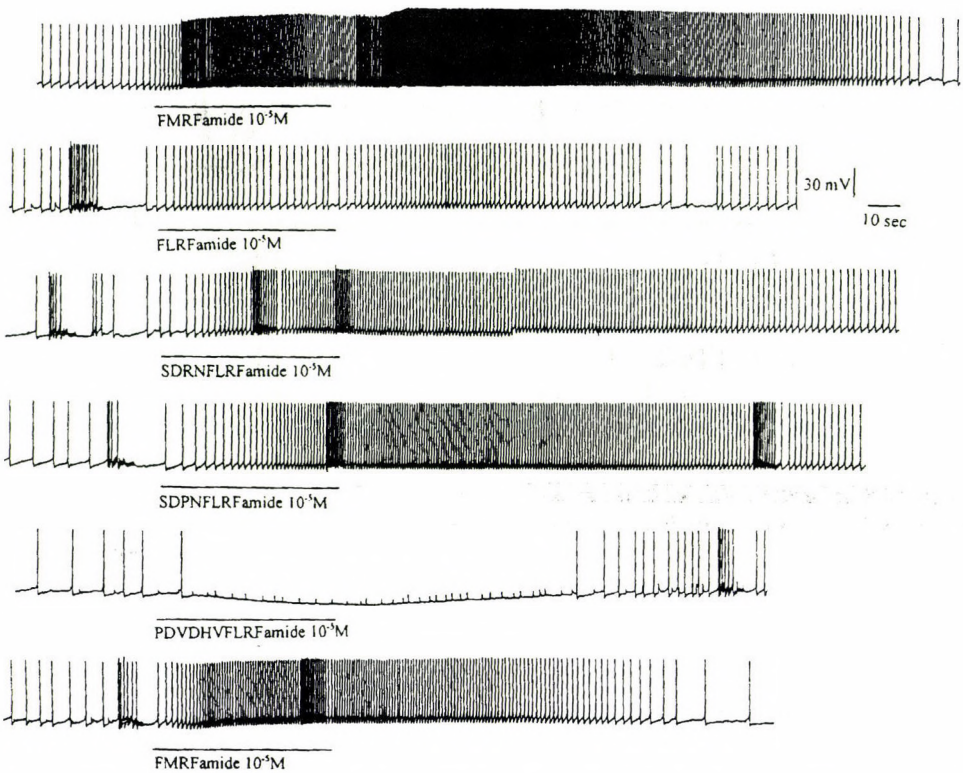

Fig. 3. Traces to show the effect of a series of FxRFamide peptides on the activity of neurone F-13. While 10 μM FMRFamide, FLRFamide, SDRNFLRFamide and SDPNFLRFamide depolarized and excited the neurone, PDVDHVFLRFamide 10 μM hyperpolarized the neurone and inhibited activity

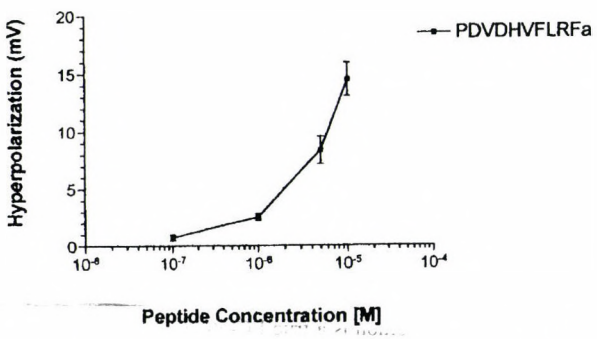

Fig. 4. Graph to show the inhibitory effect of increasing concentrations of PDVDHVFLRFamide on cell E-12. Experiments were performed on at least four preparations at each concentration. Error bars indicate S.E.M.

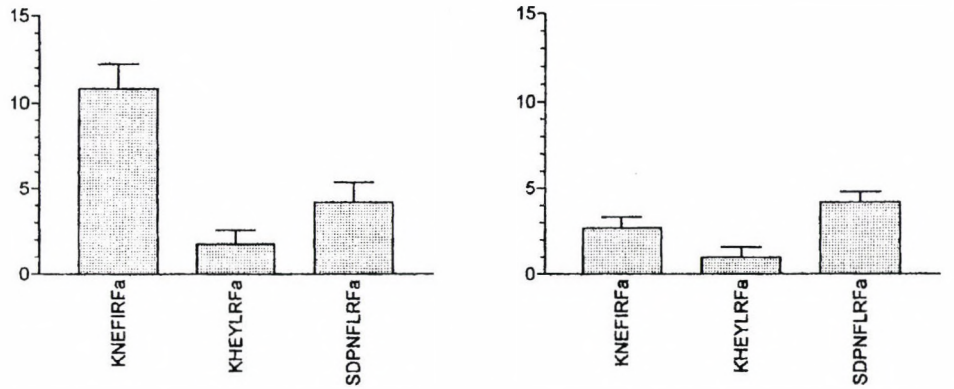

Fig. 5. A) Summary of the actions of 10 μM of the nematode peptides, KNEFIRFamide, KHEYLRFamide and SDPNFLRFamide on neurone F-2. Of the eight neurones investigated, four failed to respond to KHEYLRFamide (AF-2) and one failed to respond to SDPNFLRFamide (PF-1). Error bars indicate S.E.M. – *B)* Summary of the actions of 10 μM of the nematode peptides, KNEFIRFamide, KHEYLRFamide and SDPNFLRFamide on neurone E-14. Experiments were performed on at least five neurones. Two neurones failed to respond to KHEYLRFamide. Error bars indicate S.E.M.

The action of three nematode peptides was also investigated on *H. aspersa* neurones, Table 1. All three peptides could excite or inhibit cell activity. These data are presented as histograms in Figure 5. On cells inhibited by the peptides, KNEFIRFamide is the most potent (Fig. 5A), while on cells excited by the peptides, none show much activity (Fig. 5B). A concentration-response graph for KNEFIRFamide inhibition on cell F-2 is shown in Figure 6A where this peptide has an EC-50 value of 540 nM, Table 1. An example of an experiment where acetylcholine and FMRFamide both excite while KNEFIRFamide inhibits is shown in Figure 6B. As can be seen in this figure, KNEFIRFamide is potent on this cell, with 10 nM inhibiting cell activity. Higher concentrations produce clear hyperpolarization with the effects being concentration-dependent. Clearly FMRFamide and KNEFIRFamide act on different receptors on cell F-2.

The effects of changing external ion concentrations on the hyperpolarization induced by KNEFIRFamide and DNFLRFamide were determined. The results are summarised in Figure 7. As can be seen in Figure 7A, both low external chloride, 16 mM from control of 108 mM, and high external potassium, 16 mM from control of 4mM, significantly reduced the hyperpolarization induced by KNEFIRFamide ($p < 0.005$). However, neither sodium-free nor potassium-free significantly altered the response. For comparison, the effect of changing external ion concentrations on the hyperpolarization induced by DNFLRFamide was also investigated and the results summarised in Figure 7B. High external potassium greatly reduced the response to DNFLRFamide ($p < 0.001$) while potassium-free enhanced the response but this was not significant ($p = 0.1$). Further support that the hyperpolarization induced by this peptide is mainly a potassium-mediated event came from testing the

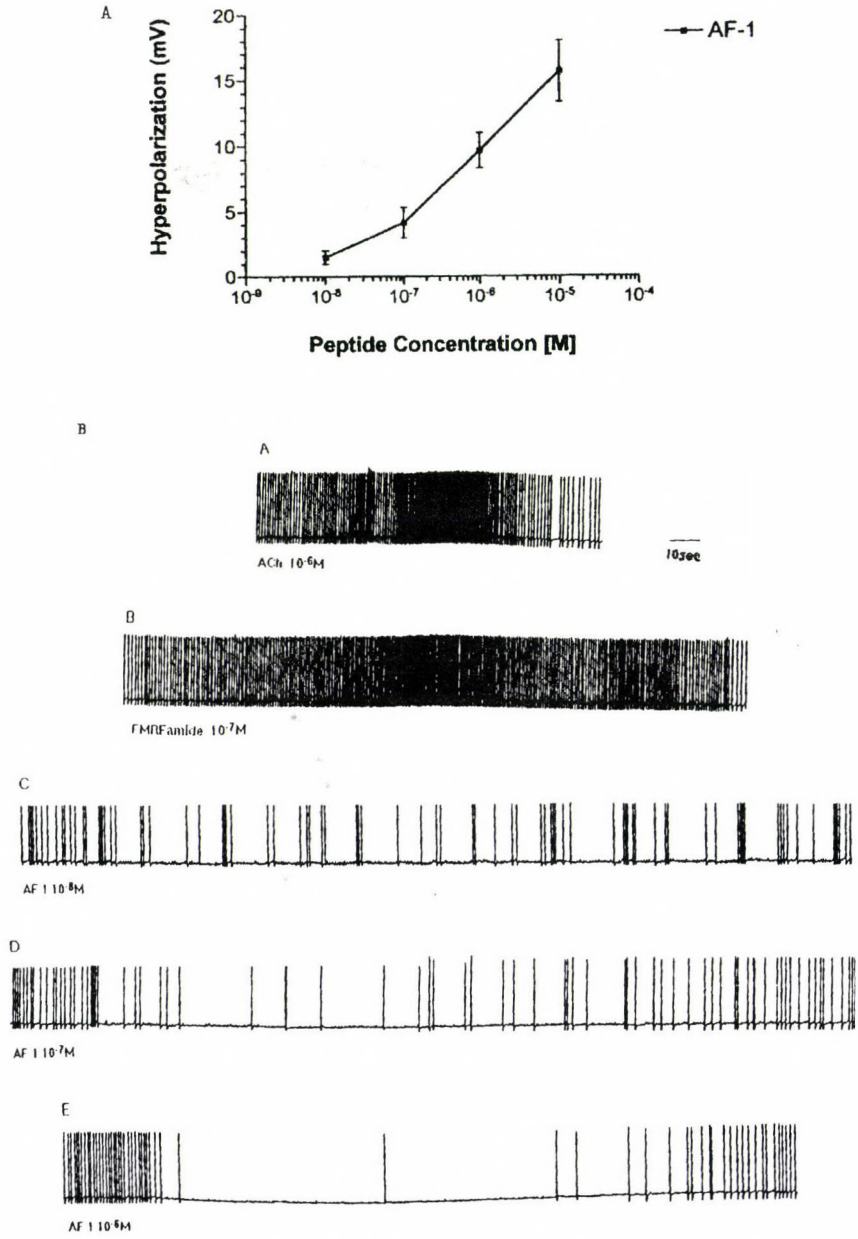

Fig. 6. A) Graph to illustrate the concentration-dependent responses to KNEFIRFamide (AF-1) on cell F-2. Experiments were performed on at least six preparations. Error bars indicate S.E.M. – *B)* Traces to show the effect of acetylcholine (ACh), FMRFamide and increasing concentrations of AF-1 on neurone F-2. Threshold inhibition due to AF-1 application was 10 nM. While ACh 1 μM and FMRFamide 100 nM excited the neurone, AF-1 inhibited cell activity with clear hyperpolarization

effects of TEA and 4-AP. Both TEA, 10 mM, and 4-AP, 250 μM, both almost completely blocked the hyperpolarization response. An example of this is shown in Figure 8. While the response to DNFLRFamide recovered following removal of TEA, the response did not recover after 4-AP application. In contrast neither low chloride nor low sodium had any effect on DNFLRFamide hyperpolarization.

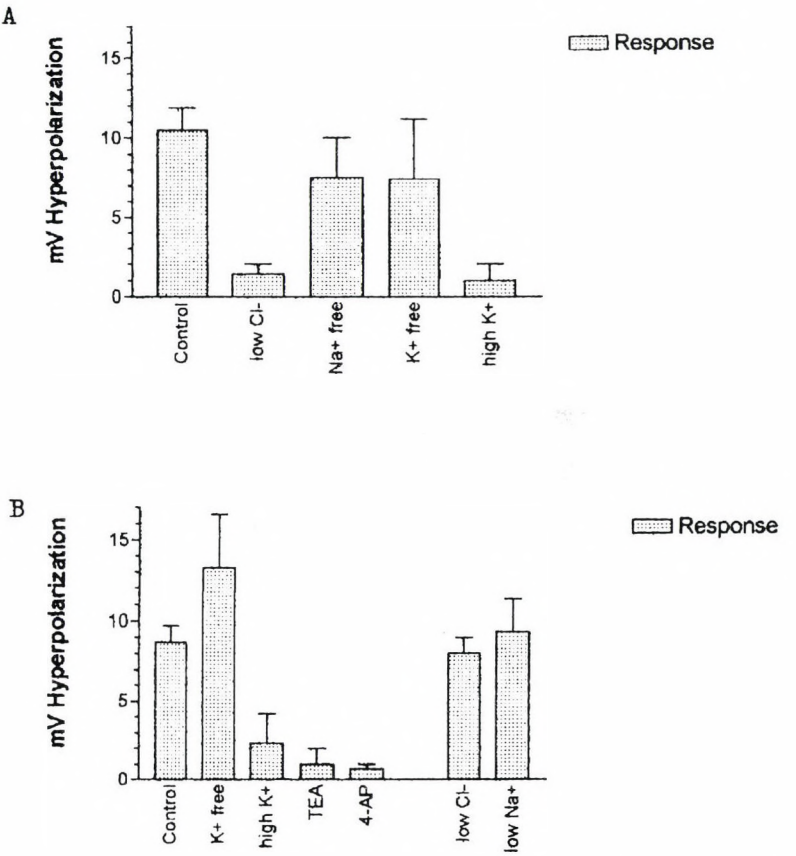

Fig. 7. A) Summary of the results of changing the concentration of ions in the external saline on the hyperpolarizing responses to AF-1, 10 μM. The experimental saline was applied at least five minutes prior to application of AF-1. Each experiment was performed at least four times. Error bars indicate S.E.M. Low chloride was 16 mM and high potassium was 16 mM. – *B)* Summary of the results of changing the concentration of ions in the external saline on the hyperpolarizing responses to DNFLRFamide, 50 μM. The experimental saline was applied at least five minutes prior to application of the peptide. Each experiment was performed at least four times. Error bars indicate S.E.M. High potassium was 16 mM, TEA was 10 mM, 4-AP was 250 μM, low chloride was 16 mM and low sodium was 20 mM

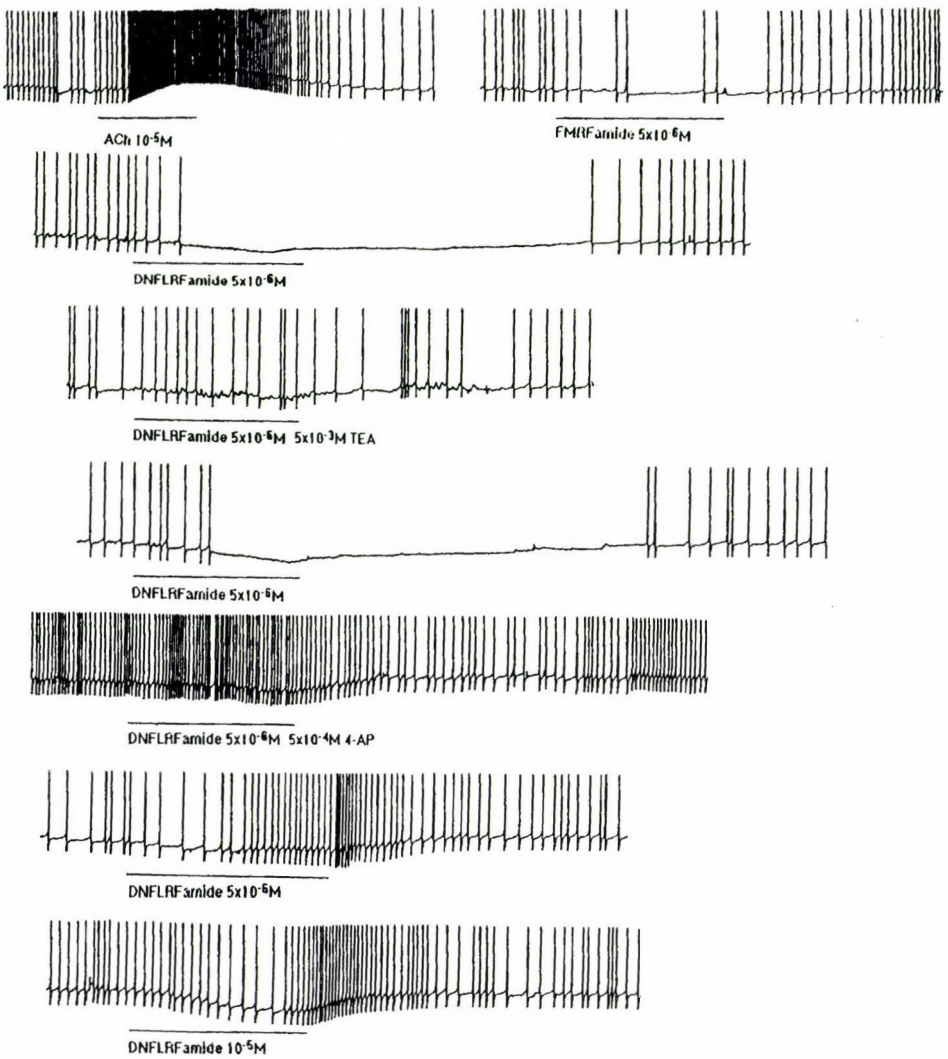

Fig. 8. Traces to show the effect of 5 mM TEA and 500 μM 4-AP on the inhibitory effect of 5 μM DNFLRFamide. While the effect of TEA was reversible, that of 4-AP only reversed to a small degree

DISCUSSION

The peptides investigated in this study can be divided into several groups. Firstly there are peptides which occur in molluscs, such as, FMRFamide and FLRFamide. Secondly there is a peptide which occurs in insects, PDVDHVFLRFamide and three peptides which occur in nematodes, i.e. SDPNFLRFamide (PF-1) and KNEYLRFamide (AF-2). Thirdly there is a peptide which has been identified in crustacea, SDRNFLRFamide and finally a synthetic peptide, DNFLRFamide. All these extended peptides, with the exception of KNEFIRFamide, possess a C terminal LRFamide. One question to be addressed concerns the number of receptors which are activated by these peptides.

The ionic mechanisms and receptor subtypes associated with FMRFamide and extended peptides have been investigated by Cottrell and his group [2, 3, 4]. These authors found that tetrapeptides were active on slow potassium hyperpolarization responses and sodium-mediated depolarizations while extended peptides were active on fast potassium-mediated hyperpolarization responses and depolarizations mediated by a reduction in potassium conductance. It is difficult to interpret the present results in the light of the earlier findings. Clearly the ionic mechanism for PDVDHVFLRFamide should be investigated though on cells F-5/6 and E-14, FMRFamide is far more active than PDVDHVFLRFamide, suggesting this hyperpolarization may be via a slow potassium-mediated event.

There have been several decapeptides identified in arthropods, e.g. pQDVDHVFLRFamide in cockroaches [11] and PDVDHVFLRDFamide in locusts [22]. This latter peptide has potent inhibitory effects on a range of tissues in the locust including the heart and oviduct. A related decapeptide occurs in the moth, *Manduca*, pEDVVHSFLRFamide [13]. In contrast, a nonapeptide based on FMRFamide has been identified in *Drosophila*, DPKQDFMRFamide [23]. In the present study PDVDHVFLRFamide was overwhelmingly inhibitory with only weak excitation on cells E-14/16. This peptide clearly activates a different receptor on F-13/16 to either tetrapeptides or octapeptides since it is inhibitory on these cells while the other peptides, including both tetrapeptides and octapeptides, were excitatory. It would be interesting to identify the structure of the endogenous peptide in *Helix* which activates this receptor.

Octapeptides have been identified in crustacea with the sequences, SDRNFLRFamide and TNRNFLRFamide [14, 24] and heptapeptides, NRNFLRFamide and DRNFLRFamide [17, 18]. These may be equivalent to the extended heptapeptides found in *Helix* and *Lymnaea*, such as, SDPFLRFamide. Certainly SDRNFLRFamide was very weak on cells depolarized by RFamides. The actions of SDRNFLRFamide have been investigated on motor patterns in the crab stomatogastric ganglion [26]. SDRNFLRFamide and the related peptide, TNRNFLRFamide, both activate this preparation with a threshold in the low pM range. The evidence would suggest that these peptides were mainly excitatory on stomatograstric neurones. In contrast, on *Helix* neurones, SDRNFLRFamide appears to be mainly inhibitory with an apparent

EC-50 value of 70 μM and a threshold in the high nM range. This is considerably less potent than the situation in the stomatogastric ganglion.

Two of the nematode peptides studies in the present investigation, KNEFIRFamide (AF-1) and KHEYLRFamide (AF-2) were first identified in *Ascaris* [6, 7]. The third nematode peptide studied here is SDPNFLRFamide (PF-1) and first identified in *Panagrellus* [1]. This latter peptide has potent relaxing and inhibitory actions on nematode body wall muscles and is closely related to the crustacean peptide, SDRNFLRFamide, already mentioned. In contrast, both AF-1 and AF-2 are mainly excitatory on *Ascaris* body wall muscle. However, AF-1 is inhibitory on motoneurone activity in *Ascaris* [7]. AF-1 proved to be a potent inhibitory peptide on *Helix* neurones and may act on a specific receptor for this peptide. If this is the case, then there must be an endogenous *Helix* peptide which activates this receptor. AF-2 was relatively inactive on cells potently inhibited by AF-1, suggesting a different receptor subtype. PF-1 is similar in structure to SDRNFLRFamide and is of a similar potency and so may activate the same receptor subtype. From the ion studies, it would appear that both potassium and chloride are involved in the AF-1 response but further studies are required to resolve this. In this context it would be very interesting to investigate the action and ionic mechanism of another inhibitory peptide from *Panagrellus,* KPNFIRFamide (PF-4) [16], on *Helix* neurones. In contrast to other RFamide inhibitory responses on gastropod neurones, which are generally potassium-mediated, the inhibitory action of PF-4 on *Ascaris* body wall muscle is mediated mainly through an increase in chloride conductance [10]. This PF-4 induced hyperpolarization is a fast response, resembling the action of GABA on the *Ascaris* muscle but does not act on GABA receptors to produce this inhibition.

The synthetic hexapeptide, DNFLRFamide, proved to be both inhibitory and excitatory on *Helix* neurones. The ionic mechanism for the inhibitory phase clearly involves an increase in permeability to potassium and it would be interesting to explore the type of receptor which this peptide is activating. However, the response to changing external potassium was very variable which makes interpretation of the results difficult.

In conclusion, this study shows that peptides isolated from both arthropods and nematodes are active on *Helix* central neurones. Evidence is presented which suggests that one or more of these peptides may act on a specific FxRFamide receptor subtype. This would suggest that a peptide with a similar structure to these non-molluscan FxRFamide peptides may be present in *Helix.* However, considerably more work needs to be undertaken before this can be confirmed.

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EFFECT OF VOLATILE ANAESTHETICS ON THE ELECTRICAL ACTIVITY AND THE COUPLING COEFFICIENT OF WEAKLY ELECTRICALLY COUPLED NEURONES*

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1. The application of the volatile anaesthetics, halothane and isoflurane (1% v/v and 2% v/v), to the CNS of *Lymnaea* reduced the firing frequency of the small weakly coupled pedal A cluster (PeA) neurones, which eventually become quiescent. There was no change in their resting membrane potential.
2. Met-enkephalin significantly increased the coupling coefficient between PeA neurones.
3. The volatile anaesthetics decreased the coupling coefficient even in the presence of met-enkephalin.
4. These effects were dose dependent and the effects of halothane were more rapid than those of isoflurane, reflecting their different anaesthetic potencies.

Keywords: Volatile anaesthetics – met-enkephalin – *Lymnaea* – electrical coupling

INTRODUCTION

Opinions are divided as to the cellular mechanisms of general anaesthesia. Anaesthetic agents are generally believed to interact with hydrophobic regions of the plasma membrane, but it is unclear whether the hydrophobic sites are the membrane lipids themselves or the membrane proteins. Given that anaesthetics have a multiplicity of cellular effects it is, however, unlikely that only the membrane protein or lipids are modified by them. All membrane components are possible targets including nerve terminals. Indeed it is widely believed that anaesthetic actions on chemical synapses are of great importance in anaesthesia. Electrical synapses have not so far been studied in detail with respect to anaesthesia, although gap junctions in the heart have been investigated [28]. Here it was shown that halothane decoupled cell pairs isolated from guinea pig ventricles.

Electrical coupling is of great importance for synchronisation of the activity of cells in many different tissues such as heart muscle [10] and neurones [29]. The first electrical synapse was discovered by Furshpan and Potter [12] between nerve cells

*Dedicated to Professor János Salánki for his 70th birthday.

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of crayfish. Through these synapses, sub-threshold currents can pass from one cell into the other. Furthermore, it is generally accepted that communication via electrical synapses is mediated by gap junctions [for review see 6, 10, 16, 25]. The CNS of *Lymnaea* contains many different electrically coupled systems e.g. VD1/RPD2, the cerebral giant cells (CGC's) and PeA systems [17, 27, 30]. The degree of coupling varies from one system to another within the same tissue. Sometimes it is very strong and the activity of the neurones is in almost perfect synchrony, such as between the neurones VD1 and RPD2 and the CGC's. Conversely, in other systems, electrical coupling between neurones can be weak, such as between Pedal A cluster (PeA) neurones.

The Pedal A-cluster neurones of *Lymnaea* are weakly coupled. They were found to be distinguished from other pedal neurones on the bases of their morphology and their electrical characteristics [27]. It has also been reported that these neurones contain the neurotransmitter serotonin [3, 8]. Syed [26] showed that they act as ciliomotor neurones modulating the activity of the locomotor cilia of *Lymnaea* which are excited by the injection of serotonin into the headfoot.

PeA neurones are located on the medial edges of the largely symmetrical left and right pedal ganglia [20, 27]. The morphological and electrophysiological characteristics of PeA neurones were studied intensively by Syed and Winlow [27]. The effects of direct synaptic connections onto the cells by the peptidergic neurone VD4 have been described [27] and the effects of anaesthetics on these chemical synapses are presented elsewhere [23, 24].

In this paper the effects of the volatile anaesthetics, halothane and isoflurane (1% and 2%) on the electrical activity and the coupling coefficient between Pedal A neurones of the pond snail *Lymnaea stagnalis* are demonstrated in the presence and the absence of Met-Enkephalin. These investigations were performed mainly on the coupling coefficient between adjacent small PeA neurones.

MATERIAL AND METHODS

The methods used are as previously reported [5]. Briefly, simultaneous intracellular recordings were made from either the neurones VD1 and RPD2 or from two PeA neurones. These recordings were performed when the snail brain was perfused with HEPES buffered saline (HBS), or HBS-containing the anaesthetic agents. Data were captured and transferred to a PC using a C.E.D. 1401 interface and analysed using a spike2 software package.

Two different concentrations of each of the volatile anaesthetics, halothane and isoflurane were used. They were prepared with the aid of vaporisers (Fluotec 3 for halothane and Isotec 3 for isoflurane, Ohmeda). The length of the plastic tubing between the vaporisers and the glass bottles was minimised to reduce any possible absorption of the anaesthetics. The flow rate of air was adjusted to 2.5 litres per minute and the concentration of the anaesthetic was adjusted to the required concentration. The vaporised anaesthetic was bubbled into the HBS through a filter stone

and the gas was allowed to equilibrate for 20 minutes at ambient temperature. The excess of the anaesthetic was drawn off into the fume cupboard [13, 14]. All anaesthetic solutions were prepared immediately prior to the experiment. Different flasks, tubing and filters were used for different anaesthetic to minimise the risk of contamination. The concentrations of the anaesthetics quoted in the text are the initial reservoir concentrations.

However, various concentrations (in mM) of the two volatile anaesthetics present in the recording dish were determined by extracting the anaesthetic into carbon tetrachloride and analysed using gas liquid chromatography (GLC). The final measurement concentrations were as in Table 1 and this compares well with previously recorded values in this laboratory [21, 31] and are well within the clinical range (0.20–0.78 mM [9]), at least for halothane. Similar clinical data is not available for isoflurane as far as we are aware.

To investigate the effect of general anaesthetics on coupling coefficient between small PeA neurones, the following procedure was used:

Preparations were kept perfused with HBS and were left to stabilise for at least 30 min. Then simultaneous intracellular recordings were obtained from two small adjacent PeA neurones. Hyperpolarising current pulses (0.3–1.5 nA) were injected into one of the neurones for 1–2 seconds every 5–10 seconds. This current-pulse was maintained throughout the experiment. After recording of the control, the preparation was perfused with HBS-containing 1% halothane followed by HBS-containing 2% halothane and where possible followed by HBS-containing 1% or 2% isoflurane. In all cases, preparations were washed with HBS after the application of HBS-containing the anaesthetic. In a few experiments, the order of the application of anaesthetic concentrations was varied, but the results obtained were not statistically different from those in the original sequence. In 10 experiments, before and after recording normal neuronal activity and calculating the coupling coefficient between the two neurones, they were perfused with HBS-containing met-enkephalin (10^{-5} mM) followed by HBS-containing met-enkephalin (10^{-5} mM) and the above anaesthetics (in the above concentrations) at the same time. Washing the CNS with HBS-containing met-enkephalin (10^{-5} mM) always took place after the application of HBS-containing both met-enkephalin (10^{-5} mM) and the anaesthetic.

RESULTS

Effects of anaesthetics on spontaneous activity of Pedal A cluster neurones

a) Halothane

In all of the isolated whole brain preparations, the neurones of PeA (16/16) were found to fire spontaneously. However, they did not show any synchronisation in their electrical activity and they fired irregularly (Figs 1a, d, 2a, d).

Table 1
Comparison between anaesthetic concentrations in the flasks and the recording dish

Anaesthetic	Concentrations in flasks		Concentration in recording dish
	%	mM (m ± sd)	mM (m ± sd)
Halothane (n = 4)	1	0.71 ± 0.05	0.43 ± 0.07
	2	1.14 ± 0.08	0.77 ± 0.10
Isoflurane (n = 4)	1	0.65 ± 0.09	0.37 ± 0.05
	2	1.2 ± 0.12	0.78 ± 0.09

These results demonstrate that the concentrations used are within the same range of other results obtained in the same laboratory and found to be within the clinical range.

Table 2
The effective (Te) and recovery times (Tr) of general anaesthetics on small PeA neurones

Anaesthetic	Te (min) (m ± sd)	Tr (min) (m ± sd)
1% halothane	7.3 ± 1.3 (6)	7.8 ± 1.7
2% halothane	2.8 ± 1.0 (6)	10.4 ± 2.2
1% isoflurane	8.9 ± 1.5 (5)	6.4 ± 0.7
2% isoflurane	4.1 ± 0.8 (5)	7.5 ± 1.2

Although the time taken for the anaesthetic to have its maximum effect (Te) of 2% halothane was more than 60% and for isoflurane was approximately 50% shorter than 1%, the recovery time (Tr) was approximately 30% longer for both of them. The number between brackets = n.

1% halothane reduced the firing frequency of coupled PeA neurones, eventually producing quiescence. Similar results were gained with 2% halothane, but again the effect was faster (Table 2 and Fig. 1). However, there was no change in the resting membrane potential (–46 ± 3.5 mV; n = 5) after application of either concentration.

b) Isoflurane

The effect of the volatile anaesthetic isoflurane on the spontaneous electrical activity of different *Lymnaea* neurones has been examined on 22 different preparations. As with halothane, it was applied to the CNS by vaporisation in HBS as used by Girdlestone et al. [14], and as described the methods section.

In 10 different preparations the electrical activity of these neurones was altered by 1% or 2% isoflurane. 1% isoflurane decreased the frequency of their spontaneous fir-

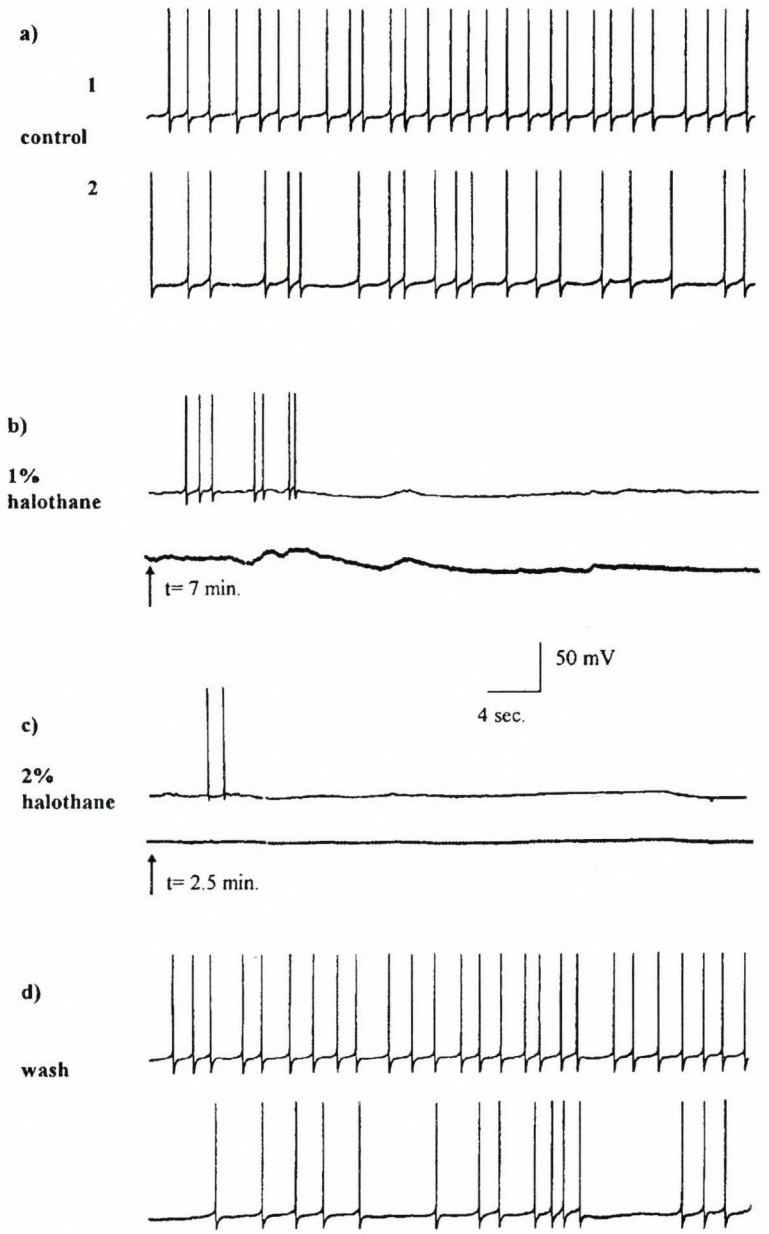

Fig. 1. Effect of halothane on the electrical activity of PeA neurones. *a)* Normal electrical activity of two small neurones of PeA. Even though they are electrically coupled, they are not firing in synchrony. *b)* and *c)* the application of 1% and 2% halothane, respectively, reduced the firing frequency and eventually they became quiescent. The effect of 2% halothane was faster (t = time from application). *d)* the effect was reversible. There was no change in the membrane potential from its resting values

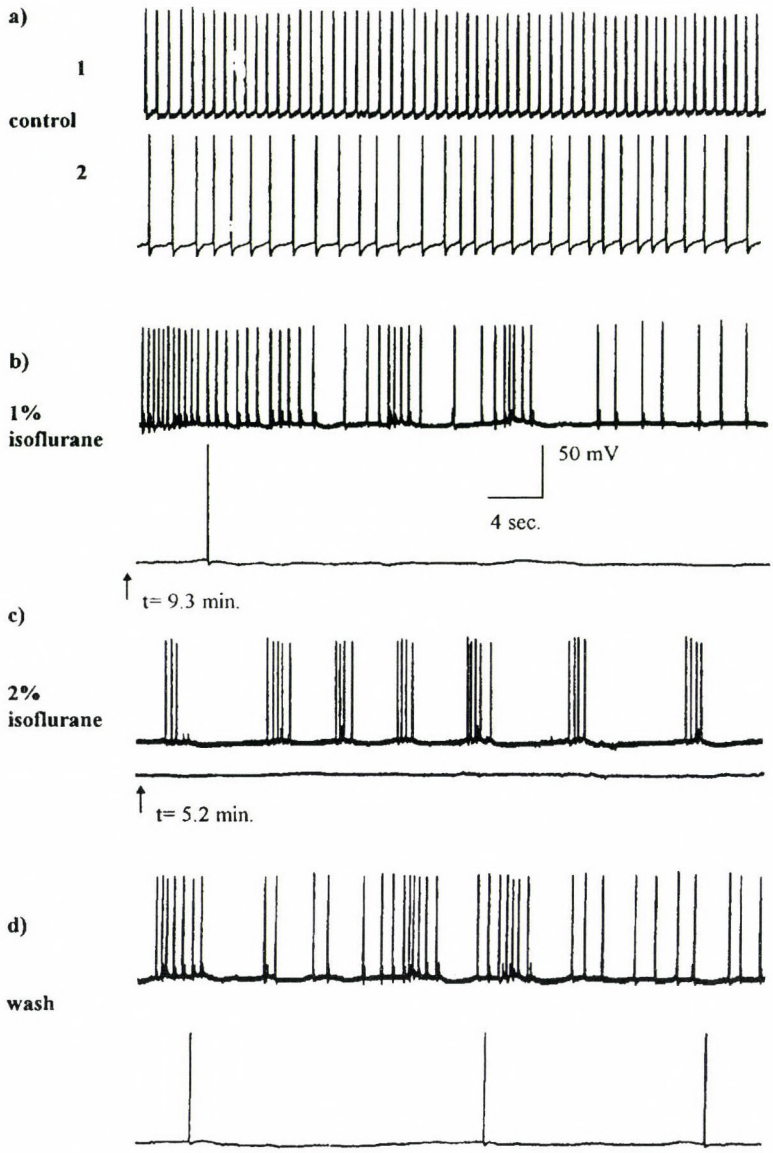

Fig. 2. Effect of isoflurane on the electrical activity of PeA neurones. *a)* Normal electrical activity of two small neurones of PeA. Even though they are electrically coupled, they are not firing in synchrony. *b)* and *c)* the application of 1% and 2% isoflurane, respectively, reduced the firing frequency and eventually they became quiescent. The effect of 2% isoflurane was faster (t = time from application). *d)* the effect was reversible. There was no change in the membrane potential from its resting values

ing after 8.9 ± 1.5 minutes but did not lead to quiescence unless left for a prolong period (i.e. over 30 minutes). 2% isoflurane produced similar effects but more rapidly and lead to quiescence in a shorter time (around 4.1 ± 0.8 minutes) (Fig. 2, Table 2). Isoflurane, at both concentrations, did not alter the resting membrane potential of PeA neurones.

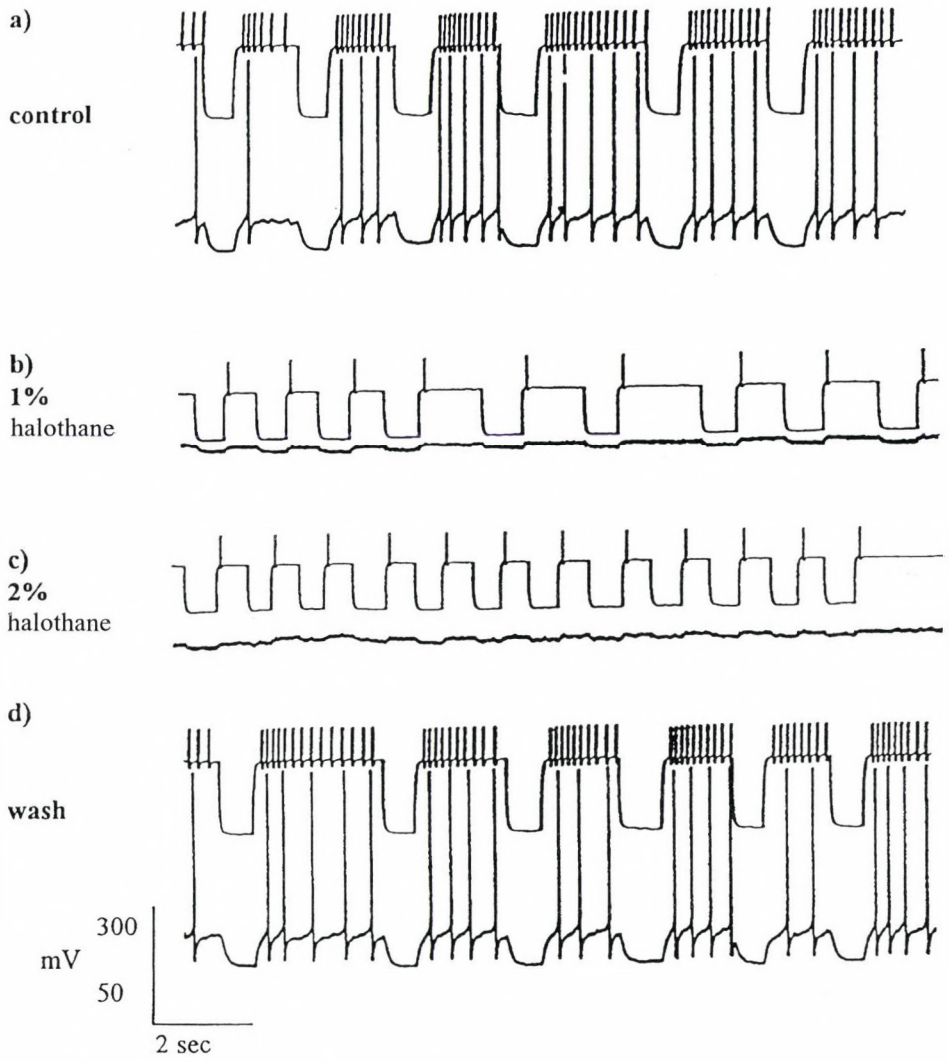

Fig. 3. Effect of halothane on the coupling coefficient between PeA neurones. *a)* The coupling coefficient between PeA neurones was calculated to be 7.58 ± 0.23% (n–16). *b)* and *c)* the application of 1% and 2% halothane reduced the coupling coefficient significantly (5.19 ± 0.23% and 2.86 ± 0.14%, respectively. *d)* the reduction was abolished when the CNS was perfused with fresh HBS again

Effect of volatile anaesthetics on the coupling coefficient between Pedal A cluster neurones

PeA neurones fire with a patterned discharge and sometimes they fire irregularly. Syed and Winlow [27] demonstrated that they are electrically coupled in two ways. Small-sized neurones are weakly electrically coupled to their adjacent cells, while giant and medium-sized neurones make stronger electrical connections with their contralateral homologues as well as to their adjacent cells. Here, we investigated the effect of volatile anaesthetics on the coupling coefficient between the small PeA neurones in both left and right pedal ganglia.

a) Effect of halothane

Normally, the coupling coefficient between PeA neurones was $7.58 \pm 0.52\%$ ($n = 16$). When preparations were perfused with HBS-containing 1% halothane continuously the coupling coefficient decreased significantly to 5.19 ± 0.47. Perfusing CNS of *Lymnaea* with HBS-containing 2% halothane further reduced the coupling coefficient to 2.86 ± 0.21 (Figs 3, 5). These effects of different concentrations of halothane on the coupling coefficient were completely reversible.

b) Effect of isoflurane

Isoflurane affected the coupling coefficient significantly. When the CNS was perfused with HBS, the coupling coefficient between these neurones was equal to $8.1 \pm 0.61\%$ ($n = 16$). Perfusing the CNS with HBS-containing 1% isoflurane caused a significant reduction ($6.85 \pm 0.36\%$). Furthermore, when the CNS was perfused with 2% isoflurane the reduction in coupling coefficient was even more ($4.59 \pm 0.24\%$) (Figs 4, 5).

Effect of volatile anaesthetics on the coupling coefficient between Pedal A cluster neurones in the presence of met-enkephalin

In electrically coupled systems the strength of electrical coupling can be increased by the exogenous application of neurotransmitters or their precursors [15]. Dyakonova et al. [11] were able to facilitate the strong electrically coupled neurones VD1 and RPD2 by the application of serotonin and met-enkephalin. In the presence of met-enkephalin only they were able to enhance the coupling between the weak electrically coupled serotonin-containing PeA neurones. The effects of volatile anaesthetics, halothane and isoflurane on coupling coefficient between small PeA neurones were investigated under control conditions during the application of 10^{-5} M met-enkephalin.

a) Effect of halothane

The CNS of *Lymnaea* was perfused with HBS-containing 10^{-5} M met-enkephalin for the rest of the experiment after recording the normal control ($7.22 \pm 0.46\%$) ($n = 10$). After 30 minutes from the application of 10^{-5} mM met-enkephalin, their coupling coefficient was found to increase significantly to $10.54 \pm 0.72\%$ ($n = 10$,

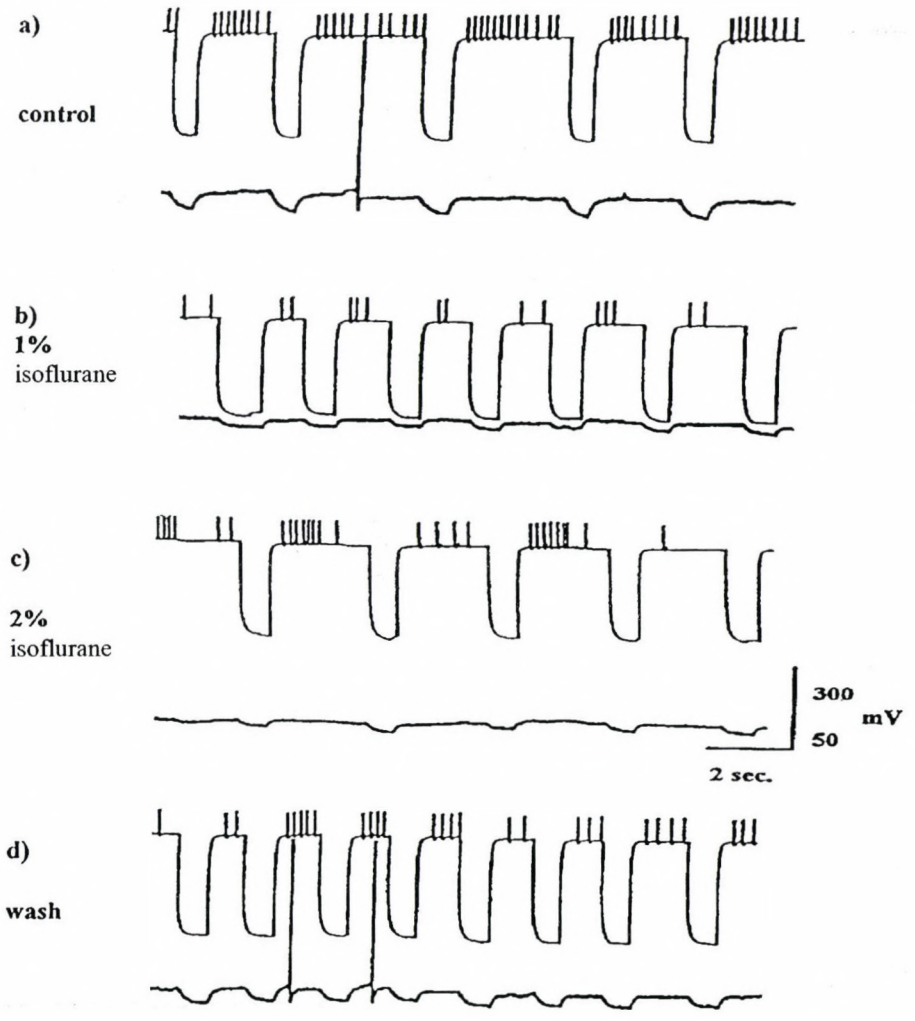

Fig. 4. Effect of isoflurane on the coupling coefficient between PeA neurones. *a)* The coupling coefficient between PeA neurones was calculated to be $8.62 \pm 0.27\%$ (n–16). *b)* and *c)* the application of 1% and 2% isoflurane reduced the coupling coefficient significantly ($6.85 \pm 0.17\%$ and $4.59 \pm 0.24\%$, respectively. *d)* the reduction was abolished when the CNS was perfused with fresh HBS again

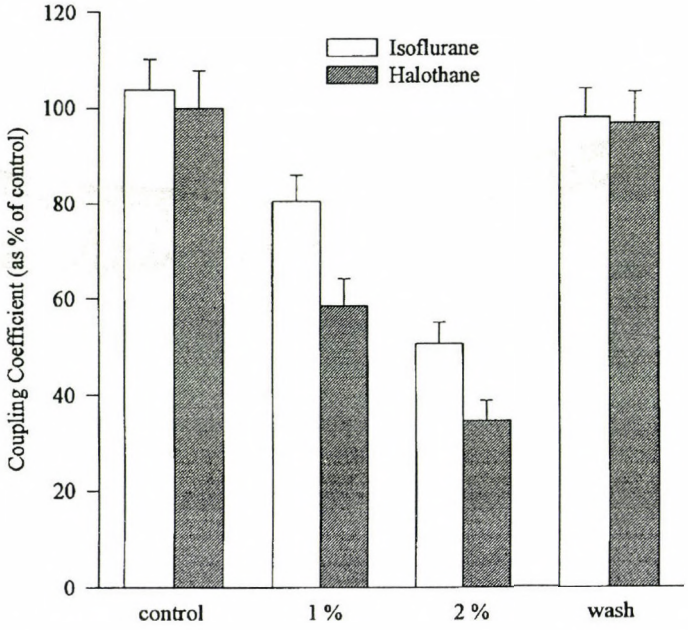

Fig. 5. Effect of volatile anaesthetics on the coupling coefficient between Pedal A cluster neurones. The coupling coefficient between small PeA of *Lymnaea* was significantly decreased by 1% and 2% isoflurane (n = 16) in a dose dependent manner. 1% and 2% halothane induced an even greater reduction in the coupling coefficient in comparison with isoflurane (n = 16). Bars represent mean ±SE

Figs 6, 8). When the brain was perfused with HBS-containing met-enkephalin (10^{-5} mM) and 1% halothane, the coupling coefficient between PeA neurones was significantly decreased to the value of 5.50 ± 0.34% (n = 10, Figs 6, 8). The anaesthetic effect was completely reversible when the CNS was perfused with HBS-containing 10^{-5} M met-enkephalin. The reduction in the coupling coefficient between small PeA neurones was even greater (2.95 ± 0.22%), but still reversible, when the CNS was perfused with HBS-containing 10^{-5} M met-enkephalin and 2% halothane (Figs 6, 8).

b) Effect of isoflurane

As in the above section 10^{-5} M met-enkephalin was found to increase the coupling coefficient between PeA neurone from 7.13 ± 0.61% during the control where the CNS was perfused with HBS to 10.93 ± 0.85 (n = 10) where it was perfused with HBS-containing 10^{-5} M met-enkephalin. The application of HBS-containing 10^{-5} M met-enkephalin and 1% isoflurane caused a significant reduction in their coupling

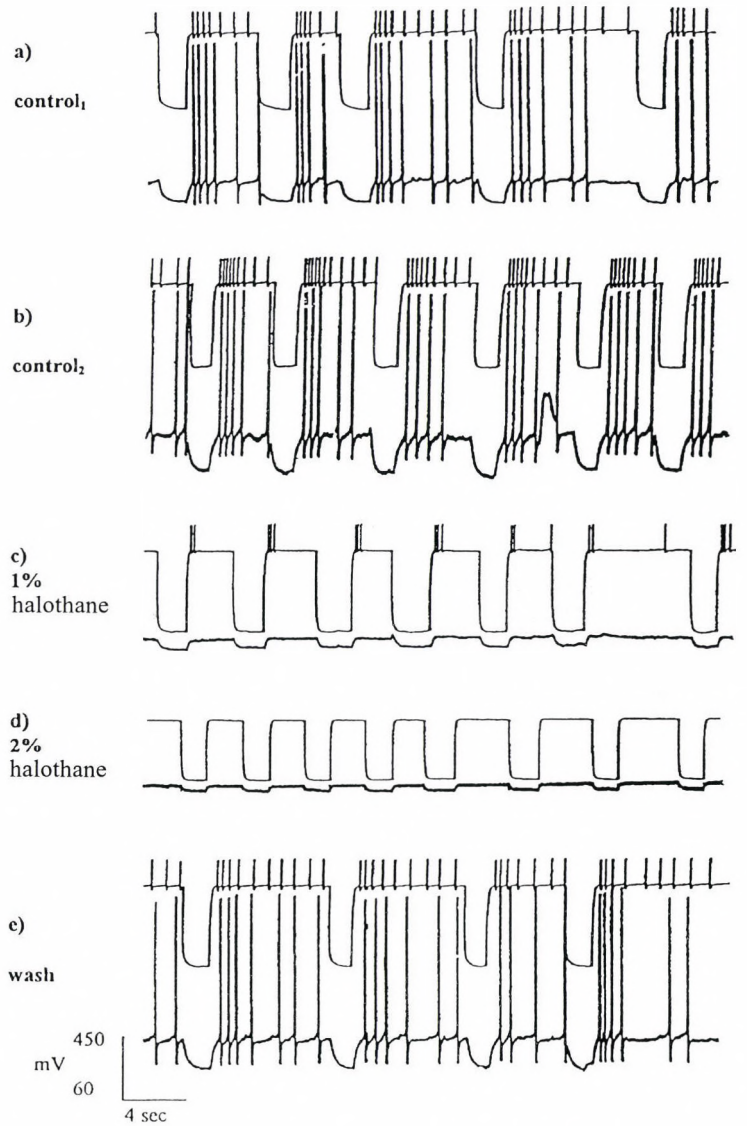

Fig. 6. Effect of halothane on the coupling coefficient between PeA neurones in the presence of met-enkephalin. *a)* The coupling coefficient between PeA neurones was calculated to be 7.22 ± 0.16% (n–10). *b)* a significant increase in their coupling coefficient was demonstrated when the CNS was perfused with HBS-containing 10^{-5} M met-enkephalin (10.54 ± 0.16%; n = 10). *c)* however, when the CNS was perfused with HBS-containing 10^{-5} M met-enkephalin and 1% halothane, a significant reduction in their coupling coefficient was demonstrated (6.85 ± 0.17%) which was reduced to less than its original control value. *c)* this reduction was even greater (4.59 ± 0.24%) when the CNS was perfused with HBS-containing 10^{-5} M met-enkephalin and 2% halothane. *d)* however, the reduction caused by halothane was abolished when the CNS was perfused again with 10^{-5} M met-enkephalin

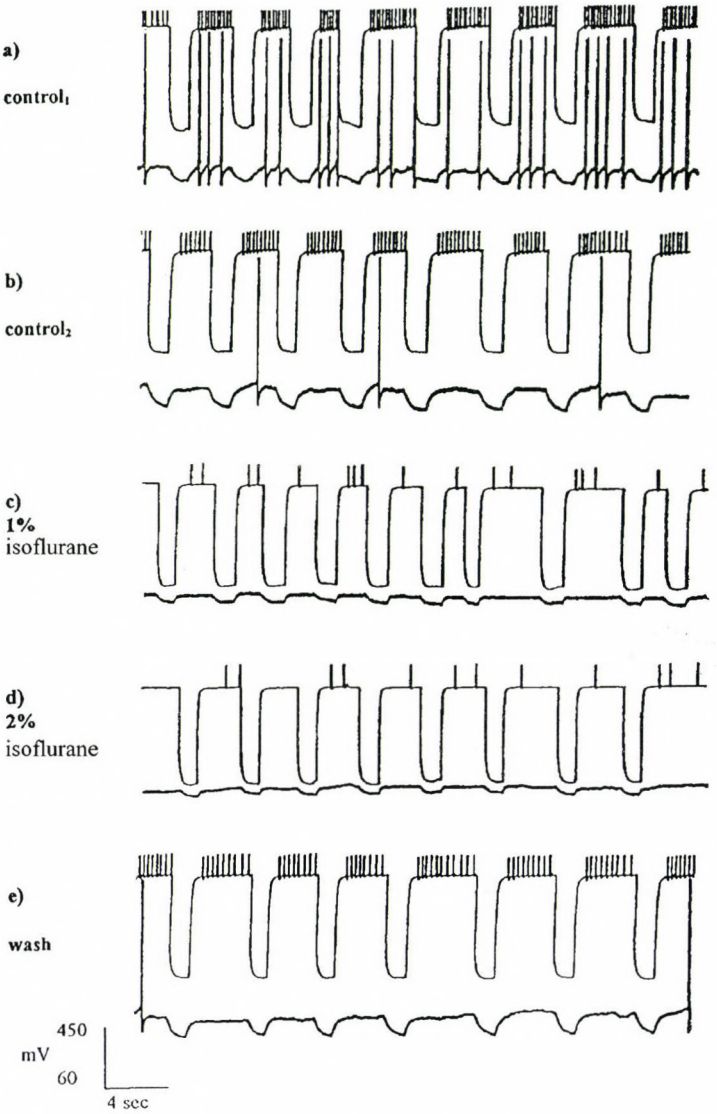

Fig. 7. Effect of isoflurane on the coupling coefficient between PeA neurones in the presence of met-enkephalin. *a)* The coupling coefficient between PeA neurones was calculated to be 7.13 ± 0.09% (n–10). *b)* a significant increase in their coupling coefficient was demonstrated when the CNS was perfused with HBS-containing 10^{-5} M met-enkephalin (10.93 ± 0.10 %; n = 10). *c)* however, when the CNS was perfused with HBS-containing 10^{-5} M met-enkephalin and 1% isoflurane, a significant reduction in their coupling coefficient was demonstrated (6.37 ± 0.07%) which was reduced to less than its original control value. *c)* this reduction was even greater (4.10 ± 0.05%) when the CNS was perfused with HBS-containing 10^{-5} M met-enkephalin and 2% isoflurane. *d)* however, the reduction caused by halothane was abolished when the CNS was perfused again with 10^{-5} M met-enkephalin

coefficient (6.37 ± 0.45%) (n = 10, Figs 7, 8) and a greater reduction was produced in the coupling coefficient when the CNS was perfused with HBS-containing 10^{-5} M met-enkephalin and 2% isoflurane (4.10 ± 0.31%) (n = 10). In all cases the effect of isoflurane was completely reversible (Figs 7, 8).

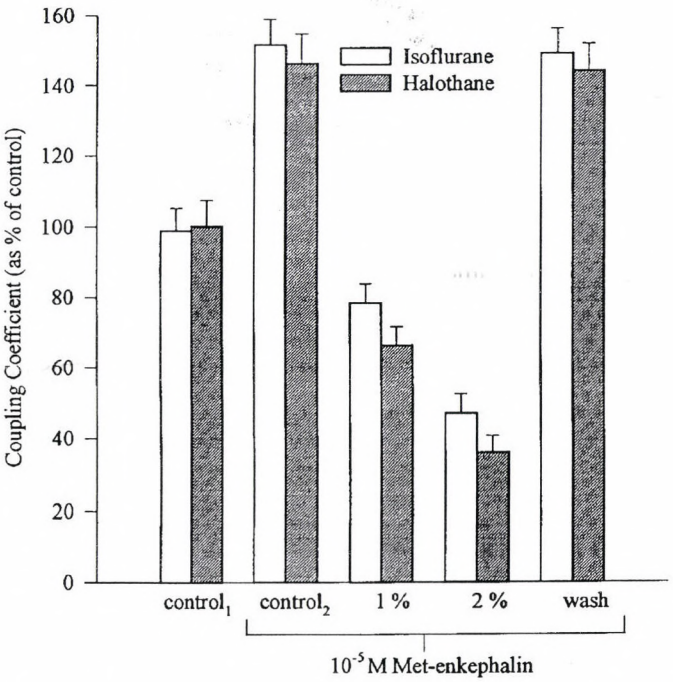

Fig. 8. Effect of volatile anaesthetics on the coupling coefficient between Pedal A cluster neurones in the presence of met-enkephalin. The coupling coefficient between small PeA of *Lymnaea* was significantly increased by the application of 10^{-5} M met-enkephalin. However, its value was reduced significantly to a value below its original one when the CNS was perfused with 1% and 2% halothane or isoflurane (n = 10) in a dose dependent manner. 1% and 2% halothane induced an even greater reduction in the coupling coefficient in comparison with isoflurane (n = 10). Bars represent mean ±SE

DISCUSSION

It is clear from the data presented here that the two volatile anaesthetics halothane and isoflurane at clinical concentrations (Table 1) reduce the firing frequency of all the PeA-cluster neurones tested and the time to both maximum effectiveness and recovery is related to the concentration of anaesthetic applied (Table 2). Furthermore, halothane is more potent than isoflurane in line with their known clinical effects.

The PeA cells are weakly electrically coupled [11] and both the volatile anaesthetics reduce their coupling coefficients still further. Again, these effects are dose dependent and halothane is more potent than isoflurane. The effect on coupling coefficient appears to be related to the fact that the anaesthetics also reduce the input resistance of many PeA neurones [18, 19], while having little effect on others [18, 23]. A reduction in the input resistance will allow current to leak preferentially across the cell membranes to earth rather than passing across the coupling resistance between them. These effects may be caused by increasing the leakiness of the membrane, perhaps by altering the processes that generate the leakage currents. Another factor that may be involved in decoupling the neurones is that the volatile anaesthetics raise intracellular calcium concentration which is known to de-couple electrically connected cells [12, 16, 28]. Simultaneous electrophysiological recordings and measurements of intracellular calcium concentration need to be carried out to determine the importance of changing calcium concentration in these cells.

We have previously documented the effects of met-enkephalin on electrically coupled molluscan neurones [11, 22]. Met-enkephalin probably enhances electrical coupling by increasing input resistance in PeA neurones, but this effect is totally abolished by both halothane and isoflurane. Comparing the histograms in Figures 5 and 8 show that the anaesthetics reduce the coupling coefficient of the neurones to levels expected for the anaesthetics alone. The mode of action of met-enkephalin is yet to be determined.

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RAINBOW TROUT *(ONCORHYNCHUS MYKISS)* EXPOSED TO OXYGEN SUPERSATURATION AND HANDLING STRESS: PLASMA CORTISOL AND HEPATIC GLUTATHIONE STATUS*

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Three groups of one summer old rainbow trout were exposed for 22 days either to normoxia (100%) or moderate oxygen supersaturation; 120% and 140%. After the exposure, all groups were transported for three hours in hyperoxic conditions (123% O_2) thus simultaneously experiencing density and handling stress. The recovery of rainbow trout to multiple stressors was measured in normoxic conditions. Moderate oxygen supersaturation did not have any negative effects on growth, feed conversion and blood hematology measured over 22 days. On the other hand, the combined effects of the stressful environment in the fish farm and oxygen supersaturation resulted in a 3-fold increase in plasma cortisol levels in those with 100% and 120% O_2 supersaturation and a 2-fold increase in the 140% supersaturation group. Furthermore, the stress response after transportation was lowest in the 140% group 24 hours after recovery but highest after 70 hours. Moderate hyperoxia or transportation stress did not change glutathione concentrations in liver indicating that routine sampling does not affect hepatic glutathione status. Our results indicate that moderate O_2 supersaturation (<140%) could be considered as feasible in cultivation of rainbow trout since no harmful effects were found.

Keywords: Hyperoxia – stress – glutathione – cortisol – rainbow trout

INTRODUCTION

Oxygen supersaturated water is routinely used in fish transportation to provide adequate oxygen concentrations for the fish. Recently several fish farms have also invested in oxygen supplementation systems in their production facilities in order to maximise production capacity. The use of oxygen supersaturated water is a potential alternative for land based fishfarms to ensure adequate rearing conditions especially during the summertime when the water volume is restricted and the dissolved oxygen concentrations are low. Higher oxygen concentrations in tanks make it possible to reduce water consumption and to increase water recirculation. Additionally, it

*Dedicated to Professor János Salánki for his 70th birthday.

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allows more fish per rearing unit and covers possible oxygen depletion after feeding [4]. If the water current in tanks cannot be increased, the easiest way to guarantee that the oxygen supply will be sufficient throughout the day is to maintain high, constant oxygen supersaturation in the rearing tanks. However, this additional oxygen is an extra production cost and there is no evidence that high concentrations of oxygen enhance fish growth or feed conversion [8]. On the contrary, hyperoxic conditions may act as a physiological stressor for fish and thus lead to detrimental tissue damage in their gills [13].

Under normal resting conditions, mammalian mitochondria utilise 95% of oxygen for producing energy and water. According to Singal and Kirschenbaum [22] 3–5% of this oxygen could be consumed in alternative oxygen reduction pathways and form reactive oxygen species (ROS). Those reactive intermediates are capable of causing oxidative tissue damage (e.g. protein oxidation, lipid peroxidation and DNA fragmentation). The tripeptide glutathione plays a central role in cellular protection by scavenging, decomposing toxic oxygen metabolites and maintaining a favourable redox environment for other antioxidants within the cell [14]. The state when the endogenous antioxidant defence system is overwhelmed by prooxidant forces (e.g. ROS) is called oxidative stress. In the aquatic environment, oxidative stress was thought to be due to environmental pollutants [7], but recent studies have shown that changes in antioxidant status occur during embryonic development are probably also linked to altered respiration rates [17]. Therefore the consequences of high oxygen concentrations in fish should perhaps be seen as changes in the antioxidant status of fish adapting to varying environmental conditions.

In this study, classical hematological stress indicators and plasma cortisol measurements along with hepatic antioxidant potential have been used as indicators of moderate oxygen supersaturation and acute handling stress in rainbow trout. The experiments were conducted in a commercial fish farm to clarify the combined effects of oxygen supplementation (<140%) and transportation stress in aquaculture.

MATERIALS AND METHODS

The combined effects of constant supersaturated oxygen and transportation on physiological and haematological responses in rainbow trout were examined in two consecutive studies. In the first experiment, the effects of two constant hyperoxic environments, 120% and 140%, were compared to normoxic conditions (100%) during a 22 day growth experiment. In the second experiment, the fish from the previous experiment were exposed to 123% oxygen supersaturation, netting and density stress caused by a three hour transportation period in oxygen supersaturated water. After the transportation, the fish were transferred to normoxic conditions and physical parameters were measured during the recovery period.

Growth experiment

Both experiments were performed in October in the commercial fish farm, Nilakkalohi Ltd., located in central Finland. One summer old rainbow trout (*Nilakkalohi*-strain) were acclimated for two weeks in the same tanks under normoxic conditions where the study would be conducted. After the adaptation period the fish were either held at normoxic conditions (100%) or exposed to 120 or 140% oxygen supersaturation for 22 days. Initially, fish averaged 37.1 g (± SE 1.2 g) in weight before the experiment. Each test group consisted of two 1000 L tanks with central drainage. The tanks were placed on the floor without any cover on the top, so that fish were exposed to environmental stress associated with the daily routines in fishfarm. The average fish biomass in tanks increased from 11.6 kg · m^{-3} (± SD 0.1 kg · m^{-3}) to 15.9 kg · m^{-3} (± SD 0.3 kg · m^{-3}) during the test period. In the growth experiment, fish were fed for the first 14 days at 1.5% body weight per day and for 8 days at 1.3% with a diet of salmon dry food (Ecoline 4.0 mm, BioMar AS, Norway) which was dispensed for 12 hours daily by belt feeders. A constant 12L : 12D light rhythm was used during the 22-day growth experiment.

The average oxygen concentrations measured from tank outlets were 9.0 mg · L^{-1} (± SD 0.05) in normoxic groups, 12.6 mg · L^{-1} (± SD 0.02) in 120 % groups and 15.2 mg · L^{-1} (± SD 0.03) in 140% groups. Oxygen supersaturated water was generated in a sealed, packed column where pure oxygen and water were mixed. The desired oxygen concentrations were established for each tank by mixing supersaturated water with normoxic water. The total gas pressure did not exceed 102 % and partial pressure of nitrogen was kept under 100%. Total gas pressure and pN^2 were measured with a Weiss saturometer (Eco Enterprises, Seattle, USA). Water temperature decreased in each group during the growth experiment from 10.5 to 7.2 °C. Measurements of water temperature and dissolved oxygen concentrations were performed every second day with a portable oxygen meter (Oxy Guard, Denmark).

Due to the large number of fish (340 individuals per tank), whole fish biomass in tanks was estimated as wet weight biomass. This biomass was converted to average weight estimates of individual fish since the number of fish in each tank was known. The fish were netted and placed into the pretared water volume using one netting per measurement. The gain in biomass was used to calculate the feed conversion coefficient (FCC, equation 1). The number of individuals was counted manually after the experiment and the calculated, average individual weight was used as basis for the specific growth rate (SGR (% ww·d^{-1}, equation 2).

$$FCC = \text{feed offered (kg)/weight gain (kg)} \qquad \text{Equation 1}$$

$$SGR = [(\ln(ww_2) - \ln(ww_1))/dt) \times 100], \qquad \text{Equation 2}$$

where

ww_1 = wet weight at the beginning (g)
ww_2 = wet weight at the end (g)
dt = time of the experiment (days)

Transportation experiment

In the transportation experiment, test groups from the previous growth experiment were collected by netting from the growing tanks and placed into one 1000 L transportation tank. The transportation tank was divided into 6 equal sections to separate the different groups. Oxygen supersaturation during the three hours transportation was kept at 123% (± SD 2.3%) by aeration and injection of pure oxygen. During the transportation, the fish density was 100 kg · m^{-3} in each section and temperature was maintained at 8.0 °C. After the transportation, the groups were kept apart and placed into normoxic conditions to observe the physiological changes evoked by the stress. During the recovery period the dissolved oxygen concentration remained constant (11.2 mg · L^{-1}), total gas pressure stayed under 101% and partial pressure of nitrogen was under 102 %. Water temperature was 7.2 °C (± SD 1.1 °C) during 70 hours recovery period. The samples were collected before the transportation and during the recovery period at 0.5, 2, 24 and 70 hours after the transportation.

Sampling and sample preparation

At the time of sampling, 8 fish per tank (16 fish per oxygen treatment) were stunned by a blow to the head and their wet weight and length were measured. Blood samples were collected with Na-heparinzed syringes from the caudal vein. A drop of blood was taken for micronucleus testing, spread on an acid-washed slide and dried in air before fixation. Hematocrit (Hct; 3-min centrifugation) was measured immediately using a microhematocrit centrifuge (Compur M 1100). For hemoglobin (Hb) measurements, 150 µl of whole blood was kept on ice until analysed (< 4 hours). The remaining blood was centrifuged (1000 *g* for 5 min at 4 °C) to obtain plasma. Plasma samples were frozen in liquid nitrogen and stored to –80 °C for cortisol analysis. In the assessment of hepatic glutathione status liver was removed, weighed and frozen in liquid nitrogen.

The oxidised (GSSG) glutathione and total (tGSH) glutathione levels were determined by homogenizing frozen liver samples in ice-cold 10% 5-sulfosalicylic acid and 5% 5-sulfosalicylic acid with a Potter–Elvehjem-type homogenizer, respectively in a ratio of 1 : 4 (weight : volume). The homogenates were centrifuged at 10,000 *g* for 5 minutes at 2 °C (Sorvall Instruments RC5C) and supernatants were stored at –80 °C until analysed.

Assays

Plasma cortisol was validated for rainbow trout [19] and measured using a RIA kit (Cortisol [^{125}I], Orion Diagnostica, Finland). Blood hemoglobin was converted to cyanomethemoglobin and measured spectrophotometrically using a commercial kit (SPR Veripalvelu, Finland). The mean cellular Hb concentration (MCHC) was cal-

culated from the hemoglobin/hematocrit value ratio. Erythrocyte micronucleus samples were prepared using the method described by Carrasco et al. [3]. The micronucleated erythrocytes (MN) were counted in each smear sample (6 fish per group) with the same person examining two subsets of 1000 cells per fish at 1000 X magnification. The number of micronucleated red cells was calculated as a percentage of 1000 erythrocytes.

Total glutathione was determined as described by Sen et al. [21] in which the 5,5'-dithiobis (2-nitrobenzoic acid) reduction rate is correlated to the sum of oxidised and reduced glutathione. Oxidised glutathione was measured as described by Griffith [10] using a double beam spectrophotometer (Perkin Elmer Lambda 2 UV). Both glutathione analyses were carried out at 23 °C. The amount of reduced glutathione (GSH) was calculated from subtraction of GSSG from tGSH in GSH equivalents.

Statistics

The assumption of equal variances was tested with the Shapiro–Wilk-test, while the assumption of normal distribution of the variables was tested with the Kolmogorov–Smirnov-test. When both assumptions were valid ($p > 0.05$) one-way analysis of variance or Students *t*-test were used. The differences between groups were observed by contrasts (ANOVA). When heterogeneity of variables ($p < 0.05$) was present the non-parametric Kruskall–Wallis one-way analysis and Mann–Whitney U-test with Bonferroni's correction were applied. Comparisons between regression coefficients were done with χ^2-test using Bonferroni's correction. All statistical analyses were carried out with SPSS 7.5-program (SPSS, Chicago, USA).

RESULTS

Growth experiment

Body weight ($p < 0.01$), body length ($p < 0.001$) and condition factor ($p < 0.001$) were significantly increased (*t*-test) during the growth period. However, oxygen concentration did not affect the growth (Table 1). Measurements of the whole biomass showed that specific growth rates were at equal level 1.24% · d^{-1} (± SD 0.01% · d^{-1}) in each group and food conversion coefficients were equivalent in all groups 0.84 (± SD 0.07). No mortalities were observed in any group during the experiment.

Plasma cortisol levels were equal in each group at the beginning of the experiment. At the end of the growing period plasma cortisol concentrations were elevated significantly ($p < 0.001$, *t*-test) in the 100% and 120% groups in comparison to respective control values (Fig. 1). The highest values were recorded in the 120% group and lowest values in the 140% group. Nonetheless, no significant differences

Table 1
Physical parameters of one summer old rainbow trout grown in constant oxygen supersaturation

	Body weight (g)	Length (cm)	Liver weight (g)	CF	LSI
At the beginning 100%	37.1 ± 1.2	13.5 ± 0.2	0.5 ± 0.03	1.51 ± 0.02	1.45 ± 0.1
At the end 100%	45.5 ± 2.9[1]	14.8 ± 0.3[2]	0.7 ± 0.007	1.37 ± 0.02[2]	1.34 ± 0.08
120%	43.5 ± 2.2[1]	14.7 ± 0.2[2]	0.6 ± 0.04	1.36 ± 0.03[2]	1.35 ± 0.06
140%	44.9 ± 2.4[1]	14.8 ± 0.3[2]	0.6 ± 0.04	1.36 ± 0.02[2]	1.35 ± 0.04

Data represent mean activity ±SE; n = 16–24. CF: condition factor (weight/body length^3)*100.
LSI: liver somatic index (liver weight/body weight)*100.
[1,2]: significantly different from the control group ($p < 0.01$; $p < 0.001$, respectively).

were observed between test groups ($p > 0.070$, Contrast) at the end of the experiment. Cortisol values did not alter in 140% group during the growth period.

Hematocrit values were significantly decreased in all groups ($p < 0.001$, *t*-test) during the growth experiment as presented in Table 2, but oxygen supersaturation did not induce any significant differences between groups. Similarly, hemoglobin con-

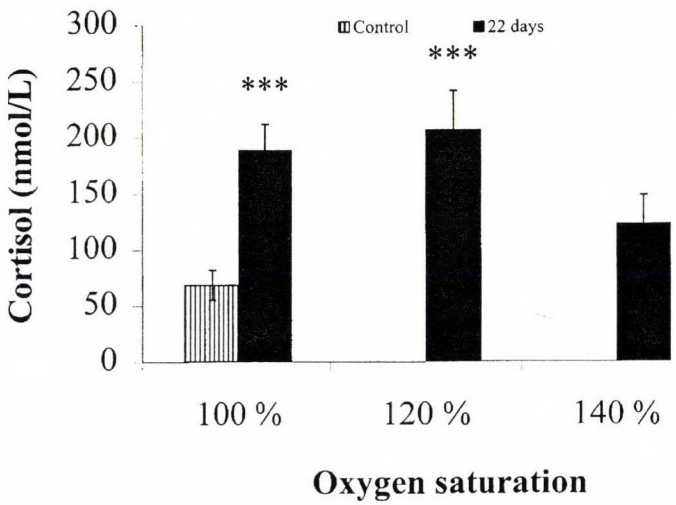

Fig. 1. Plasma cortisol concentrations (nmol/L) in one summer old rainbow trout grown in three different oxygen concentrations. *** indicates significant difference between initial and 22-day samples at level $p < 0.001$. Vertical bars indicate S.E.

Table 2
Hematological parameters and hepatic glutathione concentrations of one summer old rainbow trout after 22 days growth in oxygen supersaturated water

	Control	Oxygen supersaturation		
		100%	120%	140%
Glutathione measurements				
GSSG (nmol/g)	121.2 ± 14.9	107.0 ± 24.1	116.6 ± 21.4	122.8 ± 25.8
GSH (μmol/g)	1.30 ± 0.09	1.25 ± 0.06	1.19 ± 0.07	1.25 ± 0.05
tGSH (μmol/g)	1.54 ± 0.08	1.47 ± 0.03	1.42 ± 0.07	1.45 ± 0.06
Hematological measurements				
Hemoglobin (g/dL)	7.58 ± 0.24	7.84 ± 0.39	7.53 ± 0.27	7.42 ± 0.25
Hematocrit (%)	35.4 ± 1.1	28.1 ± 1.6[2]	28.9 ± 0.8[2]	28.3 ± 0.9[2]
MCHC (g/L)	205.3 ± 6.2	290.8 ± 19.1[1]	261.3 ± 8.6[1]	264.5 ± 9.1[1]

Data represent mean ±SE; n = 3–8; [1,2] significantly different from the control value at level $p < 0.01$; $p < 0.001$, respectively

centrations did not differ between the various treatment groups at the end of the growth experiment. The mean cellular Hb concentration (MCHC) was significantly higher ($p < 0.004$, *t*-test) in the 100% group in comparison to the control value, but no further changes were observed between different test groups at the end of the experiment. Oxygen supersaturation did not have any effect on the levels of micronucleated erythrocytes. The number of micronuclei varied between 0.0 to 0.2% (data not shown).

Both total glutathione and GSH did not change during the growth experiment in liver samples. Hepatic oxidised glutathione (GSSG) was 12% lower in the 100% group during the 22 days. On the other hand, GSSG concentrations in the 120% and 140% groups were elevated by 9% and 15% in comparison to corresponding control group (Table 2), respectively. However, the increase in both groups was not statistically significant due to extensive individual variation.

Transportation experiment

During the recovery period following the transportation plasma cortisol levels showed a similar decreasing tendency in each group for the first 24 hours (Fig. 2). According the regression analysis the difference between the 100% and the 120% group was biggest, but not significant ($p < 0.1254$, χ^2-test). The regression equation for the 120% group was: cortisol (nmol/L) = $84.61 \times e^{-\text{Time(h)} \times 0.077}$ ($p < 0.0001$, $R^2 = 0.79$, $n = 16$). Plasma cortisol values were lowest in the 140% group after the

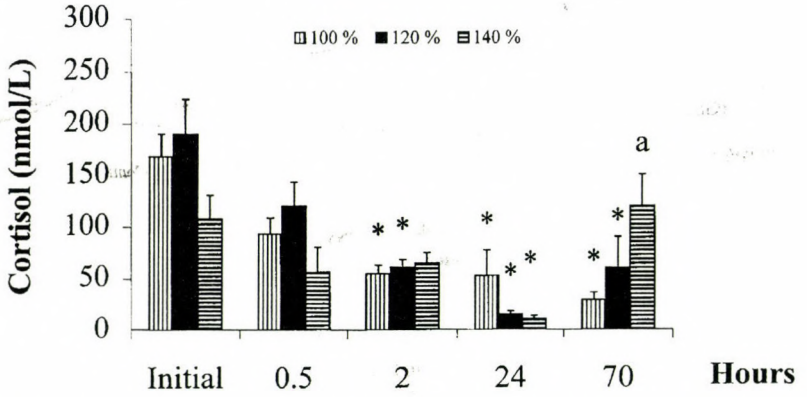

Fig. 2. Plasma cortisol concentrations (nmol/L) of one summer old rainbow trout during the recovery period in a normoxic environment. Fish were acclimated for 22 days in three different oxygen concentrations and then exposed to three hours transportation at 123% oxygen saturation level."a" indicates significant difference between 100% and 140% groups at the same time of recovery ($p < 0.05$). Further, "*" indicates the difference between inital values ($p < 0.05$). Vertical lines are S.E.

Table 3

Hepatic total glutathione (tGSH), reduced glutathione (GSH) and oxidised glutathione (GSSG) concentrations of one summer old rainbow trout after transportation in 123% oxygen supersaturation and recovery in normoxic water

	Initial	Time after transportation (h)			
		0.5	2	24	70
Total-GSH (μmol/g wet tissue)					
100%	1.47 ± 0.03	1.49 ± 0.08	1.69 ± 0.09	1.59 ± 0.16	1.61 ± 0.09
120%	1.42 ± 0.07	1.63 ± 0.06	ND ± ND	1.56 ± 0.15	1.34 ± 0.06
140%	1.45 ± 0.06	1.39 ± 0.07	1.43 ± 0.03	1.61 ± 0.09	1.39 ± 0.19
GSH (μmol/g wet tissue)					
100%	1.25 ± 0.06	1.17 ± 0.13	1.27 ± 0.24	1.42 ± 0.14	1.26 ± 0.12
120%	1.19 ± 0.06	1.31 ± 0.10	ND ± ND	1.35 ± 0.18	1.06 ± 0.19
140%	1.25 ± 0.05	1.16 ± 0.06	1.26 ± 0.12	1.26 ± 0.12	1.18 ± 0.20
GSSG (nmol/g wet tissue)					
100%	107.0 ± 24.1	158.9 ± 44.0	211.1 ± 17.1	84.3 ± 15.1	177.0 ± 59.0
120%	116.6 ± 21.4	157.4 ± 79.3	ND ± ND	105.2 ± 28.5	164.3 ± 101.2
140%	119.0 ± 27.7	117.1 ± 52.9	85.7 ± 41.5	139.9 ± 38.5	109.2 ± 37.8

Data represent mean activity/concentration ±SE; n = 3–8; ND: not determined.

transportation for up to the 24-hour point. Then, the difference between 100% and 140 % groups was highest, but not still significant ($p > 0.069$, Mann–Whitney U-test). At 70 hours of recovery, the plasma cortisol level was increased in the 120% and 140% groups but it was depressed in the 100% group. The difference between the 100% and 140% group was significant ($p < 0.030$, Mann–Whitney U-test) at the end of the recovery period.

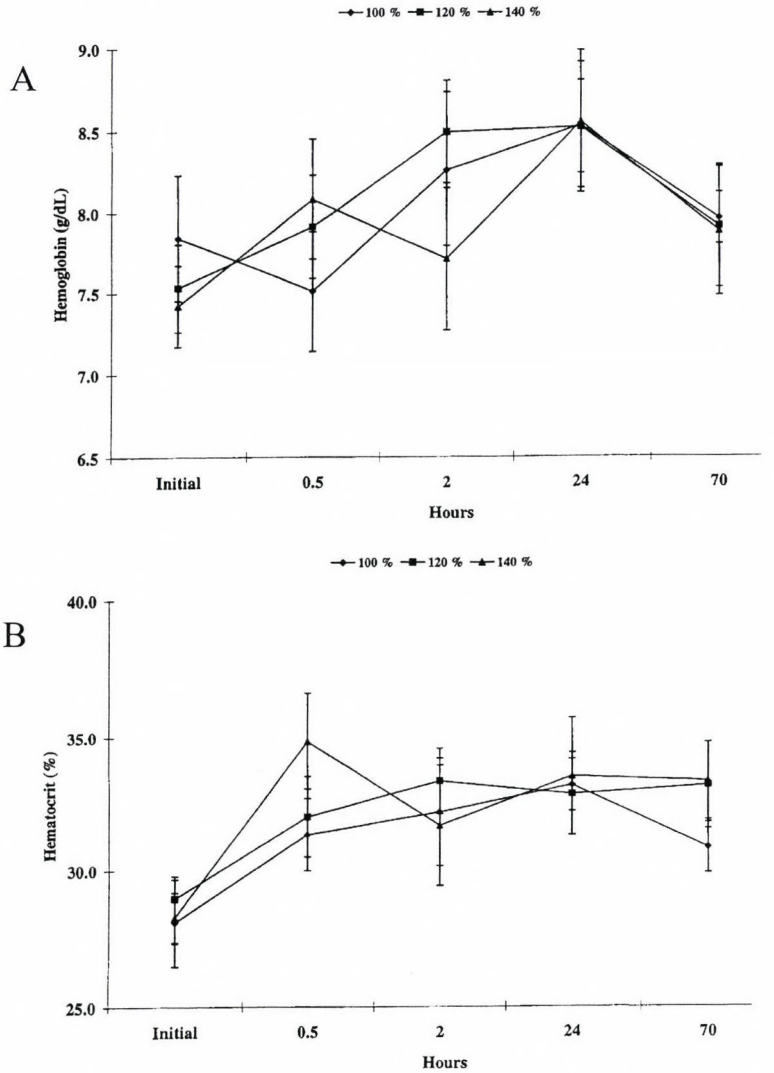

Fig. 3. Hemoglobin concentrations (g/dL) (3A) and hematocrit (%) (3B) of one summer rainbow trout during the recovery period in a normoxic environment. Fish were acclimated for 22 days in three different oxygen concentrations and then exposed to three hours transportation. Vertical lines are S.E.

Blood hemoglobin increased for 24 hours in the 120 and 140% groups during the recovery period in normoxic water (Fig. 3A). In both groups, the peak value was 8.5 g/dL, which returned to the initial levels after 70 hours of recovery. The hemoglobin concentration in the 100% group tended to decrease during the first 30 min. After this point, however, the 100% group could not be distinguished from the other groups.

Hematocrit values were slightly elevated in each group at 24 hours after transportation (Fig. 3B). Highest values were detected in the 140% and lowest in the 100% group. The hematocrit values showed a tendency to be inversely correlated with oxygen supersaturation although the differences between groups were not statistically significant ($p > 0.060$, contrast). Transportation and acute handling stress did not affect the levels of MCHC or micronucleated erythrocytes (data not shown).

As seen in Table 3, the short-term transportation stress in oxygen supersaturated water did not alter ($p > 0.41$, GLM) hepatic glutathione (GSH, GSSG or total glutathione) levels. The concentrations of GSH and tGSH were at the same level during the recovery period in each group, whereas hepatic GSSG level tended to increase in comparison to inital values.

DISCUSSION

Our results with one summer old rainbow trout indicate that moderate oxygen supersaturation did not enhance either fish growth or feed conversion. This was not due to stress since the fish raised in hyperoxic conditions had virtually normal plasma cortisol levels and hematological measurements. Oxygen supplementation can be used safely at moderate supersaturation levels (<140%) to increase the carrying capacity of on-growing tanks as there were no negative physiological effects in measured parameters. Our results confirm previous findings that moderate oxygen supersaturation does not provide any advantage to fishfarms with respect to growth [2] or feed conversion [9].

In the present study oxygen supersaturation did not act as a cumulative stressor. Instead, oxygen concentration showed slightly favourable effects at 140% saturation level in that it reduced plasma cortisol levels. Similarly, cortisol levels were lowest in 140% group at the beginning of the recovery period implying lower stress response and thus better adaptation to the transportation stress. Furthermore, the recovery in normoxic water was uniform in all three oxygen groups up to 24 hours and the plasma cortisol levels decreased to the initial level. However, the last sampling, 70 hours after the stress resulted in significantly higher cortisol levels in the 140% group, which might be a late sign of incomplete recovery or better ability to react to a new stressor. In this study, the samplings were conducted every time in the same way and fish were collected randomly to avoid variation caused by the sampling process itself. Apparently, the plasma cortisol concentration of rainbow trout has a predictable diurnal rhythm, which is influenced by feeding ratio and time [18]. The normal diurnal variation in cortisol level in rainbow trout is three-fold with a

measured maximum concentration of 100 nmol/L [18] at the feeding level used in our study. Routine maintenance work (cleaning tanks, feeding, etc.) caused major increase in plasma cortisol levels in comparison to the control group. Our results indicate that uncovered tanks should not be placed on the floor if the surrounding environment is stressful. In previous studies, the recovery after confinement or handling stress in oxygen supersaturated water have not been shown to improve the general stress response in rainbow trout [2] or even to reduce the rainbow trout's ability to tolerate added stress [1].

Oxygen supersaturation causes hypoventilation in fish, since ventilation is primarily controlled by O_2 drive [5]. Physiologically this leads to respiratory acidosis, which is compensated by acid excretion via the gills. The decrease in pH facilitates oxygen delivery from erythrocytes to the tissues. Low extracellular pH is neutralised by the build-up of HCO_3^- in the plasma, the corrections being completed by 72 hours [23]. However, when the fish are returned back to normoxic conditions this causes metabolic alkalosis which is compensated in almost 24 hours. In our growth experiment, oxygen supersaturation did not cause any significant changes in Hct or in Hb, but Hb concentrations tended to decrease as the oxygen concentrations increased. Similarly, a sign of erythrocyte hypertrophy (lowered MCHC) was seen in hyperoxic groups. Both these changes could be interpreted as consequences of hyperoxic conditions. Nonetheless, all the measured values did not differ from those in the control group and no significant differences were detected during the growth experiment. Previously it has been shown that long-term hyperoxic exposure (180%) have reduced both Hct and Hb values in rainbow trout [9]. A similar trend has been found at 130% saturation level [2], but the difference was not significant. Three hours of hyperoxia in rainbow trout decreased plasma pH, increased blood oxygen tension but maintained arterial oxygen saturation implying that the oxygen affinity of hemoglobin in the erythrocytes had decreased in hyperoxia [15].

During the recovery period after transportation the Hct was elevated and there was a concomitant increase in Hb which is interpreted as being due to an increase in the number of erythrocytes [20]. With the exeption of the finding that transportation in hyperoxic conditions caused slight decrease in Hb values after 30 min in 100% group, all groups had similar types of adaptation responses to the stress. In another study a six hours confinement challenge of rainbow trout for 130% oxygen saturation caused similar stress responses in Hct and Hb values in hyperoxic group than normoxic group [2]. These authors concluded that normoxic fish recruited red blood cells to refill oxygen demand whereas hyperoxic fish utilised cellular swelling. This hypothesis was not measured directly in the present study.

Micronuclei test turned out to be laborious and an insensitive method in the evaluation of damage caused by hyperoxia. This test in fish blood erythrocytes was also an insensitive indicator of chemical pollution [3, 12].

No changes in hepatic tGSH or GSH levels were detected in the present study. GSSG concentrations were at the same level as those reported by Otto and Moon [16] and revealed no evidence of oxidative stress. In general, the glutathione concentrations did not change in any of the groups either during the growth experiment or after

transportation. Our finding implies that neither oxygen supersaturation nor acute handling stress have any effect on hepatic glutathione status. Apparently, rainbow trout can effectively control the amount of oxygen in circulation and tissues (e.g. by changing respiratory rate, heart volume and blood pH) at moderate hyperoxia (<140%) and thus they do not need to recruit their glutathione dependent protection system against oxyradicals. In mammalian studies, liver is regarded as a storage site of glutathione [6] and it reflects the overall glutathione status of the animal. Similarly in the aquatic environment, a high hepatic GSH concentration have been found to reflect the ability of the liver to protect directly against free radicals [11, 16]. Hepatic GSH concentrations can be used as a biomarker in environmental studies as well as in aquaculture.

We conclude that long-term moderate oxygen supersaturation (<140%) did not enhance specific growth rate or food conversion in one summer old rainbow trout. Plasma cortisol measurements indicate that rainbow trout need an unstressful growing environment. The fish recovering after transportation in the 140% group expressed signs of insufficient recovery time or quicker response to a new stressor. This phenomenon was not detected in other groups. No signs of oxidative stress were observed in hepatic glutathione status during the growth period or in the transportation experiment. The latter finding also implies that acute sampling stress does not have any effect on hepatic glutathione concentrations. Our results indicate that rainbow trout possess the capacity to handle stressful situations and thus cope with moderate oxygen supersaturation at temperatures near 10 °C.

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NITRIC OXIDE SELECTIVELY ENHANCES cAMP LEVELS AND ELECTRICAL COUPLING BETWEEN IDENTIFIED RPaD2/VD1 NEURONS IN THE CNS OF *LYMNAEA STAGNALIS* (L.)*

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The isolated CNS of the freshwater mollusc *Lymnaea stagnalis* was used as a model to study the role of cAMP in NO-mediated mechanisms. The NO donor, DEA/NO (10^{-5} – 10^{-3} M) increased cAMP concentrations in the cerebral, pedal, pleural, parietal and visceral ganglia. In contrast, in the buccal ganglia the same doses of DEA/NO decreased the level of cAMP production. The NOS inhibitor, L-NNA (10^{-4} M) increased cAMP concentrations in all areas of the CNS. L-arginine (1 mM), a metabolic precursor of NO, mimicked the action of the NO-donor. The coefficient of electrical coupling between two viscero-parietal peptidergic neurons (VD1/RPaD2) was enhanced by both DEA/NO (10^{-4} M) and 8-Br-cAMP (10^{-4} M) whereas 8-Br-cGMP (2×10^{-4} M) reduced the coupling. We suggest that cAMP-dependent mechanisms are involved in neuronal NO signaling in this simpler nervous system.

Keywords: Nitric oxide – cAMP – electrical synapses – *Lymnaea* – *Aplysia* – molluscs

INTRODUCTION

The gaseous radical nitric oxide (NO) is a versatile neuromodulatory molecule widely distributed across the animal kingdom [2, 6, 12, 16]. In the CNS of the freshwater pulmonate snail, *Lymnaea stagnalis* the presence of NO synthase (NOS) activity was demonstrated in central nervous and peripheral tissues [3, 8, 11]. Putative nitrergic neurons are mainly located in the buccal ganglia [9]; there are a relatively small number of these cells in the central ganglionic ring (i.e. in the cerebral, pedal, pleural, parietal and visceral ganglia). In *Lymnaea,* NO activates buccal motor patterns [11] and is considered a mediator of chemosensory inputs to the feeding network [4]. Although in this and other grazer gastropod molluscs *(Helix* and *Aplysia),* NO acts via cGMP-dependent pathways [4, 5, 7], cAMP-dependent mechanisms can be also involved. For example, in the predatory sea-slug *Pleurobranchaea,* NO enhanced the cAMP gated sodium currents [14, 15]. This response might be mediated by the rise of intracellular cAMP.

*Dedicated to Professor János Salánki for his 70th birthday.

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Here, we compare the action of NO-donors on endogenous levels of cAMP in two parts of the *Lymnaea*'s CNS: the buccal ganglia and the central neuronal ring. Additionally, we use a pair of multifunctional peptidergic neurons (VD1/RPaD2) to investigate the effects of NO-donors and a cAMP analog on the electrical coupling between these cells. We have shown that NO indeed increases cAMP production in selected central ganglia and, therefore, cAMP-dependent mechanisms can be involved in NO signaling in the molluscan nervous system.

MATERIAL AND METHODS

Specimens of *Lymnaea stagnalis* (2–4 g) were collected locally, kept for up to 4 weeks in tapwater at 14–16 °C and feed on the lettuce. Freshly isolated CNSs were used in both biochemical and electrophysiological tests.

NO donors [diethylamine/nitric oxide sodium complex (DEA/NO), S-Nitroso-N-acetylpenicillamine (SNAP), L-arginine], and a NOS inhibitor, N^G-Nitro-L-arginine (L-NNA) – all from RBI, were prepared immediately before use in HEPES buffered saline for *Lymnaea* (in mM: NaCl – 44, KCl – 2, $MgCl_2$ – 2, $CaCl_2$ – 4, Hepes – 10, pH = 7.8)

Endogenous cAMP levels were determined by the standard radioimmune assay technique [13]. In each experiment ten pairs of the buccal ganglia or three central ganglionic rings were incubated for 20 minutes either in 0.2 ml of control solution or in the presence of the drug tested.

Intracellular recording was performed by a conventional microelectrode technique. Glass microelectrodes (10–40 MOm) were filled with 2.5 M KCl. Central neurons were identified according to their location, size, color and electrophysiological characteristics (see the map in [1]). The coupling coefficient (CC) was measured as $CC = \Delta V_{RPaD2}/\Delta V_{VD1}$, where ΔV_{RPaD2} and ΔV_{VD1} are changes in the membrane potential of RPaD2 and VD1, respectively, and the hyperpolarizing current (0.5 nA) was injected in VD1. During the experiments the neurons were slightly hyperpolarized to prevent spontaneous spiking activity. All drugs tested were added into the experimental chamber via a perfusion system, and all concentrations indicated in the text are the final concentrations.

RESULTS

Effect of NO on cAMP production

The summary data are presented in Table 1 and Fig. 1. Incubation of the central ganglia in the presence of the NO donor, DEA/NO induced a significant increase of the endogenous cAMP in the central ganglionic ring. A precursor of NO synthesis, L-arginine, mimicked the action of the DEA/NO. It seems that even small concentrations of DEA/NO (10^{-5} M) resulted in a nearly maximal effect and higher concen-

trations of the NO donor (10^{-3} M) did not produce any further rise in the cAMP levels (Fig. 1A). In contrast, in the buccal ganglia the same small doses of DEA/NO (10^{-5} M) induced minor changes, but higher doses of the donor (10^{-3} M) markedly reduced the cAMP production (Fig. 1B).

NOS inhibitor, L-NNA (10^{-4} M) increased cAMP levels in all parts of the CNS (Table 1).

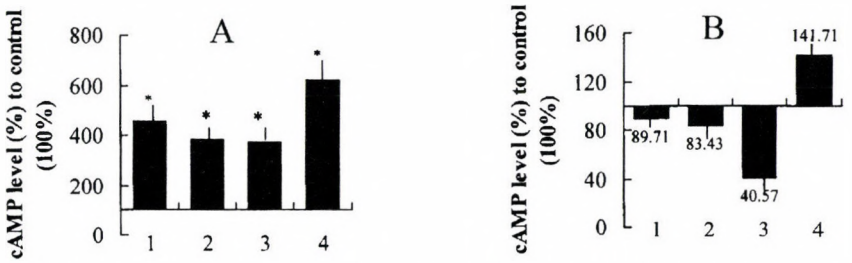

Fig. 1. The effects of NO donors and NOS inhibitors on cAMP concentrations in the different parts of the *Lymnaea*'s CNS. A) Central ganglionic ring (n = 4); B) Buccal ganglia (n = 4). *P < 0.05. 1 – L-arginine (1 mM); 2 – DEA/NO (10^{-5} M); 3 – DEA/NO (10^{-3} M); 4 – L-NNA (10^{-4} M)

Table 1
The effects of NO donors and inhibitors of NO synthase on cAMP levels in the different parts of the CNS of *Lymnaea stagnalis*

	cAMP concentration in the central ganglionic ring (fmol/ml)	cAMP concentration in the buccal ganglia (fmol/ml)
Control (0.2 ml HBS)	64.0 ± 14.46	17.5 ± 2.50
L-arginine (10^{-3} M)	292.0 ± 78.48*	15.7 ± 1.70*
DEA/NO (10^{-5} M)	246.7 ± 91.97*	14.6 ± 0.83*
DEA/NO (10^{-3} M)	237.7 ± 56.51*	7.1 ± 1.35*
L-NNA (10^{-4} M)	395.7 ± 94.09*	24.8 ± 1.42*

Data present mean values SEM. *P < 0.05 vs control (Student's *t*-test)

Effect of NO and cyclic nucleotide analogs on the identified electrical synapse

Both DEA/NO (Fig. 2) and SNAP at 10^{-4} M increased the electrical coupling coefficient between VD1 and RPaD2 by about 20%. L-arginine (10 mM) induced no detectable changes in the coupling coefficient (n = 3), whereas 10^{-4} M of 8-Br-cAMP enhanced the electrical coupling between these two neurons by 31.6% (n = 4). These concentrations of the cAMP analog also caused a steady-state hyperpolarization of

VD1 (13.2 ± 2.6 mV, n = 4) and RPaD2 (8.3 ± 1.8 mV, n = 4). Interestingly, the cGMP analog, 8-Br-cGMP, at a similar concentration range (2×10^{-4} M) reduced the coefficient of electrical coupling by 31.1% (n = 5), and strongly depolarized both VD1 (43.2 ± 5.6 mV, n = 4) and RPaD2 (10.7 ± 2.8 mV, n = 4) neurons. All effects were reversible after 15 minutes of washing in the control Ringer solution.

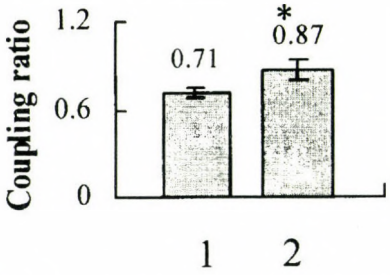

Fig. 2. Effects of NO-donors on the coupling ratio between VD1/RPaD2. 1 – control; 2 – DEA/NO (10^{-4} M). Data present mean values SEM. *P < 0.05 vs control (Student's *t*-test), n = 5

DISCUSSION

Although preliminary observations indicate the involvement of cAMP-dependent pathways in neural NO signaling, the detailed mechanisms are unknown. The suppression of cAMP production in the buccal ganglia in the presence of NO donors is correlated to the very opposite changes in cAMP levels in the rest of the CNS. The NO-induced enhancement of cAMP levels in the major central ganglia of *Lymnaea* is an unusual phenomenon requiring additional studies. On the other hand, both NO and the cAMP analog acted alike, increasing the coefficient of electrical coupling between visceral (VD1) and parietal (RPaD2) neurons. In contrast, at a similar concentration 8-Br-cGMP reduced the coupling, suggesting that the action of NO was not cGMP-dependent and can be connected to the increase of intracellular cAMP.

Elphick et al. (1995) [4] suggested that in the buccal ganglia of *Lymnaea* NO acts by enhancement of cGMP synthesis. Thus, in this system the decrease of the cAMP levels might reflect the reciprocal relationships found between cAMP and cGMP-dependent pathways. Moreover, in the buccal ganglia (but not in the central ganglionic ring) the effect of the NOS inhibitor was opposite to those induced by NO donors, suggesting that the L-NNA can suppress tonic enzymatic NO production. However, in the ganglionic ring L-NNA has an action similar to that described for NO. One possible explanation can be a potential non-enzymatic release of NO from this L-arginine analog under certain reduced conditions [10]. Since cAMP production in the ganglionic ring can be enhanced by relatively low concentrations of NO, even a small non-specific release of NO from L-NNA may contribute to the rise of the intraganglionic cAMP.

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ELEMENTARY AND COMPOUND POSTSYNAPTIC POTENTIALS IN THE DEFENSIVE COMMAND NEURONS OF *HELIX LUCORUM**

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The present communication concerns with the analysis of elementary and the compound excitatory postsynaptic potentials (eEPSPs and cEPSPs) recorded by intracellular microelectrode from an identified defensive command neuron of the snail *Helix lucorum*. The eEPSPs were evoked by single presynaptic action potentials (APs) elicited by cationic current injection into one of the identified sensory neurons synapsing on the respective command neuron. The cEPSPs were elicited by local brief tactile stimuli on the skin or internal organs. It was shown that the cEPSPs amplitudes depend mainly on the number of activated sensory neurons. Compound EPSPs depend also on frequency and the number of APs in the bursts occurring in a single neuron. Presynaptic APs having frequency 2–10 Hz evoke high frequency depression of that eEPSPs after an interval is followed by post-tetanic potentiation of single eEPSPs. Preceding stimulation of a pneumostom area facilitates the cEPSPs elicited by repeated stimulation of viscera. The eEPSPs from the same visceral area demonstrate no heterosynaptic facilitation in experiments with double parallel intracellular recording from responsive sensory and command neurons. The different types of the eEPSPs plasticity are discussed according to their contribution cEPSPs plastic changes.

Keywords: Elementary EPSP – compound EPSP – synaptic plasticity – high frequency depression – post-tetanic potentiation – heterosynaptic facilitation – habituation

INTRODUCTION

Postsynaptic potentials (PSPs) recorded intracellularly from whole cells can be divided into three main types according to their origin: miniature PSPs correspondent to quantal transmitter release; elementary PSPs elicited by single presynaptic spikes and compound PSPs generated by excitation of a set of the presynaptic sensory neurons. Single-channel potentials recorded by patch clamp method that cannot be separated from whole cell potentials are not regarded here. The task of the present study was to compare the elementary excitatory postsynaptic potentials (eEPSPs) with the compound excitatory postsynaptic potentials (cEPSPs) recorded from the same iden-

*Dedicated to Professor János Salánki for his 70th birthday.

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tifiable neuron. Special emphasis was laid on the comparison of their plasticity when the eEPSPs constitute components of the cEPSPs.

A convenient experimental object to solve the problem is an identified command neuron of defensive behavior of the land snail *Helix lucorum.* The command neurons were studied for many years by different groups of researchers [for review see 24]. The defensive command neurons respond to mechanosensory stimulation of any point of their giant mechanoreceptive fields by compound EPSPs [5, 22], which are changed in the process of leaning [23]. The synaptic plasticity was extremely selective suggesting contribution of parallel channels. Several local mechanosensory neurons from this parallel encoding having monosynaptic inputs to the command neurons have been identified in the left parietal ganglion of *Helix* [15]. The identified sensory neurons had in different regions of viscera local excitatory receptive fields surrounded by inhibitory areas. Such inhibitory areas occupy the total mechanoreceptive surface of the giant receptive field [18]. The identification of pre- and postsynaptic neurons enables recording in the same command neuron the eEPSPs evoked by single presynaptic spikes from one sensory neuron and the cEPSPs elicited by sensory stimulation. The experiments with double intracellular recordings from the identified sensory and command neurons on semi-intact preparations of *Helix lucorum* present data concerning their relationship.

METHODS

Adult land snails *Helix lucorum* of Crimea population were used in the study. Experiments were made on the semi-intact preparations consisted from the central ganlia, dissected foot, pallium and viscera. Physiological solution contained (in mM): 80 NaCl, 4 KCl, 8 $CaCl_2$, 5 $MgCl_2$, 4 Tris-HCl (pH 7.8). Solution contained twofold increased concentration of Mg^{2+} and Ca^{2+} ions was used to inhibit polysynaptic connections.

The postsynaptic potentials were recorded intrasomatically by conventional technique for intracellular whole-cell current recording. Double intracellular recording was used to record simultaneously activity of presynaptic and postsynaptic neurons. The microelectrodes filled with 2.5 M KCl had resistance 10–20 MΩ for presynaptic and 1–5 MΩ for giant postsynaptic command neurons. Intracellular potentials were amplified by MEZ-8201 (Nihon Kohden, Japan) amplifiers and capture on computer by using AD transformer and program DS (Digiscope, Moscow) for monitoring, recording and following analysis.

A scheme of the experiments is shown in Fig. 1B and Fig. 3B. Elementary EPSPs in a command neuron were evoked by single presynaptic APs elicited by short (5–25 ms) cationic current pulses (1–10 nA) injected in a sensory neuron by a bridge circuit of the amplifier. Compound EPSPs were evoked by brief (20–50 ms) tactile stimulation of internal organs (test stimuli) and mantle collar (extrastimuli). Duration of the extrastimuli was chosen to elicit a component of defensive behavior – pneumostome closure [32]. Calibrated hairs (0.1–0.2 mm, 0.4–1.5 G) connected with

solenoid and operated by electrostimulator were used for tactile stimulation. For rhythmic stimulation in the frequency range from 0.1 Hz to 10 Hz electrostimulators MSE-3R (Nihon Kohden) were used.

Morphology of the identified neurons and their synaptic connections were studied by intracellular injections of cobalt and nickel ions [1, 21]. Parallel injection of different dyes into physiologically identified presynaptic and postsynaptic neurons was used to study their synaptic connections [3]. Complete image of the identified synapse has been reconstructed from 20 μm slices after their silver intensification [17, 20].

Data from the identified presynaptic sensory neurons LPa7 and LPa9 and the postsynaptic command neurons LPa3, LPa2 and RPa3, RPa2 studied systematically were obtained in about 100 experiments.

RESULTS

Synaptic contacts between sensory and command neurons

A morphological study of synaptic contacts between monosynaptically connected neurons was based on identifiable neurons injected by dyes. Morphology of the identified command neurons was studied by intacellular cobalt ions injection [1]. The injection into physiologically identified presynaptic sensory neurons has shown that the soma and an area of multiple branching localized in a region of multiple branching of the LPa3 command neuron. Parallel injection of different dyes in the LPa7 sensory neuron and in the LPa3 command neuron has shown that the sensory neuron build up 8–9 boutons on the dendrites of the command neuron (Fig. 1A) [17, 19].

Elementary EPSPs in the command neuron

The eEPSPs elicited in the command neurons by APs arising in the identified sensory neurons have an average amplitude of about 1 mV (200 μV – 2 mV) and average duration of about 100 ms (Fig. 1B). The differences of the eEPSPs amplitudes recorded in the different experiments reflect a multicomponent nature of the eEPSP composed from miniature components reflecting participation of different synaptic boutons [17]. The miniature EPSPs contributing to generation of eEPSP were termed subelementary EPSPs (seEPSPs) [26]. Thus, the eEPSPs are resulted from summation of seEPSPs arising in single boutons of a monosynaptic contact. Polysynaptic components of the eEPSPs have also been detected [19]. Both above-mentioned monosynaptic and polysynaptic components of the eEPSPs are the fast components. Despite of these fast components the late tonic components of eEPSPs were recorded in several preparations (Fig. 3).

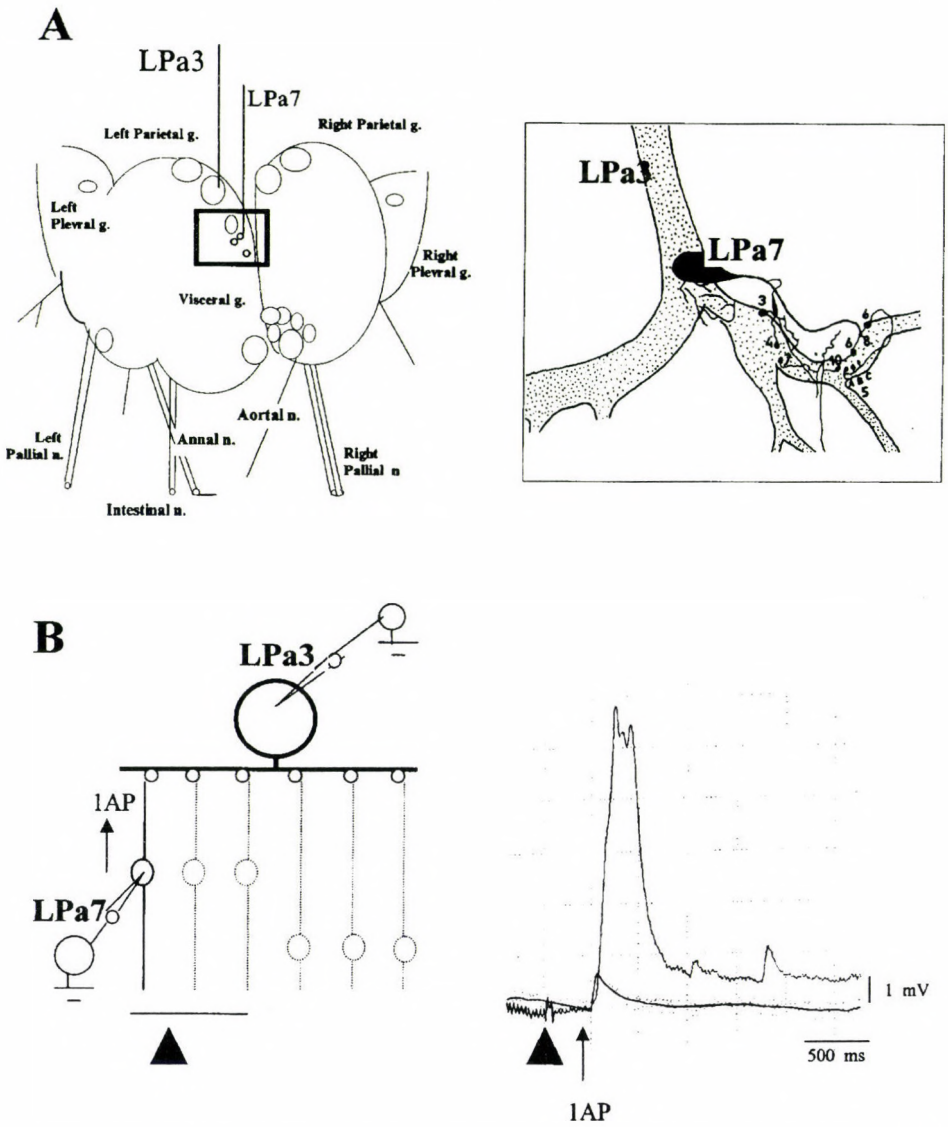

Fig. 1. An identifiable monosynaptic connection in *Helix. A)* A region of contacts between a presynaptic sensory neuron (LPa7) and a postsynaptic command neuron (LPa3) is marked on a scheme of the visceroparietal complex [10]. The details of the synaptic connection are shown on the right [17, 19]. The synaptic connection consists from several boutons marked by numbers. *B)* A simplified diagram of the synaptic connections between sensory neurons and a command neuron. The LPa3 command neuron and the LPa7 sensory neuron have been identified in the experiments with simultaneous intacellular recording. An elementary EPSP evoked in the LPa3 neuron by single presynaptic action potential (AP, fine arrow) elicited in the LPa7 neuron by current injection is show on the right. The eEPSP is presented in comparison with a compound EPSP elicited by brief local tactile stimulation of viscera (large arrow) that is an excitatory receptive field of the LPa7 sensory neuron

Compound EPSPs in the command neuron

Amplitude of a cEPSP elicited in a command neuron by a local brief tactile stimulation depends on a place of the stimulus application within its gigantic receptive field. Compound EPSPs of minimal amplitude arise under local stimulation of the internal organs [22, 23]. Thus, visceral surface represents a periphery of the mechanoreceptive field of the command neuron while excitatory focus is located near pneumostom [26, 27]. Excitatory areas of the identified presynaptic sensory neurons were located on the visceral surface [18]. A brief local stimulation of viscera (liver) activates sensory neurons such as identified presynaptic sensory neurons LPa7 and LPa9. The average amplitude of the eEPSPs evoked by single presynaptic APs in the identified command neurons was 5–8 times smaller than the average amplitude of cEPSPs elicited by a local tactile stimulation of the viscera (Fig. 1B). Because the onset time of eEPSPs closely corresponds the latency of the first peak of the cEPSP, only several (about 5–10) sensory neurons take part in a compound EPSP generation in response to the local stimulation of visceral area. An averaged cEPSP elicited in a command neuron by a brief mechanosensory stimulation has a duration of about 0.5–1 s and demonstrates two maxima with latencies about 100 ms and 400 ms. Duration and shape of a cEPSP depend on the patterns of spikes within a presynaptic neuron. The patterns were generated in the presynaptic neuron by repeated stimulation of an identified sensory neuron to simulate dynamics of eEPSPs evoked under natural stimulation.

Homosynaptic plasticity of eEPSPs

Short cationic current pulses were injected into an identified presynaptic neuron to elicit APs following with low (0.01 Hz), middle (0.1 Hz and 1 Hz) and high (10 Hz) frequency. The amplitudes of the eEPSPs decreased under all the frequencies despite of about 1 Hz. The eEPSPs amplitudes fall during first 5–15 presynaptic APs and then become stabilized at the levels different for the different frequencies. High-frequency stimulation resulted in more pronounced depression of eEPSPs than the middle and low-frequency stimulation (Fig. 2). Thus, decreasing amplitude of the eEPSPs during repeated presynaptic stimulation in high- and low-frequency range looked like a classical habituation curve [13, 28]. Surprising was, however, that the stimulation frequencies around 1 Hz demonstrated no habituation. We suggested that the reason might be a contribution of post-tetanic potentiation [16].

High-frequency stimulation of an identified presynaptic neuron was presented during the low-or middle-frequency stimulation series. It has been shown that the amplitudes of the eEPSPs were decreased under presentation and were increased after high frequency stimulation (Fig. 2). An effect was detectable even after two spikes following with a short (200 ms) interval. An increase of the number of presynaptic APs elicited by high frequency stimulation prolonged and evoked more pronounced potentiation of the eEPSPs.

Irregular presynaptic APs generated during background activity of a sensory neuron or elicited by current pulses injections evoked the eEPSPs with amplitudes correlated intervals between preceded APs.

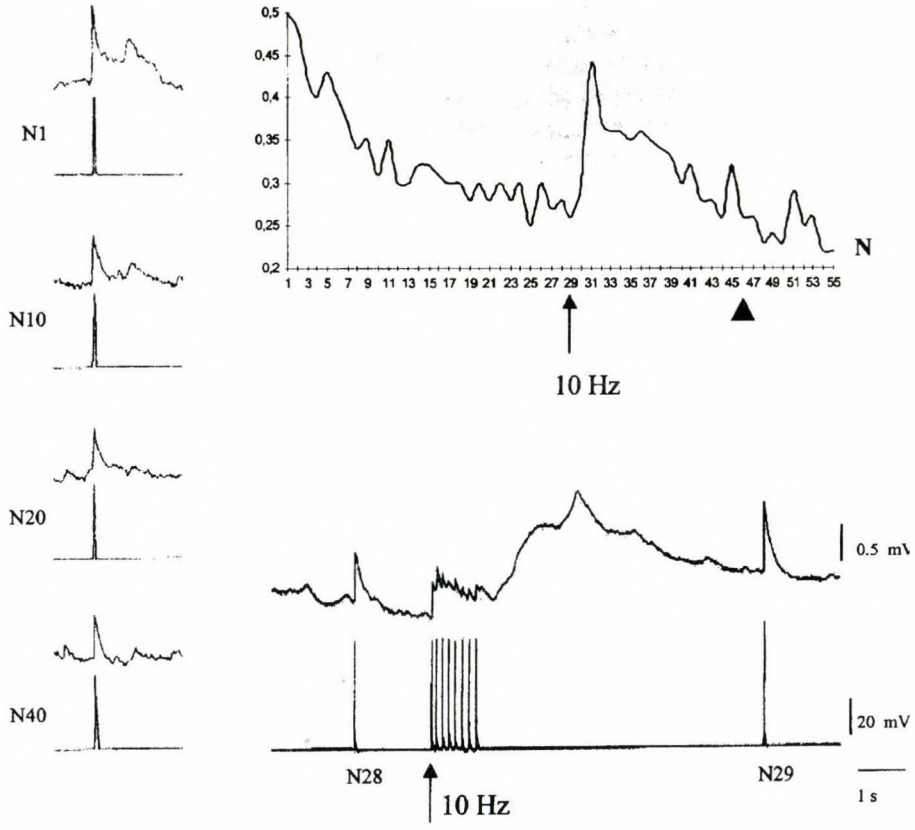

Fig. 2. Plasticity of an identified synaptic connection. A diagram illustrates low-frequency depression (habituation) of eEPSPs elicited by presynaptic APs evoked in an identified sensory neuron by low-frequency (0.1 Hz) intracellular stimulation. The diagram presents averaged (n = 5) eEPSPs amplitudes recorded in the command neuron. The elementary EPSPs elicited by 1st, 10th, 20th and 40th low frequency stimuli are shown on the left. High-frequency (10 Hz, fine arrow) stimulation of the presynaptic neuron during low-frequency stimulation results in a high-frequency depression of eEPSPs followed with an increase of eEPSPs amplitudes elicited by low-frequency stimuli. An example of high-frequency depression followed with posttetanic potentiation of the eEPSPs is shown in the bottom of the figure. A large arrow marks a moment of an extrastimulus presentation (see Fig. 3)

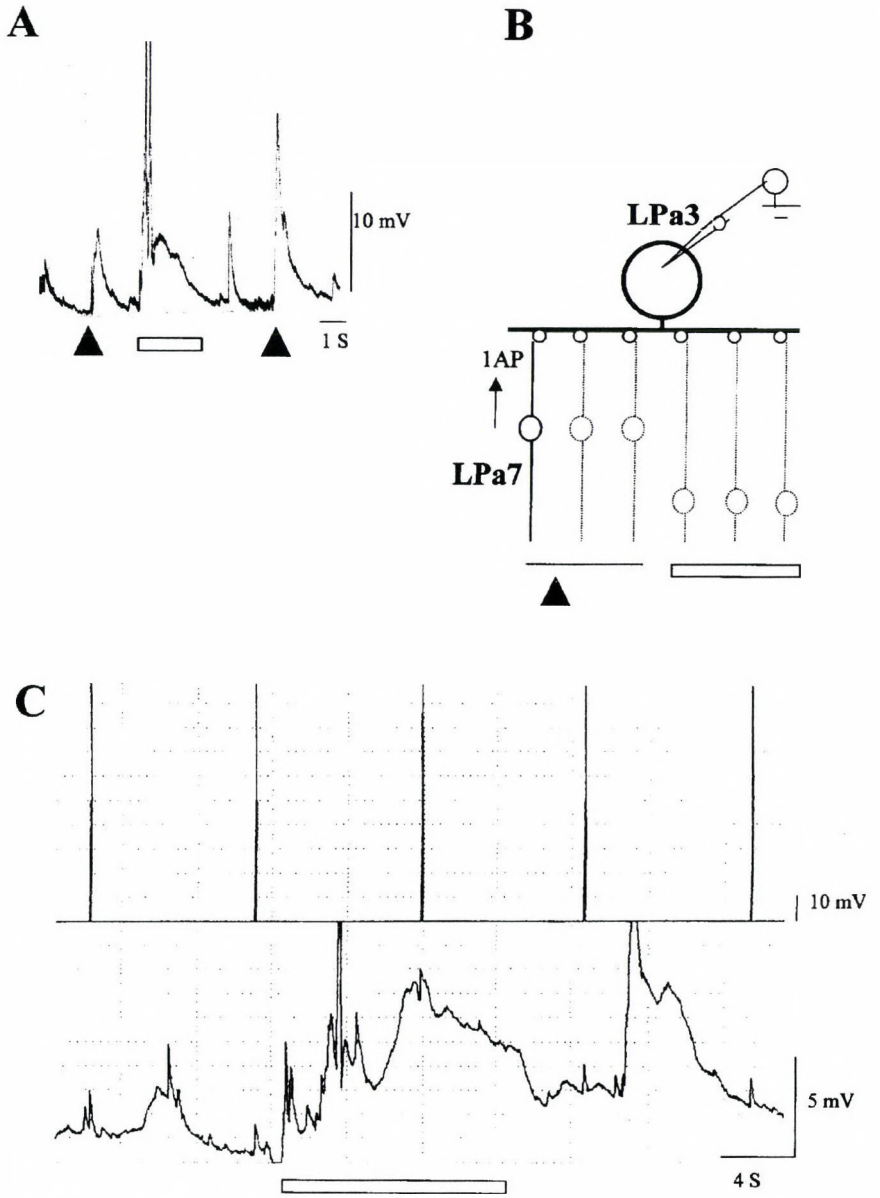

Fig. 3. Heterosynaptic facilitation in the command neuron. An effect of an extrastimulus to the cEPSPs (A) and the eEPSPs (C). A scheme of the experiment is shown in B: an extrastimulus (rectangular) – tactile stimulation of pneumostom with duration to evoke its closure – has been presented during seria of repeated test stimulation (0.1 Hz). A: the test stimuli (large arrow) are local brief (20 ms) tactile stimulations of viscera. The amplitude of cEPSP has been twofold increased after the extrastimulus. C: the test stimuli are presynaptic APs elicited by brief (20 ms, 2 nA) current injection into the LPa7 sensory neuron. The extrastimulus does not affect on eEPSPs amplitudes

Plasticity of the cEPSPs under repeated sensory stimulation

The cEPSP in an identified command neuron is a very convenient object to study synaptic plasticity [4, 5, 25, 26]. Dynamics of the cEPSPs amplitudes during repeated sensory stimulation was studied in details in several experiments [23, 30, 31]. New data obtained in our experiments concerns with contribution of post-tetanic potentiation in middle-frequency range and with an absence of cEPSPs depression during repeated stimulation with frequency around 0.1 Hz.

Heterosynaptic plasticity of the cEPSPs and the eEPSPs

Heterosynaptic facilitation is a simplest case of associative plasticity [6, 7, 11, 12]. It has been suggested that in *Helix* heterosynaptic facilitation is a result of modulatory influences from the serotoninergic system [30, 31]. Recording of the cEPSPs was a bases of this suggestion. An effect of an extrastimulus presentation has been tested in our experiments with the cEPSPs and the eEPSPs recordings.

A scheme of the experiment is shown in Fig. 3B. An extrastimulus was a tactile stimulation of pneumostom area with duration sufficient to evoke pneumostom closure. The extrastimulus has been presented during series of repeated low-frequency (0.01 Hz) or middle-frequency (0.1 Hz) sensory stimulation. The test stimuli were local brief tactile stimulations of viscera. The amplitude of cEPSP was increased after the extrastimulus. Two waves of the cEPSPs amplitudes rise were obtained. The first wave followed immediately after extrastimulus presentation (Fig. 3A). A late wave of facilitation began after short time of decreasing of the cEPSPs amplitudes. The stimuli to test heterosynaptic facilitation of eEPSP were presynaptic APs elicited by cationic current pulses injected into an identified sensory neuron. An extrastimulus was the same stimulation as for the cEPSPs. The main result is that the extrastimulus used for heterosynaptic facilitation does not change the eEPSPs amplitudes (Fig. 3C) while cEPSPs elicited by sensory stimuli were facilitated.

DISCUSSION

Monosynaptically connected sensory and command neurons identified in several invertebrate species are intensively studied with respect to synaptic plasticity [2, 3, 6, 7, 9, 14]. An advantage of the monosynaptically connected neurons identified in *Helix* is that the excitatory areas of the presynaptic sensory neurons receptive fields are located on a periphery of the receptive fields of the command neurons, so that only several sensory neurons take part in a compound EPSP generation. It is assumed that amplitudes of cEPSPs in the command neuron mainly depend on the number of the activated presynaptic sensory neurons, but not on the number of APs generating eEPSPs. It was demonstrated, however, that patterns of presynaptic spikes evoked in a sensory neuron by a sensory stimulus strongly influence the shape of cEPSP

recorded in a postsynaptic neuron. Plasticity of eEPSPs during neuronal response to a single sensory stimulus has been termed "immediate plasticity". Immediate plasticity is a compound effect of high frequency depression (HFD) and post-tetanic potentiation (PTP) of the eEPSPs.

The frequency and the number of APs in a sensory neuron burst evoked by a sensory stimulus increase the amplitudes of cEPSPs in the postsynaptic command neuron elicited by a sequence of sensory stimuli. This effect is due to post-tetanic potentiation of the eEPSPs following initial HFD. The well-known differences between habituation curves for sensory stimulation of different intensity and frequency [29] might be explained by the eEPSPs plasticity. HFD of eEPSPs arises because of limitation of presynaptic transmitter vesicles release [13]. A recovery lasts less then several seconds and thus HFD cannot influence the dynamics of the cEPSPs elicited by sensory stimulus following with intervals more than recovery time.

A mechanism of post-tetanic potentiation of eEPSPs (PTP) in the command neurons has possibly a presynaptic localization [16]. PTP of the eEPSPs might be detected even several minutes after a high-frequency train of presynaptic spikes [3]. The bursts evoked in the sensory neuron by a local sensory stimulation consist from several high-frequency spikes and, thus, the amplitudes of cEPSPs elicited by sensory stimuli following with intervals in a range of 0.1–0.01 Hz could be increased by PTP evoked by previous sensory stimuli. Duration of PTP depends on the frequency and the number of presynaptic spikes. The frequency and the number of APs in a burst of a sensory neuron depend on the intensity of sensory stimulus. Rise of intensity prolongs the effect of post-tetanic potentiation. Rise of sensory stimulation frequency attenuates low frequency depression evoked by desensitization of postsynaptic receptors [8, 25]. A state of modulatory systems [30, 31] contributes to heterosynaptic facilitation. Modulatory neurons activated by defensive stimulation produces sensitization of sensory neurons making them responsive to local stimulation.

Thus, plasticity of the cEPSPs reflects a complex interaction of different types of plastic changes of the eEPSPs: high-frequency short-term depression, post-tetanic potentiation and low-frequency depression.

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INHIBITION OF MICROGLIAL EGRESS IN EXCISED GANGLIA BY HUMAN INTERLEUKIN 10: IMPLICATIONS FOR ITS ACTIVITY IN INVERTEBRATES*

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We studied the effects of recombinant human interleukin-10 (IL-10) on invertebrate immunocytes and microglia. The present report demonstrates that the spontaneous activation of invertebrate immunocytes can be specifically inhibited by recombinant human IL-10. Induced immunocyte activation by fMLP can also be significantly diminished by IL-10. This inhibition becomes apparent over hours and causes ameboid cells to become round and nonmobile. Furthermore, *Mytilus edulis* pedal ganglia maintained in culture, over the course of 24 hours, emit microglia. IL-10 significantly reduces this microglial egress, an action that can be diminished by concomitant exposure of the excised ganglia to an antibody specific to IL-10 as well as IL-10. The anti-IL-10 alone is without effect. Active-ameboid microglia that egress become round and inactive following IL-10 exposure, an action prevented by anti-IL-10. Lastly, a substance immunoreactively similar to human IL-10 can be detected in pedal ganglia homogenates. Taken together, and since the immunocytes and microglia are responding to IL-10, it implies that an IL-10-like substance could be present in invertebrates. In conclusion, the study demonstrates that both invertebrate immunocytes and microglia respond to IL-10, suggesting an early evolution of this generally inhibitory cytokine.

Keywords: Recombinant human IL-10 – microglia – immunocytes – fMLP – invertebrate – *Mytilus edulis*

INTRODUCTION

Ongoing studies in our laboratory have demonstrated that intercellular signalling involving neuropeptides has been conserved through evolution due to the findings that messenger molecules and their receptors similar to the human counterpart are present in invertebrates. This is especially true for opioid peptides and opiate alkaloids [9, 17–19, 25, 26]. Recent studies indicate this is equally true for cytokines [19]. Interleukin (IL)-1-, Tumor Necrosis Factor (TNF)- and IL-6-like materials have also been found in invertebrates, specifically in the marine mollusc *Mytilus*

*Dedicated to Professor János Salánki for his 70th birthday.

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edulis [6, 7, 13]. Furthermore, these cytokine-like molecules were found to alter the behavior and conformation of immunocytes from *Mytilus* in a fashion similar to human monocytes [6, 7, 19]. This activity involves a stereoselect alteration of immunocyte conformation (i.e. conversion from a circular to an ameboid shape) as well as a stimulation of migration [21, 22, 26].

Given the strong parallelisms in intercellular communication seen in man and molluscs, in spite of 500 million years of divergence, we sought to investigate the effects of another cytokine, IL-10, on invertebrate microglia and immunocyte behaviors. IL-10 appears to have the ability to diminish the phenomenon of microglial egress from excised invertebrate ganglia maintained in culture [5, 16]. IL-10 also has the ability to suppress both spontaneously active and fMLP stimulated immunocytes. Furthermore, an immunoreactively similar substance can be found in the ganglia preparation. The results demonstrate for the first time that IL-10 can inhibit both invertebrate glia and immunocyte activities and also suggests that this cytokine and its function have also been conserved over evolution.

MATERIAL AND METHODS

Mytilus edulis were harvested from the shores of Long Island Sound at Montauk Point, Long Island, NY. The animals were maintained for periods of up to three months in an internal marine aquarium system developed to reduce bacteria [27].

For *in vitro* observations of microglia, pedal ganglia of *Mytilus* were bathed for 1–24 h in sterile physiological saline containing antibiotics (50 mg streptomycin, 30 mg penicillin, 50 mg gentamicin in 100 ml, pH 7.5) and then transferred to a microwell culture dish in which a 1 : 1 ratio of centrifuged cell-free hemolymph and physiological saline plus antibiotics was added 50% by volume. The hemolymph was inspected by light microscopy to ascertain its cell-free status, then subjected to UV radiation for 1 h to further eliminate bacteria. Following the desired incubation period ganglionic explants, IL-10, anti-IL-10, alone and in combination was added. Explants were then examined by image analysis [16]. The number of microglia which migrated from the excised ganglia was then quantified.

For the examination of microglial and immunocyte conformational changes, ganglia or immunocytes were placed on a glass slide within a ring of petroleum jelly covered with a coverslip. Cell shape changes following addition of IL-10 were then determined by image analysis at a magnification of ×100 [16, 21, 22, 25, 26]. Cells treated with fMLP (10^{-9} M, 30 min., then washed three times) were later exposed to IL-10 for periods of up to 24 h and measured similarly.

Morphological analysis

The determination of the degree of activation in glial cells (ameboid vs. round conformation) that egress from lesioned ganglia as well as that of peripheral immunocytes was based on measurements of cellular area and perimeter by use of the Oncor,

Inc. (San Diego, CA) and Image Analytics (Hauppauge, NY) image analysis software [21, 22]. A 400 μm viewing diameter was selected for determining the number of cells having moved into the culture dish, and for frame grabbing.

Reagents

Il-10 and its antibody were obtained from Biosource International. Both were dissolved in artificial sea water (Instant Ocean). Anti-IL-10 also contained bovine serum albumin (0.5%). fMLP was obtained from Sigma, Inc.

Enzyme linked immunosorbent assay

Pedal ganglia from 200 *Mytilus* were removed and treated with vehicle (non-stressed) or LPS 1 nM (stressed) for 6 h. Following treatment ganglia were homogenized in phosphate buffered saline (30% w/v). Debris free homogenates were then subjected to enzyme linked immunosorbent assay (ELISA) for human IL-10. ELISA (Biosource International, Camarillo, CA) were performed in microtiter plates according to the manufacturers instructions. Plates were rigorously washed between each procedural step. Optical densities of the indicator solution were determined at 450 nM on an automated ELISA reader (BioRad). All values obtained were adjusted for nonspecific binding.

Statistical analysis was by way of Student's *t*-test. Each curve was compared to a control performed at the same time using cells obtained from the same animal in order to minimize variations. Each evaluation measured or counted 67 to 112 cells. Experiments were replicated three times and the resulting mean was graphed ± (Standard Error of the Mean).

RESULTS

We have previously shown in *Mytilus edulis* that a proportion of freshly harvested immunocytes will become spontaneously activated over a period of time (Fig. 1). Treatment of these cells with human IL-10 (100 U/ml) significantly reduces the spontaneous activation ($P < 0.05$). This effect was specific for IL-10 since the 1) IL-10 was recombinant and 2), monoclonal antibody blocked the effect (Fig. 1.). As previously shown with other substances that inhibit spontaneous activation of immunocytes (e.g. morphine and melanocyte stimulatory hormone), IL-10 also induced ameboid, mobile cells to become round and immobile [20–22, 26] (Fig. 2). This activity only became significant after 6–12 hours of exposure to IL-10 ($P < 0.01$; Figs 1, 2), whereas nonexposed cells continued to increase their spontaneous activation.

Since IL-10 inhibited and prevented the gradual increase of the spontaneous activation of immunocytes, we determined if it was capable of inhibiting induced activation. Thus, after exposure of the cells to fMLP (10^{-9} M) and their subsequent washing, IL-10 (100 U/ml) was added. Figure 1 shows a significant inhibition by IL-10 in

the number of fMLP activated immunocytes when compared to those exposed to fMLP alone ($P < 0.01$). Again anti-IL-10 blocked this effect (100 U/ml; Fig. 1). Inhibition of fMLP action also took place during the extended time period (Fig. 1). These results indicate that IL-10 diminishes the stimulation induced by a well-established immunocyte activating agent.

It was reported that morphine inhibits the microglial egress from excised invertebrate and vertebrate ganglia [16]. Since morphine and IL-10 have been shown to inhibit activation of human immunocytes [16], as well as induce them to become round and inactive in the process, we determined if it would also inhibit invertebrate microglial egress. Figure 3 shows that the egress of microglia from excised invertebrate ganglia does not occur at a constant rate during the initial 24-hour observation period. There is a lag lasting approximately 6 hours followed by a period of significant microglia egress of up to 24 hours (Fig. 3) at which it appears to peak [16]. We found that IL-10 significantly (83.4 ± 8.4 vs. IL-10 [100 U/ml] exposed $= 43.7 \pm 6.2$; $P < 0.05$) reduced the egress of microglial cells in a concentration dependent manner

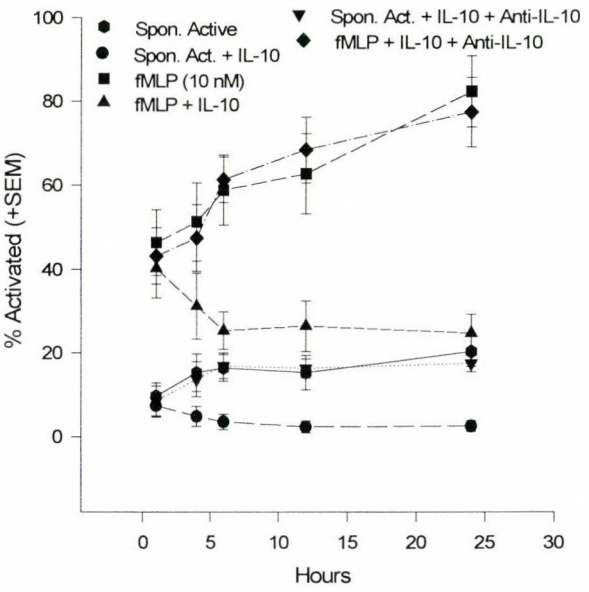

Fig. 1. IL-10 (100 U/ml) induces spontaneously active immunocytes and those stimulated by fMLP to become round and inactive over a 24-hour incubation. This immunocyte downregulating action can be antagonized by coincubation with anti-IL-10 (100 U/ml). The experiments were replicated 4 times. The mean value of these trials is represented along with the standard deviation. The percent active immunocyte number was determined by counting cells exhibiting a form factor ($4 \times \pi \times$ area/perimeter^2) of less than 0.5. Statistical significance ($P < 0.01$) was determined by a one-tailed Student's *t*-test comparing the ff at 1 hour with that obtained at 24 hours. For IL-10 treatment compared to controls $p < 0.01$

(Fig. 4). This effect was blocked by anti-IL-10 (Fig. 4). In all of the above experiments, anti-IL-10 alone, has no noticeable effect (data not shown).

Of the cells that respond to ganglionic surgical excision by leaving the tissue, about 46% normally become ameboid (form factors of 0.6 or less) and active while the rest appear round (form factors of more than 0.8) and inactive. IL-10 treatment resulted in a decrease in the number of ameboid cells to below 16% ($P < 0.01$). Control cells or those treated with IL-10 and anti-IL-10 exhibited a modest increase in the number of ameboid cells to 53% and 48%, respectively (Fig. 5). Thus, it would appear that IL-10 has specific, yet general immune down regulating properties in this system.

Since xenogeneic human IL-10 acted on the microglia we next wanted to determine if an immunoreactive (ir) similar substance could be detected in excised ganglia. Thus pedal ganglia were excised from *Mytilus* and treated with LPS (1 nM) , as a stressor to possibly induce the production of ir-IL-10. Ganglia were then homogenized and the homogenates tested for IL-10 immunoreactivity. Ir-IL-10 can be detected in both stressed and nonstressed ganglia. A higher level is seen in the stressed ganglia versus the nontreated control (78.5 pg/ml vs. 27.3 pg/ml, respectively).

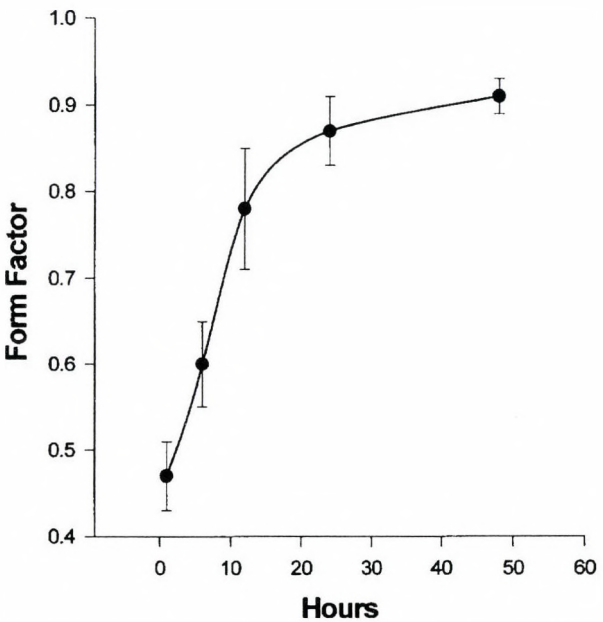

Fig. 2. IL-10 (100 U/ml) slowly induces active (mobile and ameboid) microglia to become round and inactive. The experiments were replicated 4 times. The mean value of these trials is represented along with the standard deviation. Form factor is equal to 4 X π X area/perimeter^2. Statistical significance ($P < 0.01$) was determined by a one-tailed Student's *t*-test comparing the ff at 1 hour with that obtained at 24 hours. In control samples 46 ± 5.9% of the cells were in the ameboid-active conformation. It is these cells that were analyzed for the IL-10 induced rounding action over the time period. IL-10 reduced this level of activation to 13.7 ± 2.5%

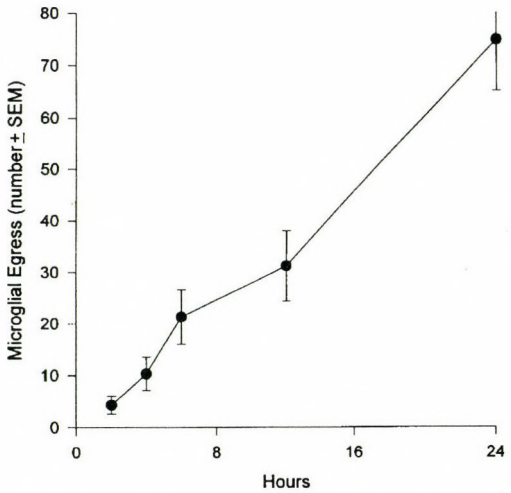

Fig. 3. Microglial exodus over a 24-hour period. The experiments were replicated 4–6 times. The mean value of these trials is represented along with the standard deviation. Statistical significance ($P < 0.01$) was determined by a one-tailed Student's *t*-test comparing migration numbers at one hour to 24 h

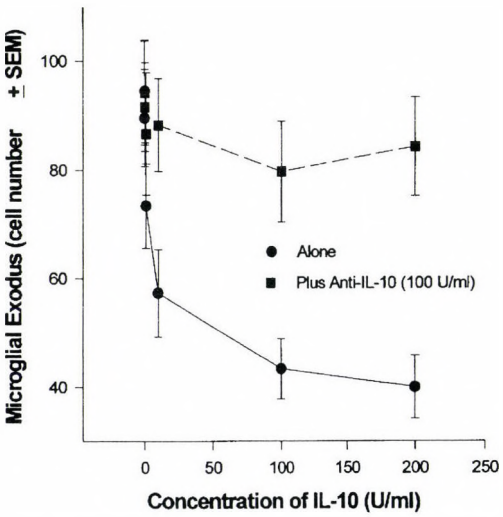

Fig. 4. The effect of IL-10 on microglial exodus at 24 hours and its antagonism by concomitant IL-10 antibody exposure. The experiments were replicated 4–5 times at doses of the cytokine previously shown to be effective in altering immunocyte shape (Fig. 1). The mean value of these trials is represented along with the standard deviation. Statistical significance ($P < 0.01$) was determined by a one-tailed Student's *t*-test comparing IL-10 migration numbers to that of the controls. $p < 0.005$ at 200 U/ml compared to anti-IL-10 (100 U/ml)

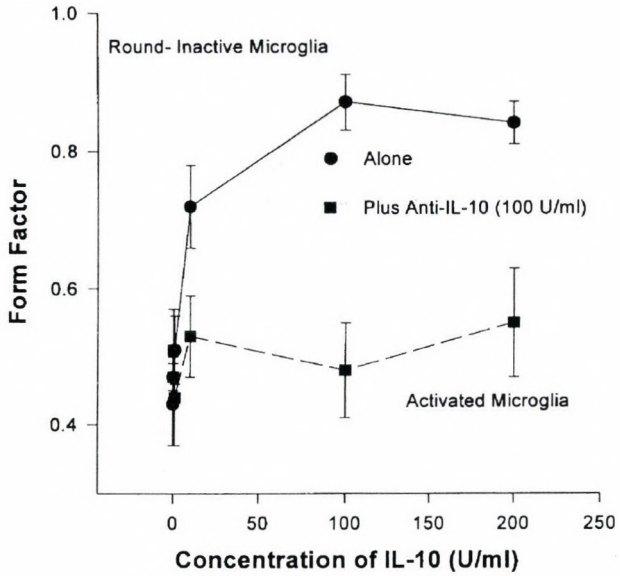

Fig. 5. IL-10, in a concentration dependent manner, induces active (mobile and ameboid) microglia to become round and inactive, an effect blocked by IL-10 antibody co-incubation. The experiments were replicated 4–6 times. The mean value of these trials is represented along with the standard deviation. Form factor is equal to 4 X π X area/perimeter^2. Statistical significance ($P < 0.01$) was determined by a one-tailed Student's *t*-test comparing treatment with IL-10 at 100 & 200 U/ml to that in the presence of Il-10 antibody. In control samples 46 ± 5.9% of the cells were in the ameboid-active conformation. It is these cells that were analysed for the IL-10 induced rounding action. IL-10 (100 U/ml) reduced this level of activation to 13.7 ± 2.5%. The IL-10 antibody did not initiate any observable immunocyte conformational changes

DISCUSSION

The present report demonstrates that: (1) the spontaneous activation of invertebrate immunocytes can be specifically inhibited by recombinant human IL-10; (2) induced immunocyte activation by fMLP can be inhibited by IL-10; (3) inhibition becomes apparent over hours and causes ameboid cells to become round and inactive; (4) *Mytilus* pedal ganglia maintained in culture, over the course of 24 hours, emit microglia; (5) IL-10 significantly reduces this microglial egress; (6) antibody specific to IL-10 inhibits its action on both immunocytes and microglial cells; (7) anti- IL-10 alone is without effect; (8) active-ameboid microglia become round and inactive following IL-10 exposure; and (9) a substance immunoreactively similar to human IL-10 can be detected in ganglia homogenates. Taken together and since the immunocytes and microglia are responding to IL-10 it implies that an IL-10-like substance could be present in invertebrates. This was further supported by our demonstration that an ir-IL-10 could be detected by ELISA in this system.

The assignment of microglial status to the cells that have egressed from excised invertebrate and vertebrate ganglia has been documented [16]. Briefly, the evidence supporting their microglial status is twofold. In response to surgical trauma, a subset of reactive glial cells exhibit a number of features characteristic of the *Mytilus* immunocytes. In addition, these structural and functional commonalities parallel those between microglia and macrophages observed in vertebrates under comparable experimental conditions [15]. The critical aspects of this concept can be summarized as follows: (1) in mammals and invertebrates, comparable mobile immune cells are known to enter various tissues, including nervous tissue [23, 24]; (2) in the process of transformation to resident glia, these immunocytes appear to undergo similar conformational changes and some biochemical dedifferentiation [23]; (3) during egress from lesioned neural tissue, the microglia regain some of their inherent characteristics, including an ameboid conformation, adhesiveness, and motility [16]; (4) an important criterion for the immunoregulatory potential of these mobilized microglial cells and their counterpart in vertebrates is their phagocytotic activity which is used for the uptake and removal of degenerating neural structures in both mammals [2, 15, 24] and invertebrates [1, 11]; (5) in both groups, the egress of reactivated microglial cells from lesioned nervous tissue and their conformational changes are counteracted by exogenous morphine, an activity which corresponds to its effect in lymphoid cells [16, 17]. Thus, it would appear that the small nonneuronal cells found mostly juxtaposed to nerve cell bodies are indeed microglia.

The data presented demonstrates that microglial cells are capable of mounting a primitive immune response since these cells also accumulate at severed nerves [22]. At present we assume the glial mobility is initiated by neuropeptides and cytokines generated by damage due directly to the tissue trauma [5, 6, 7, 21, 22]. The data in the present report also suggests that as in mammals [4, 10], IL-10 may downregulate an immune response by inhibiting the synthesis of the signal molecules such as IL-1 and TNF thereby effectively blocking the response to tissue trauma seen in control preparations. This conclusion is also supported by the fact the antibody to IL-10 specifically blocked IL-10 action. Furthermore, as part of IL-10's macrophage downregulating activity it induces a "rounding" of the cells [3] which has now also been demonstrated in the present study. Videomicrograph examination of immunocytes exposed to IL-10 showed the rounding phenomenon and its inhibition by antibody to IL-10. The delayed onset of IL-10 action in immunocytes and microglia when compared to other inhibitory substances such as melanocyte stimulating hormone [21, 22, 26], may reflect its mammalian role in inhibiting cytokine synthesis. This could occur not only during trauma but also during the observed spontaneous activation of immunocytes. Furthermore, our data showing the presence of an ir-IL-10 indicate that an endogenous cytokine synthesis inhibitory mechanism may be occurring.

Data indicating that IL-10 could play a role in nervous tissue has been reported in vertebrates. We have shown that IL-10 acts in the nervous system of rats to reduce sleep and that IL-10 is both produced and acts in the neuroendocrine system in mice and humans [8, 12, 14]. Thus the findings of IL-10 acting on invertebrate microglia in pedal ganglia from *Mytilus edulis* has parallels in the vertebrate system.

In conclusion, our data suggests that IL-10's ability to inhibit invertebrate immunocyte activation has been conserved through evolution since it parallels its activity in mammals. The study also demonstrates the suitability of *Mytilus edulis* as an alternate and economical animal model for studies involving IL-10 as well as general studies of immune function involving cells having characteristics of monocyte/macrophages. The study also has important implications for immune disorders associated with cellular hyperactivity [23].

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THE ANTICONVULSIVE EFFECT OF NON-NMDA ANTAGONIST GYKI 52466 ON 3-AMINOPYRIDINE-INDUCED PRIMARY AND SECONDARY CORTICAL ICTAL ACTIVITY IN RAT*

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The effect of GYKI 52466, a selective, non-competitive antagonist of the AMPA glutamate receptor subtype was investigated on the development, expression and propagation of 3-aminopyridine-induced cortical ictal activity, both in the primary and secondary focus. In one group of animals GYKI 52466 was administered intraperitoneally, 20 minutes prior to the local application of the convulsant the surface of the cortex of anaesthetized rats. Control animals were injected by physiological solution. Different parameters of electrographic ictal discharges were measured under the influence of the antagonist and compared to control values. The results demonstrate that GYKI 52466 exerts anticonvulsive effects on both the induction and the expression of primary and secondary electrical ictal activity, by delaying the onset of the first ictal period, shortening the duration of ictal activity and decreasing the amplitudes of epileptiform discharges. However, the seizure propagation was not influenced significantly. It is suggested that the initiation, maintenance and the propagation of spontaneous seizures may be controlled by separate mechanisms and that changes can occur in one of the procedures without parallel changes in others. The observations of the present study extend those reported previously by others, namely that activation of non-NMDA receptors is significantly involved in the initiation and maintenance of cortical epileptiform activity.

Keywords: AMPA receptors – antiepileptic – experimental epilepsy

INTRODUCTION

Fast excitatory neurotransmission involving glutamate plays an important role in the initiation, maintenance and spread of seizure activity [7, 15]. There are three different subtypes of glutamate receptors: N-methyl-D-aspartate (NMDA) and non-NMDA ones, which are receptor-ionophore complex, and the third subtype which is a metabotropic receptor. Furthermore, non-NMDA receptors are classified into α-amino-3-hydroxy-5-methyl-isoxazole-4-propionate (AMPA) and kainate receptors [23, 22]. A question of great significance is what role do the specific subtypes of glu-

*Dedicated to Professor János Salánki for his 70th birthday.

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tamate receptors play in epileptogenesis and the expression of seizures? It was shown in hippocampal slices that afterdischarges and electrical seizures are mainly dependent on NMDA receptor activation [1, 8, 16] whereas non-NMDA and metabotropic receptor activation regulates electrical interictal bursts [2, 3]. *In vivo* studies suggest that the NMDA receptor subtype is involved in the development of kindling [5, 13] whereas the non-NMDA subtype is important in the maintenance and expression of fully kindled seizure [2, 10].

We were interested to know, whether non-NMDA receptors are involved in initiation, maintenance and propagation of focal periodic ictal epileptic activity in the rat neocortex *in vivo*. Examination of the effects of specific receptor blockers on the parameters of cortical ictal seizure activity may provide important clues about the initiation and termination of epileptic discharges, and the roles played by different receptors in these phenomena. We have chosen to use GYKI 52466 (1-(4-aminophenyl)-4-methyl-7,8-methylene-dioxy-5H-2,3-benzodiazepine.HCl), a highly selective, non-competitive antagonist of the AMPA/kainate glutamate receptor subtype responses [9, 21], as a pharmacological tool to study the importance of these receptors in the induction, maintenance and expression of cortical ictal epileptic discharges.

As an experimental seizure model, the *in vivo,* 3-aminopyridine (3-Ap)-induced primary and secondary cortical ictal activity was chosen, because its characteristic electrocorticographic (ECoG) manifestation of primary and secondary paroxysmal discharges resemble human partial epilepsy [18, 19, 20]. The secondary epileptic foci, including the mirror focus (Mf), are useful places to study intrinsic mechanisms of epileptic activity, since their underlying events are less influenced by the experimental procedure. Different parameters of electrographic periodic ictal discharges were measured under the influence of the drug and compared to control values.

MATERIALS AND METHODS

Experiments were carried out on adult male (n = 9) and female (n = 9) Wistar rats (weighing 240–280 g) of our feeding animal-room. The animals were anesthetized by intraperitoneal injection of sodium pentobarbital 45 mg/kg. The head of the animal was fix in a stereotaxic instrument. Four small holes of about 2 mm diameter were drilled on the skull to space recording electrodes. A small quantity (about 1 mm^3) of 3-Ap crystal was applied unilaterally to the surface of exposed somatomotor cortex, for induction of the primary focus (Pf).

The surface electrocorticographic activity was recorded by the 4 ball-tipped silver wire electrodes of about 0.5 mm diameter. One electrode was placed at the site of 3-Ap application, an other one on the contralateral homotopic cortex (mirror focus, Mf) and two others 5 mm behind to them. The position of the electrodes is shown in the Figure 1. The reference silver wire electrode was attached to the nasal bone. The recording electrodes were connected to an 8 channel electroencephalograph (EEG 81, Medicor) with a low frequency filter at 0.1 Hz and high frequency filter at 70 Hz. ECoG activity was monitored continuously.

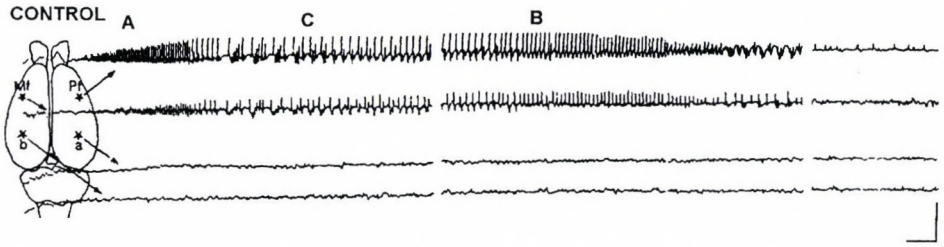

Fig. 1. The 3-aminopyridine-evoked electrocorticographic seizure activity of a control rat. The figure shows the position of the four recording electrodes, this arrangement is valid for all recordings. **A, B, C** indicate the three different firing patterns of epileptiform discharges (based on the frequency and wave forms). **Pf:** primary focus; **Mf:** mirror focus, **a** and **b** the recording points behind the Pf and Mf. The time breaks on the control recordings represent 6 sec, respectively. Calibration bars: 1 sec, 1 mV

Wound margins and pressure points were infiltered with 2% Novocain. The exposed cortical surface was kept warm and wet by physiological solution. The body temperature of the animal was maintained at 37–39 °C by a heating lamp. During experiments, the general state (level of anesthesia, pupil size) of the rats were regularly checked. Recordings started after one hour resting time, following initial surgery and ran continuously at least 60 min after the occurrence of the first ictal events.

Treatment with GYKI 52466 was carried out in 10 rats (5 males, 5 females). GYKI 52466 was administered i.p. in a volume of 1.5 ml (dissolved in 0.9% sterile saline, at pH 2.0–3.0, in a dose of 20 mg/kg), 20 minutes prior to the local application of 3-Ap to the surface of the cortex. The latency between i.p. drug injection and testing was chosen according to the time of peak effect in mice as reported earlier [4]. Control animals (n = 8) were injected by 1.5 ml of 0.9% saline 20 min before application of 3-Ap.

The effects of GYKI 52466 treatment on induction, maintenance and spread of seizure activity was assessed by measuring and comparing the latency of onset of first ictal events, the duration of ictal and interictal periods, the numbers of ictal periods and analyzing the structural pattern of ictal activity (frequency and amplitude of afterdischarges) in the Pf and Mf for control (without GYKI 52466 treatment) and GYKI 52466 treated animals.

Values were measured off line and data from separate animals were averaged and comparisons across groups of animals were made. Student's *t*-test was used to test for differences in the values of seizure activity in control and GYKI 52466 treated animals with significance criterion: $P \leq 0.05$.

All procedures were conducted in accordance with the *Guidelines for the Care and use for Laboratory Animals* and the policy on the animal experiments of the American Physiological Society.

3-Ap was purchased from Sigma, GYKI 52466 was a kind gift from Tarnawa (Institute for Drug Research, Budapest, Hungary).

RESULTS

The ECoG pattern of 3-Ap-induced seizure activity has already been characterized together with the accompanying intracellular events both in the primary and mirror foci in anesthetized cats and rats [18, 19, 20]. Briefly, focal cyclic spontaneous seizure episodes appeared in the Pf within minutes after application of 3-Ap, which were followed after several repetition of ictal period followed by similar paroxysmal activity in the Mf. Three different types of ECoG patterns (***A, B*** and ***C)*** were distinguished according to the frequency (spikes/s), amplitude μV, (peak-to-peak) and configuration of epileptiform discharges. Most commonly, the seizure began with rapid, repetitive spikes of high frequency pattern *A*, (10–15 Hz, tonic-like activity), followed by pattern *B* (4–9 Hz, repetitive spikes) or with pattern *C* (1–3 Hz, spike-wave complex, clonic activity) (Fig. 1). The sequence of pattern *B* and *C* was reversed in some cases and at the termination of the ictal period we usually observed *C* pattern. But pattern *C* never occurred first. These patterns were consistently observed in both Pf and Mf, remarkably reproducible from animal-to-animal. In 17% of the control animals the originally focal seizure activity progressed to the entire cortex and became generalized.

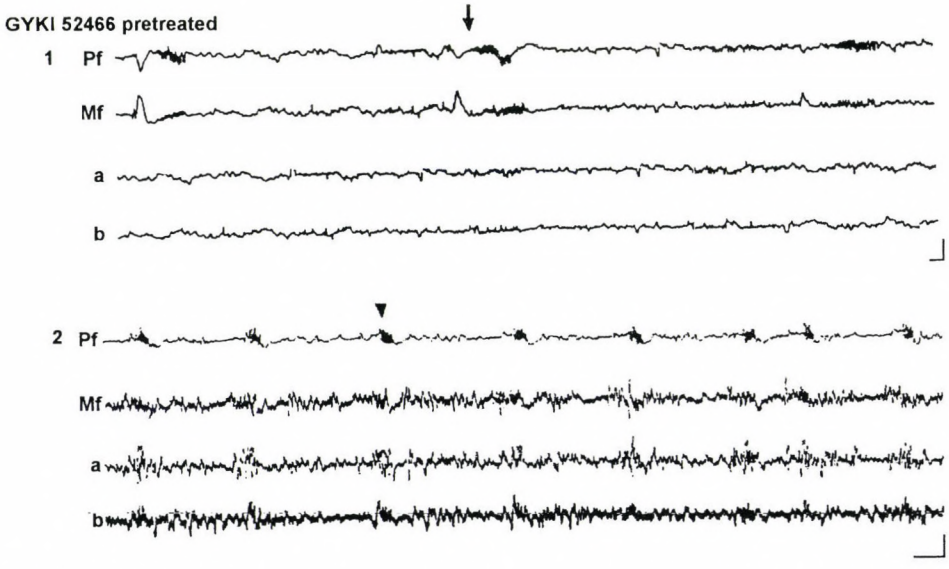

Fig. 2. The 3-aminopyridine-evoked seizure activity in two GYKI 52466 pretreated animals. Note, the more numerous, but rather short ictal events (marked by arrow) characterized by higher frequency discharges in the pretreated animal *1*, and the generalized *"spike-packages"* configurations (marked by arrowhead) in animal *2*. Calibration bars: 1 sec 500 μV

Effects of pretreatment with GYKI 52466 on the development, maintenance and expression of epileptic activity

Pretreatment of animals with GYKI 52466 injected i.p. 20 min before local application of the convulsant did not prevent the induction of epileptiform activity, however, it significantly delayed the development and depressed the manifestation of ictal seizure discharges in both the Pf and Mf foci. The ictal episodes became shorter and they appeared more frequently (Fig. 2, animal *1*) during the one hour recording time. The suppressive effects of GYKI 52466 on cortical epileptogenicity seemed to decline after about one and half an hour period. The probability rate of the generalized epileptiform activity did not differ significantly in treated animals. Since no significant differences in the results of male and female rats were observed, data were pooled and treated together.

Latency of first ictal period

Pretreatment with GYKI 52466 significantly lengthened the time till the onset of the first ictal period, about 5-times above the values determined in control animals (Fig. 3).

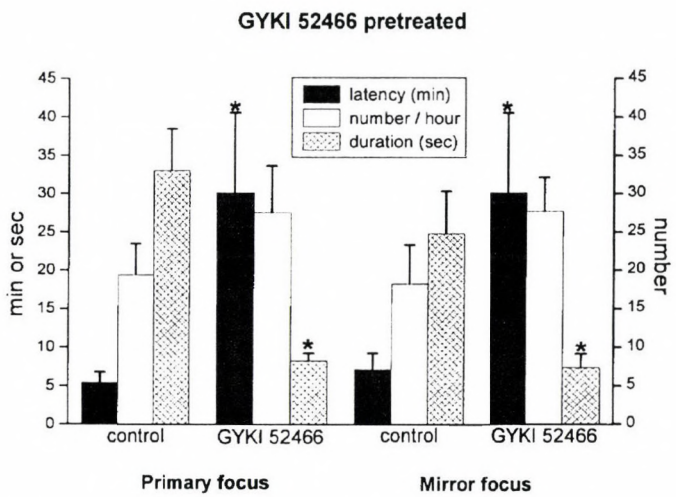

Fig. 3. Different parameters of 3-Ap-induced seizure activity (latency, the numbers of ictal periods/hour and the average duration of ictal periods) in the primary and mirror focus in the control and GYKI 52466 pretreated animals. Measurements were made during one hour inspected time. Latency represents the time elapsed till the occurrence of first ictal period after the convulsant application. Each bar represents the mean value ±SD from 8 control and 10 pretreated animals. Asterisks indicate statistically significant difference between control and treated animals ($P < 0.05$)

Numbers and duration of ictal periods

Pretreatment of animals with GYKI 52466 slightly increased the numbers of ictal events in comparison to the control values (Fig. 3). However, the mean duration of individual ictal periods was significantly reduced in both foci compared to control values (Fig. 3). As a result of these changes, the proportion of the summated ictal and interictal periods during the one hour inspected period was remarkably shifted in favour of interictal activity both in Pf and Mf. The change of summated ictal activity was from **34.19** ± 4.6% to **6.31** ± 1.34% in Pf and from **25.01** ± 6.35% to **5.65** ± 0.33% in Mf (Fig. 4).

Patterns of epileptiform electrocortical activity

In GYKI 52466 pretreated animals there was a general, significant depression in the amplitude of all types of epileptiform discharges in both foci (from ***674.9*** ± 14.67 μV to ***157.7*** ± 44.4 μV of pattern A, from ***973.6*** ± 46.3 μV to ***254.15*** ± 49.7 μV of pattern B, from ***1040.7*** ± 75.8 μV to ***287.1*** ± 41.9 μV of pattern C in Pf and from ***296.8*** ± 15.8 μV to ***98.1*** ± 14.6 μV of pattern A, from ***433.43*** ± 19.4 μV to ***121.38*** ±

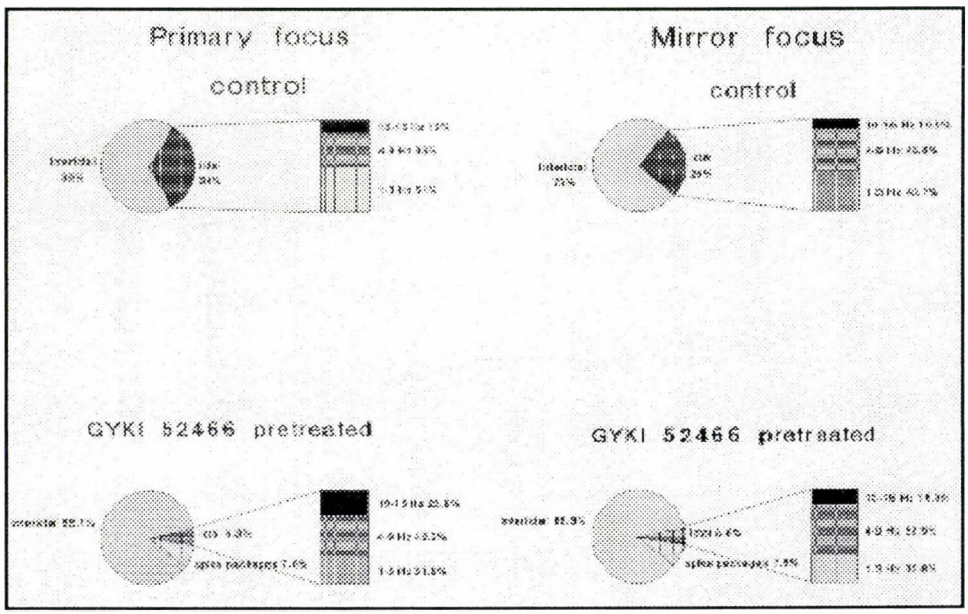

Fig. 4. Percentages of ictal and interictal activity in the primary and mirror focus during one hour of 3-Ap-induced seizure activity in control and GYKI 52466 pretreated animals. Note the significant reduction of summated ictal activity. The columns illustrate the proportion of seizure discharges of different frequencies during the averaged ictal activity of one hour inspected time. The higher frequency patterns became more dominant. Note the presence of *"spike-packages"* only in the GYKI 52466 pretreated animals

35.7 μV of pattern B, from ***274.2*** ± 14.2 μV to ***184.43*** ± 79.9 μV of pattern C in Mf) (Fig. 5).

In addition, the structure of the individual ictal periods was rearranged in such a way, that the ratio of pattern *C* discharges relatively decreased in favour of pattern *A* and *B* discharges in both foci, compared to values of control animals (from ***13.45*** ± 2.36% to ***25.86*** ± 7.37% of pattern *A*, from ***35.82*** ± 13.9% to ***42.24*** ± 7.14% of pattern *B* and from **50.75** ± 12.7% to **31.9** ± 12.68% of pattern *C* in Pf, and from ***10.51*** ± 1.75% to ***16.35*** ± 3.1% of pattern *A*, from ***45.8*** ± 15.6% to ***52.89*** ± 12.67% of pattern *B* and from **43.69** ± 16.3% to **30.76** ± 12.53% of pattern *C* in Mf) (Fig. 4).

In 70% of GYKI 52466 pretreated animals a new pattern of abnormal discharges called ***spike packages*** occurred mostly in the interictal periods, occupying ***7.63*** ± 4.97% of the one hour analyzed period. These spike packages (duration of 0.8–1.0 sec) consisted of 4–7 rhythmic spikes of 13–15 Hz frequency and 300–500 μV of amplitudes, were different from ictal events, usually generalized in occurrence and were never observed in control animals (Fig. 2, animal *2*).

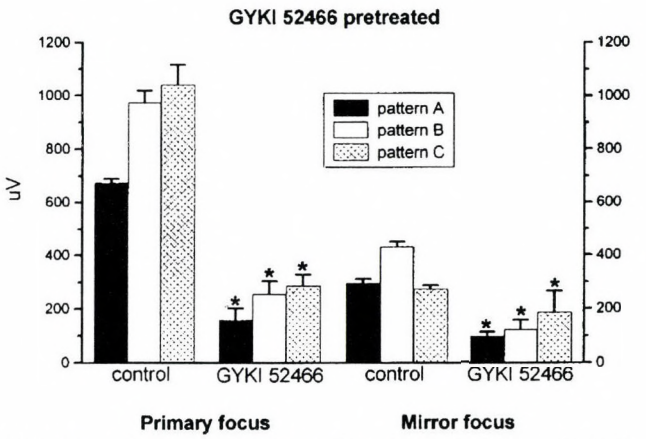

Fig. 5. Graphs show the amplitudes of seizure potentials of different frequencies in the control and GYKI 52466 pretreated animals, in the primary and mirror focus. Pattern A: 10–15 Hz, pattern B: 4–9 Hz, pattern C: 1–3 Hz discharges. Each column represents the mean value ±SD from 8 control and 10 treated animals. Asterisks indicate statistically significant difference between control and treated animals ($P < 0.05$)

DISCUSSION

The present study demonstrates that GYKI 52466, a highly selective, non-competitive antagonist of the AMPA glutamate receptor subtype, exerts anticonvulsive effects on both the development and the expression of 3-Ap-induced cortical primary and secondary electrical ictal activity of rats in *in vivo* experiments. The anticonvulsive effects were indicated by delaying the onset of first ictal period, shortening

the duration of ictal activity and decreasing the amplitudes of epileptiform discharges. The propagation of seizure activity to other cortical areas was not influenced significantly by GYKI 52466. The time elapsing before the onset of the first ictal activity is an indicator of the ease with which seizure activity can be initiated. On the other hand, the duration of individual ictal periods has been postulated to be an electrophysiological correlate of acquisition and an indicator of the ability of the system to terminate seizure activity.

The mechanism responsible for the anticonvulsive effect of GYKI 52466 might be associated with several processes. It is widely accepted that GYKI 52466 is a highly selective AMPA receptor antagonist and appears to act by a non-competitive, allosteric mechanism [9, 22]. GYKI 52466 was shown to penetrate the blood-brain barrier effectively in rat brain, when given intravenously [17], and to exert anticonvulsant effect in seizures induced by different convulsant agents. These observations suggest a role for the involvement of non-NMDA receptor subtype in the generation of seizure activity [4, 14, 15, 24]. In the present experiments, the effect of GYKI 52466 was investigated both on the development and expression of focal cortical ictal activity, resembling human epilepsy, both in the primary and secondary focus in *in vivo* experiments.

The significant delay in the occurrence of the first ictal period in the presence of GYKI 52466 suggests that beside NMDA receptors AMPA/kainate receptors contribute to the induction of epileptiform discharges. It was shown by labeling technique, that AMPA receptors are associated more clearly with terminals of thalamic origin, while NMDA receptor are mostly associated with cortico-cortical terminal sites [12]. One may conclude from these observations that AMPA receptor mediated synaptic transmission probably has an important contribution to the induction of cortical epileptic foci mainly through the activation of cortico-thalamo-cortical neuronal networks.

The fact that a non-NMDA receptor antagonist reduced the amplitude of paroxysmal discharges indicates the contribution of AMPA subtype receptor mediated synaptic responses to the maintenance and expression of epileptiform discharges. The pretreatment of animals with GYKI 52466 can influence the process which causes changes in the effectiveness, sensitivity or the number of AMPA receptors during the induction period. In the case of a local block of AMPA receptor activity in the Pf, the reduced paroxysmal activity in the Mf could be considered as the consequence of less effective synaptic bombardment originating from a weaker Pf.

It is an interesting observation that the number and duration of ictal periods seemed to change differently under the influence of GYKI 52466. After the long latency of first ictal events in GYKI 52466 pretreated animals, the ictal periods were much shorter in duration, but more numerous than in control animals. The shorter interictal periods in these cases can be the consequence of less effective postictal inhibition, following a brief ictal phase. Inhibitory neurons are probably weakly activated under the influence of GYKI 52466. This suggestion is supported by studies on excitatory synapses on fast-spiking interneurons originating from burst-firing neurons indicate a predominance of non-NMDA receptor mediated connections [12].

Therefore in the presence of GYKI 52466 these neurons are probably less effective to prevent induction of ictal events. However, GYKI 52466 is thought to act via benzodiazepine recognition site associated with AMPA receptors. It was reported that the $GABA_A$ receptors themselves are not critically involved in the anticonvulsant effect of GYKI 52466 [14]. GYKI 52466 was also shown to reduce the duration of cortical-afterdischarges without interfering with the development of kindling phenomena [10]. These results suggest that the initiation and the maintenance of spontaneous seizures may be controlled by separate mechanisms and that changes may occur in one of the procedures without parallel changes in the other.

The characteristic spike packages pattern occurring mostly in the interictal periods of GYKI 52466 pretreated animals may result from the blockade of non-NMDA receptor activation on the background ECoG activity. This kind of direct effect of GYKI 52466 on the background EEG activity was already observed in the cortex and hippocampus of rats [15]. The AMPA receptor-mediated excitatory postsynaptic current is the dominant component of many synapses (both at excitatory and inhibitory neurons) in the central nervous system. Modification in the background ECoG after blockage of AMPA receptors is the consequence of changes in the composition of synaptic components of cortical electrical activity.

AMPA/kainate-gated channels are permeable to Na^+ and K^+ and with some exceptions they have low permeability to Ca^{2+} ions [11]. However, the anticonvulsive activity of AMPA receptor antagonists was not affected by Ca^{2+} channel agonists [6]. Besides reduced excitatory function due to the block of non-NMDA glutamate receptor activation, shifts in the balance of different ion conductances as well as other anticonvulsant effects may be involved. It is also possible that some subtypes of AMPA receptors are more involved in epilepsy than the others, and that selective antagonists would be antiepileptic and yet preserve some function. Although investigations showed that receptors mediating kainate responses were not sensitive to GYKI 52466 [22], the role of the kainate receptors cannot be excluded.

As a conclusion the present study supports the idea that AMPA receptors contribute significantly to the induction and maintenance of cortical paroxysmal discharges, however the intracortical, horizontal propagation of paroxysmal discharges probably depends more on NMDA subtype of glutamate receptors.

ACKNOWLEDGEMENTS

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ABBREVIATIONS

3-Ap	3-aminopyridine
AMPA	α-amino-3-hydroxy-5-methyl-isoxasole-4-propionate
ECoG	electrocorticogramm

EEG	electroencephalogramm
GABA	γ-amino-butiric-acid
GYKI 52466	(1-(4-aminophenyl)-4-methyl-7,8-methylene-dioxy-5H-2,3-benzodiazepine.HCl)
Mf	mirror focus
NMDA	N-methyl-D-aspartate
non-NMDA	non-N-methyl-D-aspartate
Pf	primary focus

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PERIODIC AND OSCILLATORY FIRING PATTERNS IN IDENTIFIED NERVE CELLS OF *LYMNAEA STAGNALIS* L.*

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Firing patterns in identified neurons of *Lymnaea stagnalis* L. were analyzed by various mathematical methods including spike density function (SDF), interspike-interval histograms (ISI), Fourier transform and correlation analysis. Input-3 (IP3) events observed in most of the neurons of the respiratory regulatory system caused prominent changes in the firing frequency of the cells. Similarly, quasiperiodic firing patterns were observed in the neurons of buccal ganglia controlling feeding behavior. Apart from the known periodic patterns a fine oscillation of firing rate was observed in a large number of neurons in the visceral and parietal ganglia. The frequency of this oscillation varied between 0.2 and 0.4 Hz. The most obvious oscillatory patterns were found in the A-cells presumably resulted by periodically appearing synaptic excitation. Moderate intracellular hyperpolarizing current injection, low-Ca/high-Mg saline and application of d-tubocurarine failed to abolish the slow oscillations. Application of Ca-channel blocker cadmium, however, completely eliminated the oscillation in a reversible manner.

Keywords: Firing pattern analysis – Fourier spectrum – spike density function – *Lymnaea* neurons

INTRODUCTION

According to our current understanding, neuronal spike trains act as information carriers in/or between the nerve cells. It has recently been generally accepted that neuronal firing patterns needs to be characterized not only by firing rates and prestimulus histograms, but also with more sophisticated mathematical tools characterizing the precision of spike timing [9, 10, 13].

The principal question is how neurons and neuron assemblies encode information in their spike train. Two basic theories are known to provide an answer: 1 – the rate-coding theory takes into account only the averaged number of spikes (firing rate) in successive time periods or windows [13], and 2 – spike time coding theory, which suggests that timing of action potentials and precisely tuned intervals between adja-

*Dedicated to Professor János Salánki for his 70th birthday.

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cent and distant spikes serve as unitary information carriers [4]. To test these two probabilities experimentally, it is necessary to record long-term spontaneous or evoked spike trains in an adequate preparation.

Because of the relatively simple nerve system, molluscs have been widely used to investigate the neural circuits and to test directly the functional role of their constituent neurons and synaptic connections. In pulmonate snail *Lymnaea stagnalis* L. among others, the neural circuitry underlying cardiorespiratory function and feeding behavior has been identified [2, 8, 15, 16, 17]. In *Lymnaea* the neural circuitry regulating respiration, feeding and mantle cavity muscles involve a large number of central neurons including three interneurons, several motoneurons and their followers [16, 17]. As the above neural network displays well-defined rhythmic activity, and receives a large number of synaptic afferents [16, 17] it can be used for studying information processing as well. In such experiments the membrane potential and arrival time of spike events are measured and the correlations either with applied stimulus or with the firing of the partner cells are inspected [1, 4, 12].

MATERIALS AND METHODS

Preparation and electrophysiology

In our experiments we used identified neurons and members of known clusters from the isolated suboesophageal ganglionic ring of the pond snail *Lymnaea stagnalis* L. Ganglia were prepared and desheated in a Sylgard-lined dish in normal extracellular solution containing (in mM): NaCl 40, KCl 1.7, $CaCl_2$ 4, $MgCl_2$ 1.5, D-glucose 10, TRIS-Cl/HCl 10; pH was set to 7.6. In a few experiments the interneuron RPeD1 and motoneurons (in J-, H-, A-, K-clusters) of the respiratory central neural network [3, 16, 17] and in some cases two buccal motoneurons (RB2, LB2) of the feeding regulatory system [2] were used. Long-term (10–100 min) spike trains were recorded using standard intracellular microelectrode technique with two-channel current-clamp amplifiers (Axoclamp-2A and home-built intracellular amplifiers).

Data acquisition and analysis

The method of spike train recording and the digital filtering technique were described in detail in a previous paper [18]. Briefly, arrival times of the neuronal action potentials were measured by a computer program (OSQ) using voltage-discrimination method. The timestamps were saved in binary disk files and evaluated off-line using the author's computer program (OSW). Firing patterns were characterized by various mathematical tools including spike density function (SDF), interspike-interval (ISI) histograms, Fourier transform and correlation analysis. Spike density functions were calculated by convolving the spike train with a smooth and continuous kernel function, Gaussian-type in most cases. As a result of the convolution technique, fir-

ing rate of the neurons was achieved as a continuous and derivable function of time. Periodicities and oscillations of the firing rate were detected by Fourier transform of the SDF. Cumulative interspike interval histograms also called ISI-autocorrelograms were used to detect periodicities in the spike trains and/or signs of precise spike timing.

RESULTS

Respiratory and feeding rhythm

Transitions reflecting IP3 events were detected in a large number of visceral and parietal cells mediating pneumostome opening and/or mantle cavity muscle contractions. The firing rate of H- and J-cells as well as A-cells was temporarily increased as a consequence of the activation of the IP3 synaptic input. IP3-events occurred in a quasiperiodic manner resulting in characteristic peaks in the spike density function of the firing patterns of J-, H- and A-cells (Fig. 1) and in that of the RPeD1 neuron as well. Transients in the SDF were separated by periods of slower firing with vary-

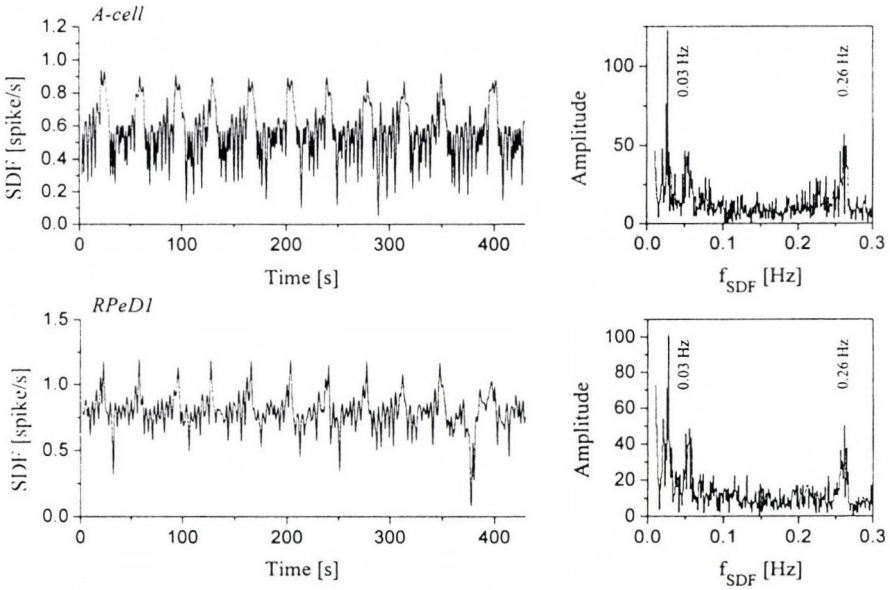

Fig. 1. Quasiperiodic firing patterns in neurons mediating respiration and mantle cavity muscle contraction. The simultaneous spike density functions (SDF) of the A-cell and RPeD1 reveal remarkable correlation between the activity of neurons. Both cells receive the IP3 synaptic input resulting prominent peaks in the SDFs. Fourier powerspectra (f) of the SDFs display sharp peaks at f = 0.03 Hz and 0.26 Hz positions. The lower frequency peak is the outcome of the periodicity of IP3-events. The 0.26 Hz peak reflects an additional slight oscillation in the firing rate. SDF and f are the same in the following Figures

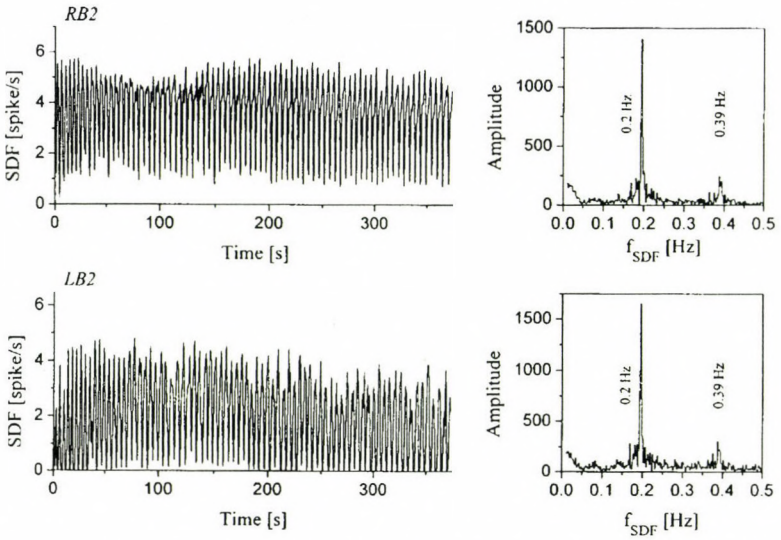

Fig. 2. SDFs and Fourier spectra calculated from the spike trains of buccal cells exhibiting regular feeding patterns (simultaneous recording). Both RB2 and LB2 neurons generated bursts occurring every 5 seconds. As a result, large amplitude waving appears in both SDFs. Fourier spectra (f) on the right display clear peaks at f = 0.2 Hz indicating the regular 5 s periodicity in the feeding rhythm

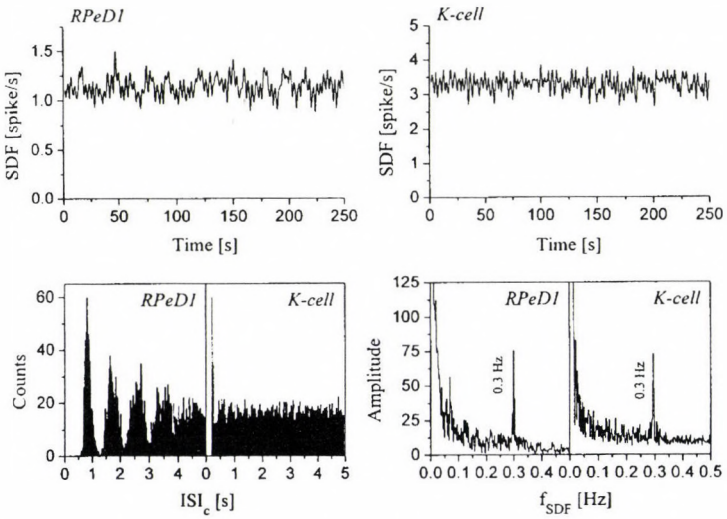

Fig. 3. Firing rate oscillations in neurons of the respiratory network. The spike trains of RPeD1 and the K-cell were simultaneously recorded. Sections of 250 s duration are displayed on the SDF plots. ISI is an interspike interval. Pair of ISI-autocorrelalograms calculated from the original ISI-sequences are shown on the bottom left panel. The Fourier spectra (f) of the SDFs reveal stable 0.3 Hz oscillation in the firing rate

ing duration (30–60 s). Figure 1 demonstrates remarkably regular firing in an A-cell and the RPeD1 neuron, and IP3-events appeared every 30 s. Accordingly, Fourier powerspectra calculated from the SDFs possessed peaks in 0.03 Hz position indicating the 30 s periodicity in the firing pattern. Another peak appearing at 0.26 Hz was the consequence of the overall network oscillation described below. IP3-transients were the most common features in the firing patterns of respiratory neurons.

Spontaneous feeding rhythm was observed only in 20% of isolated CNS preparations. Being the regular feeding rhythm active, characteristic bursts of high-frequency firing (5–20 spike/s) occurred in most of the identified buccal motoneurons including RB2 and LB2 neurons. Bursts occurred approximately every 5 s resulting in large peaks at 0.2 Hz in the Fourier spectra (Fig. 2). Usually, high variety of irregular or quasiregular firing patterns was observed in the buccal cells and spontaneous feeding rhythm developed occasionally. Periodic SDFs with 0.2 Hz frequency were observed only in the presence of regular feeding rhythm.

Synchronized slow oscillations in visceral and parietal neurons

Apart from the known periodic changes in the firing patterns, we observed slow (0.1–0.3 Hz) oscillations in a large number of visceral and parietal neurons as well as in RPeD1. Spike density function technique was very efficient in detecting such oscillations, while ISI-autocorrelalograms were less advantageous. Figure 3 demonstrates how the firing frequency of the RPeD1 neuron and a K-cell evolved in a 250 s window of simultaneous recording. Although there were seemingly irregular fluctuations in the spike density functions, Fourier spectra revealed clear oscillatory behavior. At the same time, ISI-autocorrelalograms of the two spike train differed remarkably. Periodic structure in the histogram for RPeD1 cell was observable, indicating slight periodicity in the arrival times of the spikes. The cumulative ISI-histogram for the K-neuron showed, however, no signs of long-term periodicity in the spike arrival times.

The frequency of the firing rate oscillation varied between 0.1 and 0.3 Hz in most preparations. Long-term and stable oscillations were found mostly in neurons characterized by stable, stationary firing patterns. No marked oscillations were found in cells, which expressed large fluctuations in their firing rate due to synaptic influences. On the other hand, oscillations quickly built up in respiratory motoneurons when the IP3 events were paused. A-cells in the right parietal ganglion were found to develop the most apparent oscillations. The Fourier spectra of the SDFs calculated from the spike trains of such neurons contained prominent peaks at around 0.2 Hz. A-cells typically exhibited multiplets (doublets, triplets) as well as short bursts. Figure 4 shows the overall firing properties of the A-neurons. A characteristic baseline in the ISI-sequence plot appears as a consequence of the short intraburst, interspike intervals. Longer ISI-s correspond to the time intervals between adjacent bursts. The spike density function of such cells is very often reminiscent of a complex sinusoid-like function with 0.2–0.3 Hz frequency. Peaks of various amplitude in SDF correspond to the multiplets with various spike number.

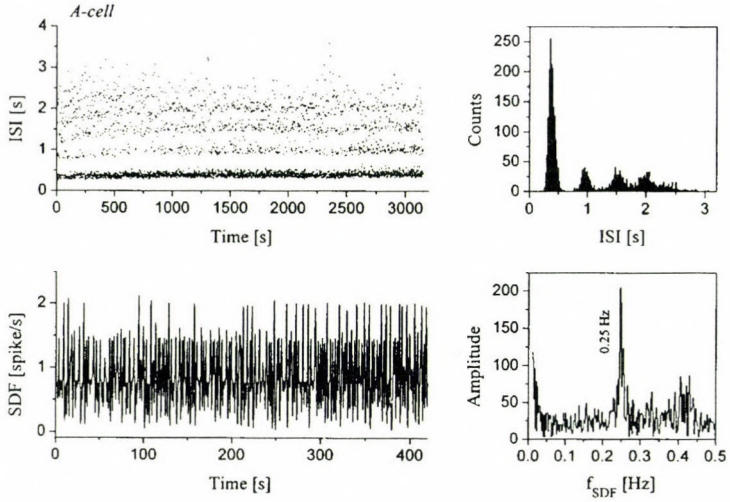

Fig. 4. The temporal properties of the firing patterns of A-cells are summarized. Instantaneous interspike interval (ISI)-plot of the upper left panel is dominated by stripes of point densities. This kind of ISI-plot is typical for regular bursting neurons. The ISI-histogram (non-cumulative) is dominated by equidistant peaks. The SDF graph in the bottom left panel consists of waves of different amplitude associated with the various multiplets. The Fourier spectra (f) on the right reveals 0.25 Hz periodicity in the firing pattern

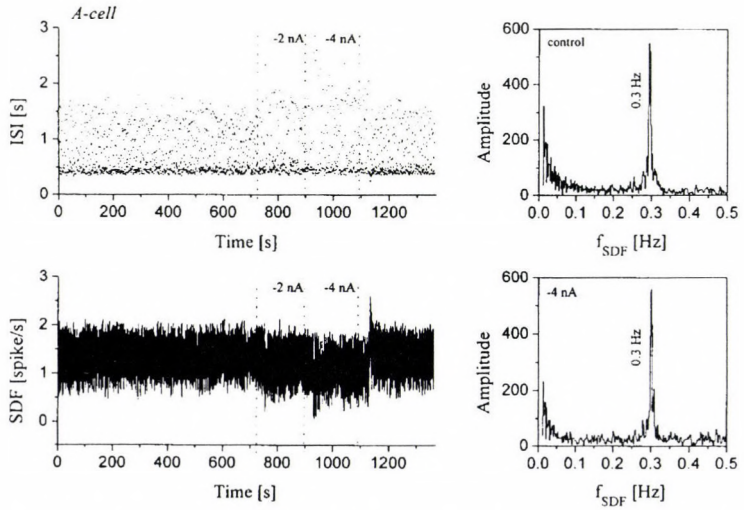

Fig. 5. The effect of intracellular hyperpolarization on the firing pattern of an oscillating A-neuron. Hyperpolarizing current with two different levels (–2 and –4 nA) was applied. Distribution of the interspike intervals (ISI) was slightly altered and the mean firing rate lowered following the current injection. However, the peak of the Fourier spectra (f) remained exactly 0.3 Hz

Effects of electrical and chemical stimuli on the oscillation

When A-cells firing in oscillatory mode were either hyperpolarized or depolarized, their firing patterns were only slightly affected. Although the mean firing rate decreased during a moderate hyperpolarizing current, the oscillation was not eliminated or shifted. This phenomenon is demonstrated in Figure 5 A-cell which was hyperpolarized at t = 750 s and again at t = 930 s of the recording. The overall shape of the SDF remained unaffected. Fourier-spectra calculated from the control part and the hyperpolarized part of the SDF revealed no shift in the frequency of the firing rate oscillation being exactly 0.3 Hz. When A-cells in current clamp conditions were hyperpolarized to a subthreshold level, EPSPs appeared, indicating a periodic excitation reaching the cell being recorded (Fig. 6). Consequently, periodicity observed in the firing pattern of such cells was the result of periodicity in the firing pattern of one or more presynaptic neurons.

In some of our experiments we used low Ca^{2+}- high Mg^{2+} containing extracellular solutions in order to decrease chemical neurotransmission over a large number of neurons. The concentration of extracellular $CaCl_2$ was lowered to 0.5 mM and that of $MgCl_2$ was increased to 5 mM. In the beginning of the experiment the preparation was perfused with normal composition solution, then washed with Ca^{2+}-poor saline. Repression of chemical synaptic transmission did not eliminate the oscillation observed in many cells, while slight depolarizing effect on the cells was typical.

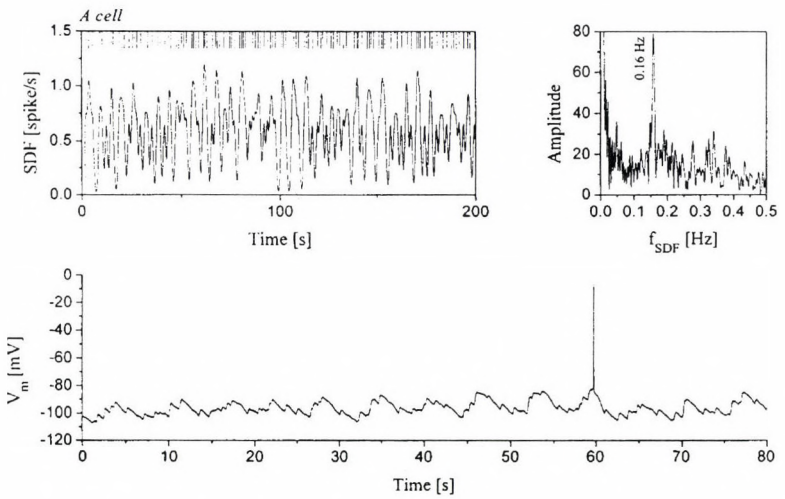

Fig. 6. Oscillatory firing patterns in an A-cell due to periodic excitatory synaptic input. The spike density function (SDF) of the A-neuron and its Fourier spectrum (f) is shown on the upper panels. When the cell was hyperpolarized by intracellular current injection apparent EPSPs were observed (lower panel). As a consequence, the membrane potential of the postsynaptic cell expressed 6 s periodicity. The suprathreshold 0.16 Hz oscillation reflects the periodicity of the EPSPs

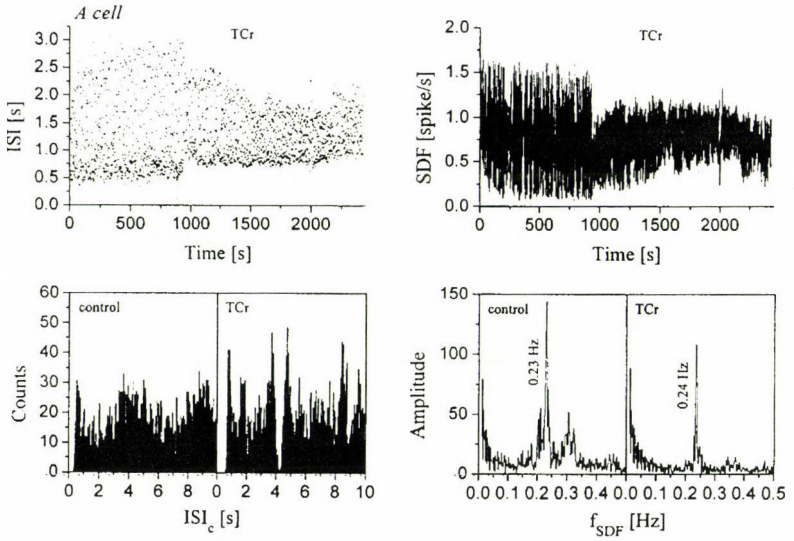

Fig. 7. The effect of D-tubocurarine (TCr) on the firing patterns of an A-cell discharging in oscillating mode. Variations in the interspike intervals (ISI) decreased markedly after the application of TCr. The amplitude of SDF fluctuations also attenuated (upper right). The ISI_c graphs calculated from the control part and the TCr part of the spike train reveals radical transformation in the timing properties of the spike train. At the same time, the network oscillation was maintained and its frequency did not change significantly (from 0.23 Hz to 0.24 Hz)

D-tubocurarine (TCr), an effective blocker for cholinergic neurotransmission was used in other experiments. Evolution of the firing pattern of an A-cell is demonstrated in Figure 7. In the control, A-cell exhibited typical oscillatory firing pattern, then at t = 900 s 0.5 mM TCr was applied. Deviations observed in the interspike-intervals decreased remarkably after the application seen in the ISI-sequence graph. However, clear 0.23 Hz oscillation of the firing rate was preserved as indicated in the Fourier spectra computed from the control part and the TCr part of the record. Interestingly, TCr caused a dramatic alteration in the shape of the ISI-autocorrelalogram. In the control, local maximum in the distribution was seen at $ISI_c = 4.3$ s corresponding to 0.23 Hz oscillation in the firing. When TCr was applied, however, an apparent minimum appeared at the same position. Appearance of sharp peaks and breaks in the ISI_c-histogram might indicate the development of a new kind of firing mode characterized by more accurate timing between distant spikes.

When cadmium, an effective blocker for voltage-gated Ca-channels and chemical synaptic transmission was applied at 0.3 mM concentration oscillation seen in A-cells was completely abolished (not shown). Firing rate of the neuron increased to three times the control level and became monotonic. After washout the firing rate of the neuron decreased nearly to the control level, but no recovery of the oscillation was observed.

DISCUSSION

The methods developed for analysis of spike trains proved to be useful for detecting synchronization of firing in various neurons and for revealing the phasic, correlating events in the firing patterns. The spike density function (SDF) reflects both the activity of the neurons and the synaptic inputs modifying their firing patterns. SDF also allows one to observe slow oscillations in neuronal spike trains, which could be troublesome to detect using other methods. Variations in the firing frequency are induced by a number of internal and external events and factors. Nevertheless, in case of non-coincident synaptic events the resulting alterations of the SDF are usually distinguishable and their temporal distribution and interrelations can be examined [18, 19].

The technique used in our present experiments provides meaningful information on the contribution of synaptic and neurochemical factors in modulating the neural activity. These methods can detect long-term changes in the dynamics of firing, and the reorganization of the synaptic inputs even when the membrane potential of the neuron seems to be unaffected on the short-term. The SDF is especially advantageous in analysis of a large set of spike data, firing corrrelations between nerve cells.

The slow oscillation (at 0.2–0.4 Hz) on the neurons studied has been revealed by SDF-technique and Fourier transform. This slow oscillation proved to be resistant to moderate membrane hyperpolarization and acetylcholine receptor inhibitor (d-tubocurarine), but was eliminated by Ca-channel blocker cadmium. Membrane oscillations with endogenous origin were described in various preparations [5, 6, 11, 14]. Moreover, slow oscillations were demonstrated before triggering action potentials [6] were evoked by modulatory substances [14], or were of endogenous origin [13]. In our experiments, periodically appearing EPSPs especially in the A-cells of right parietal ganglion suggested the presence of a wide-acting synaptic input, which might affect the function of a large number of neurons in the respiratory system of *Lymnaea*. This input differs from those previously described (IP1, IP2, IP3) [3] in several aspects. The presynaptic origin of the detected periodic excitation is unknown at this time.

Although slow membrane oscillations were found in the CNS of both vertebrates and invertebrates and resembled sinusoidal wave forms, their frequency varied across a large scale. The value registered in our studies (0.3 Hz) was the lowest known. The role of slow oscillation was suggested to be responsible for synchronization of neuron firing [13] and in learning [7].

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CHANNEL PROPERTIES OF THE PURIFIED GLUTAMATE RECEPTOR FROM RAT BRAIN RECONSTITUTED IN PLANAR LIPID BILAYER MEMBRANE*

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The properties of the purified rat brain glutamate receptor (GluR), reconstituted in planar lipid bilayer (BLM) were characterised. The single channel currents activated by glutamate and aspartate were similar. The different kinetics of current fluctuation were observed. Paroxysms of channel activity seems to be resulted from the transit of GluR through its active conformation from which it can open several times before desensitising. The effect of concanovaline A (Con A) as an agent blocking desensitisation of glutamatergic synapses was investigated. It was shown that Con A evokes high levels of conductivity and prolonged opening events of channels. Another agent, which stabilises glutamate activated conductivity, dithiothreitol (DTT), evokes "chronic" channel activity. This study demonstrates that purified GluR reconstituted in planar lipid bilayers exhibits the ion-conductivity properties that are associated with the postsynaptic membrane.

Keywords: Neurotoxin – glutamate receptor – membrane conductance – current fluctuations

INTRODUCTION

Much of what is known about the excited membrane function principles has been derived from electrophysiological studies. Sliving as the basis for more high molecular level of investigations. This level involves the studies of the participation some membrane structures in excitability process. The model systems are necessary for these investigation. One of them is BLM, incorporated with specific membrane molecules. The use of such reconstitution allows to investigate the adequacy of the induced single channels of conductivity to that which takes place on biological membranes and the identity of incorporated proteins to native receptors. This approach has been applied in the study of the acetylcholine receptor, its subunits and the excited Na-channel [13, 14, 21]. The purpose of this study is to characterise the properties of the purified rat brain glutamate receptor cation channels, reconstituted in a planar lipid bilayer.

*Dedicated to Professor János Salánki for his 70th birthday.

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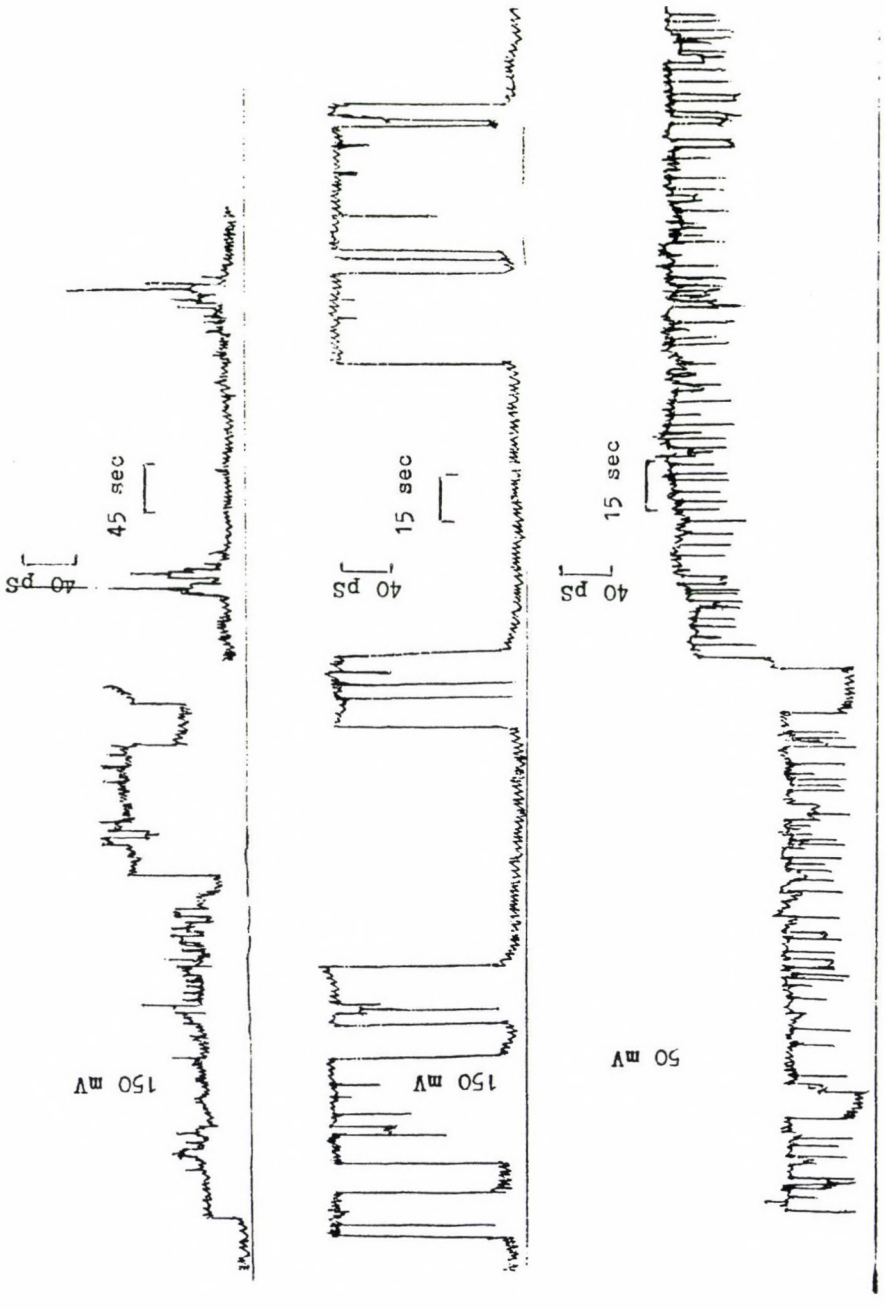

Fig. 1. Current fluctuation observed on modified BLM in the presence of glutamate ($1 \cdot 10^{-4}$ M)

MATERIAL AND METHODS

The rat brain glutamate receptor was isolated by means of affinity chromatography method with neurotoxin fraction of Argiope lobata spider venom as a ligand. The activated polyamine was used as sorbent-carrier. The isolation and solubilization of the membrane fragments, and the preparation of affinity sorbent were described in detail earlier [17, 22]. The planar membranes were obtained by the usual method [12]. Ox brain white matter phospholipids in decan as a membrane-forming solution were spread under water across a circular hole in a teflon septum. To record single channel current fluctuations the area of the hole in the teflon septum was 0.0007 cm^2. In this case a U-1-7 amplifier with an input impedans of 10^{10} om was used. The records were made by means of potentiometer KSP-4.

In all experiments, the water solution contained 100 mmol/l NaCl and 10 mmol/l tris-HCl, pH 7.5 on both sides of BLM the protein content was 10 mg/l.

The protein concentration was determined according to [7]. Ox brain white matter phospholipids were obtained according to [3].

RESULTS AND DISCUSSION

The attempt for purification of glutamate receptore by use of neurotoxin fraction of *Argiope lobata* spider venom as affinity ligand was undertaken as soon as the blocking effect of argiotoxins has been established on glutamatergic transfer [16]. We observed the receptor glycoprotein to be significantly different from the rat brain glutamate binding proteins. These isolated membrane proteins were of low molecular weight (14 kD) [10]. The glycoprotein was the large molecular (220 kD), whose base component is subunit 95 ± 5kD [19]. However, by means of radiation inactivation method it has been established that the molecule with mass of 260 kD corresponds to GluR in rat brain membranes [2]. Later by means of genetic engineering it has been shown that glycoprotein with molecular mass of 90–100 kD is able to induce glutamate-sensitive conductivity of *Xenopus oocytes* injected with rat brain RNA [5].

In our previous works it has been shown that integral conductivity of BLM, incorporated with purified glutamate receptor, can increase to 2–3 orders by the addition of glutamate. This high conductivity state is not stable. The most typical kinetics of glutamate-activated conductivity was spontaneous decrease following its maximum value [17, 18]. This may be a result of the well-known physiological desensitisation. The stable conductivity state of reconstituted system as of native membrane glutamate receptor was provided with Con A. Similar effect obtained in the presence of DTT [20].

Very diverse pictures of current fluctuation can be observed during activation of this system. Typical activations are represented in Fig. 1. It is necessary to note that fluctuation kinetics may be different at the beginning and at the end of experiments going on 1–2 hour at the same condition. It may be proposed that receptors molecule,

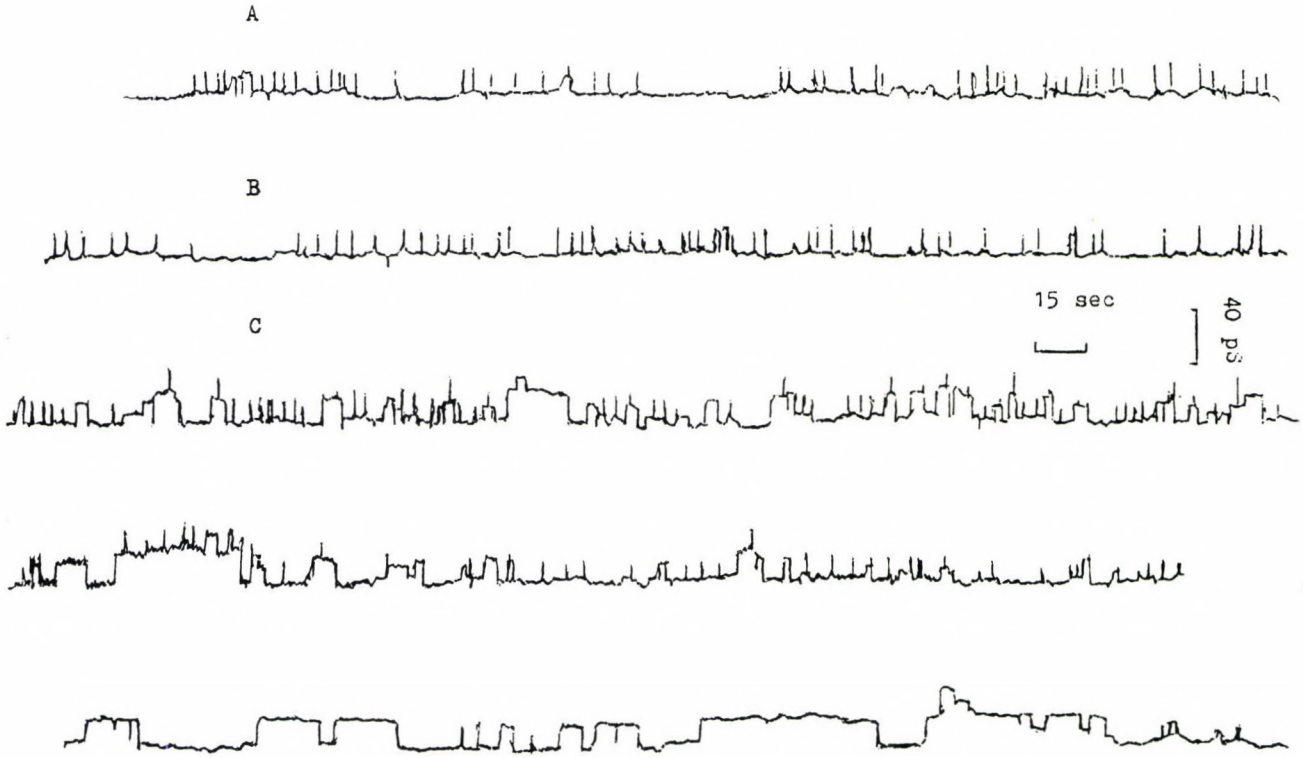

Fig. 2. The channels, evoked on modified BLM in the presence of (A) glutamate ($1 \cdot 10^{-4}$ M); (B) aspartate ($1 \cdot 10^{-4}$ M); (C) glutamate ($1 \cdot 10^{-4}$ M) + Con A ($1 \cdot 10^{-6}$ M)

incorporated in BLM accept various conformations, which determine the conductivity state.

Single fluctuations observed very rarely, are represented in Fig. 2. The amplitude histogram, obtained from this essemble of channels is represented in Fig. 3. The most probable amplitude of channel, activated by glutamate as by aspartate is 24 pS. In the presence of Con A the histogram's maximum is moved and corresponds to 20 pS. In this case, prolonged opening events and higher levels of conductivity are observed. Usually the channels are characterised by maximum level of conductivity, while a very wide spectrum of intermediate levels take place. The transitions between maximum and intermediate levels are criterion of corresponding their to sublevels of the same state. In our case during 1–2 hour interval only intermediate levels of conductivity may be observed (Fig. 2). Similar situation took place for acetylcholine receptor, sublevels have been registrated independent of base state, transition between them were not observed [4]. The observation of a number of glutamate activated channels of reconstituted system allow us to propose that minimum level of channel conductivity is 4 pS. It has been shown that the minimum level of channel conductivity is 5 pS in the CA1 region of hippocumpus [6].

If Con A increases the integral conductivity and promotes the stable active state of glutamate receptors by increasing life time of channels and evoking new, higher levels of conductivity, DTT does not increase integral conductivity and promotes con-

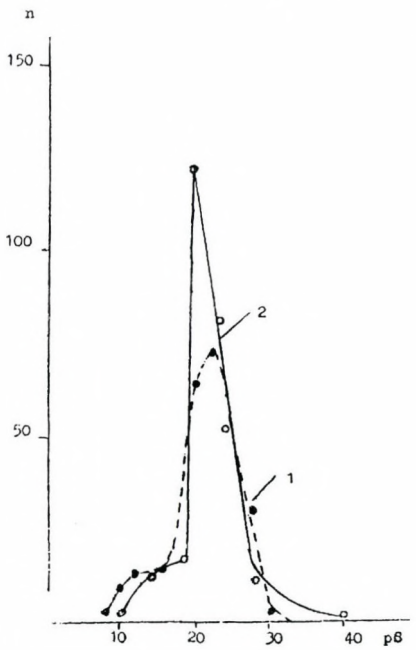

Fig. 3. Amplitude histogram of channels, recorded in the presence of (1) glutamate ($1 \cdot 10^{-4}$ M); (2) glutamate ($1 \cdot 10^{-4}$ M) + Con A ($1 \cdot 10^{-6}$ M)

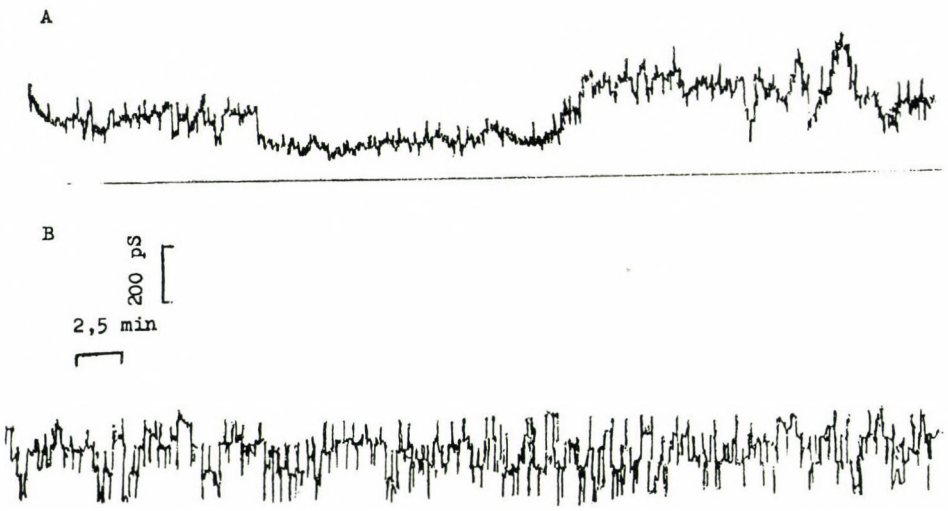

Fig. 4. The DTT influence on current fluctuations, activated by glutamate.
(A) Channels activated by glutamate ($1 \cdot 10^{-4}$ M); (B) Effec of DTT (1 mM) on this channels

ductivity stabilisation only. In this case, the chance meeting current fluctuation recorded as "burst" or "jumps" in the absence of reduced reagent were shown to become strongly marked single channels with more characteristic amplitudes 80, 120, 160 pS. The fluctuations similar to that representing in Fig. 4 (B) may be recorded during 1–2 hours of observation in the presence of 1 mM DDT. These results suggest the possibility of the participation of SH-containing amino acids in neuroreceptor desensitisation process. Linking bromacetylcholine to a sulfhydril group exposed by reduction of the disulphide bond of the acetylcholine receptor results also in a chronically activated channel [1]. Analysis of single channel activity of rat cortical glutamate receptors reveals no change in mean open times or single channel conductance on reduction with DTT but an apparent increase in frequency of single channel activity [9]. Dihydrolipoic acid (DHLA) is another substance that may influence glutamate receptor activity by modifying its redox sites. DHLA potentiates glutamate-mediated whole-cell responses in cultured rat cortical neurones and prevents to increase responses previously potentiated by DTT. Single-channel recording reveals that DHLA produces an increased channel open frequency with no change in single channel conductance or open time [15]. This is emphasizes that DTT and DHLA act only on NMDA-type of glutamate receptors in these both works [9, 15], carried out on cellular membranes. We obtained it with the help of argiotoxins. At a given time it has been shown that argiotoxins interact with all type of glutamate receptors [8].

This study demonstrates that purified GluR, reconstituted in to planar lipid belayer exhibits the ioncondactive properties that are associated with the postsynaptic

membrane. The full cycle of solubilization, purification and reconstitution of GluR can be achieved without impairment of channel function. This artificial system provides a powerful model to investigate the molecular physiology of postsynaptic transfer and to establish structure/function correlates in the GluR molecule.

Isolation of purified functional active membrane proteins does not lose its significance now that molecular genetic study of receptors are available. It has been shown that injection into *Xenopus oocytes* of purified acetylcholine receptor reconstituted in an artificial lipid matrix instead of its coding DNA or RNA will be very useful in cases where changes in the post-translational processing due to the oocyte may result in an absence or alteration of function [11].

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PRESYNAPTIC MODULATION OF TRANSMITTER RELEASE VIA α_2-ADRENOCEPTORS: NONSYNAPTIC INTERACTIONS*

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It is generally accepted that neurochemical transmission occurring at the synapse is the primary way of sending messages from one neuron to another. Neurotransmitters released from axon terminal in a $[Ca^{2+}]_0$-dependent manner act transsynaptically on the postsynaptic site.

The past 30 years have witnessed something of a revolution in the understanding of how neurons communciate with each other. It has been shown that the exocytotic release of transmitters from axon terminals is subject to presynaptic modulation via presynatic hetero- and auto-receptors. For example via stimulation of α_2-adrenoceptors expressed on varicosities and coupled to G-protein the stimulation-evoked release of different transmitters can be inhibited.

This review will focus on nonsynaptic interactions between axon terminals. The present data clearly show that transmitters released from axon terminals without synaptic contact play an important role in the fine tuning of communication between neurons within a neuronal circuit.

Keywords: Transmitter release – presynaptic – α-adrenoceptor – nonsynaptic

INTRODUCTION

The hypothesis that intercellular communication might utilize specific chemical agents, as first suggested by Elliott [18], has now been generally accepted. According to Elliott [19] "In all vertebrates the reaction of any plain muscle to adrenalin is of a similar character to that following excitation of the sympathetic (thoracico-lumbar) visceral nerves supplying that muscle".

The demonstration that acetylcholine (ACh)-mediated transmission from the vagus nerve to the heart, shown by Loewi and his colleagues [35] made this a particularly attractive preparation to provide evidence for chemical transmission and to begin an electrophysiological investigation of neuro-effector transmission. In 1934,

*Dedicated to Professor János Salánki for his 70th birthday.

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Brown and Eccles [9] discovered that if the vagal volley is set up late in a cardiac cycle then that cardiac cycle is not inhibited, the latent period of the inhibition being usually 100 ms to 160 ms. Since Sherrington [46] published his classical work on "Integrative Action of the Nervous System", it has been accepted as a neurophysiological fact [55] that the synapse, the "surface of separation" between neurones, is the primary locus for neuronal information processing. The study of synaptic transmission was revolutionised by the introduction of intracellular recording techniques, which were applied to the neuromuscular synapse by Fatt and Katz [20]. The discovery of quantal release [20], morphological [13] and neurochemical [4] evidence of vesicle was a further step in understanding of synaptic communication. Exocytosis from the axon terminal in the synapse enables transmitters to exercise a highly focal action. When the effector cell (which could be another axon terminal) is closely coupled in time, space and function with the releasing cell, we speak of a conventional synaptic interaction. Chemical transmission creates local potentials at the postsynaptic effector cell that affect the excitability of the target cell. Nerve cells respond to presynaptic nerve impulses with graded postsynaptic potentials. The action of the transmitter is restricted to the subsynaptic membrane.

It has been recently shown that in addition to neurotransmitter substances acting at close range in chemical synaptic neurotransmission, there are chemical interactions between neurons without any close synaptic contact, i.e. interneuronal modulation of transmission which operates over some distance. This would be a transitional form between classical neurotransmission and the broadcasting of neuroendocrine secretion. Transmitters released nonsynaptically may be able to reach remote target cells and control a vast ensembles of neurons [62, 64–68, 70]. Thus, when there is a loose interaction between the target cell and secreting cell this is an unconventional, nonsynaptic communication [12, 62–68].

Nonsynaptic exocytosis of neuropeptide was originally found in *Nereis diversicolor* and then in several other species of annelids [23, 22]. Similarly, nonsynaptic release was found in the CNS of the invertebrate pond snail *Lymnaea stagnalis* [10]. It was suggested that the nonsynaptic release of peptides from large dense-cored secretory granules relates to the slow rate of degradation of neuropeptides and their widespread diffusion and spheric action. Even in nonvertebrates the diffusion of endogenous ligands [53] or any heavy metals as environmental pollution [43–45, 54] plays an important role in modulation of chemical signal transmission.

In the past few years, however, several observations have been reported which suggest how the amount of transmitter released at nerve terminals may be modulated, either by a transmitter/modulator released from another nerve terminals, i.e. interneuronal modulation via activation of heteroreceptors [62], or by an autoinhibition mechanism where the transmitter released into the synaptic cleft inhibits its own release via activation of autoreceptors [31, 49–50, 62].

PRESYNAPTIC MODULATION

The term presynaptic modulation means an inhibition of the excitation of a transmission signal which results from a change of transmitter release rather than from a decrease in the postsynaptic sensitivity of effector cells to transmitters. The first evidence for the existence of presynaptic inhibition came from electrochemical studies [38]. In the early 1950s, Fatt and Katz observed that the depression of the excitatory junction potential due to stimulation of the inhibitory input was not fully accounted for by changes in postsynaptic permeability. In 1961, evidence was provided [16], that in mammals, and in crustaceans the action of the inhibitory nerve can also be exerted on excitatory axon terminals, reducing the release of the neurotransmitter.

1. Synaptic (conventional). The inhibitory neuron forms a synapse on the initial segment of the axon, or on its terminals, depressing or inhibiting transmitter release from the neuron.
2. Nonsynaptic (unconventional). Here the inhibitory or excitatory transmitter is released from axon terminals at a distance from effector cells that are devoid of synaptic morphological characteristics. The transmitter diffuses over some micrometers to exert its inhibitory or excitatory effect on a neighboring neuron. The target may be the initial segment, the axon terminals, the varicosity, and thereby only transmitter release is affected and not the sensitivity of the effector cell.

PRESYNAPTIC α-ADRENOCEPTORS

α-Adrenoceptors on cholinerg axon terminals were detected in the late sixties [3, 40, 59], followed by the recognition in seventies that sympathetic noradrenergic axon terminals also possess α-adrenoceptors [32, 49]. It has been shown that stimulation of these presynaptic α-adrenoceptors leads to a reduction of transmitter release. In addition, β-adrenoceptors at the noradrenergic axon [1, 51] can facilitate the release of noradrenaline (NA).

The discovery that there are significant differences in the pharmacological properties of pre- and postsynaptic α-adrenoceptors led to the suggestion that they be designated as α_2 and α_1-respectively. In 1972, Starke [48] stated that receptors influencing the secretion of NA are not identical with the myocardial phenylephrine receptors. Recent evidence indicates, however, that the receptor populations located pre- and postsynaptically are not homogenous; for example, α_2-adrenoceptors were found to be also present postsynaptically. Therefore, the classification of α-adrenoceptors into α_1 and α_2 groups cannot be used to reflect morphological locations and physiological functions of the receptors.

The question then, is: Is the so-called "negative feedback" mechanism a real modulation? If it just reduces the release of NA evoked by subsequent stimuli, the answer is no. Its real function is to keep the output of NA from a single neuron constant. However, when NA released from one neuron inhibits, for example, the release of

ACh from another neurone, it is a real modulation. However, the extent of modulation (reduction of ACh release) depends on the concentration of NA in the receptor area, which is, in fact, mainly influenced by the firing rate and length of noradrenergic fibres firing in concert, even though release is kept constant by "negative feedback".

α-ADRENOCEPTORS

In addition, it was also shown that the partial removal by 6-OH-dopamine [30] or by reserpine pretreatment [40] of the adrenergic restraint leads to an enhanced release of ACh. These facts support the hypothesis [61] that the release of ACh is continuously controlled by NA released from adjacent nerves. Sympathetic control of ACh release can be viewed as a kind of presynaptic inhibition, and when compared with an antagonism at the effector level, offers the physiological advantage of economy in transmitter release. This conclusion provides complete functional corroboration of the anatomical demonstration by Jacobowitz [27] that the noradrenergic fibers are located next to the cholinergic fibers without directly innervating the smooth muscle. In the Auerbach plexus no-axo-axonal contacts were found to be present between noradrenergic and cholinergic neurons.

Electrophysiological evidence [39] has also been obtained which confirms the previous results: NA acts presynaptically when it reduces the release of ACh from the Auerbach plexus. Direct intracellular recordings from myenteric neurones in the guinea-pig have shown that both noradrenaline [39] and sympathetic nerve stimulation reduce or suppress excitatory postsynaptic potentials without affecting the electrical properties of the postganglionic neurones.

In addition, evidence has been presented that in the cerebral cortex the release of ACh is continuously controlled by NA released from locus coeruleus nerve terminals [64]. In our earlier experiments [60, 61] it has been found that NA added to the bathing medium reduces the release of ACh from cerebral cortex slices evoked by ouabain.

The fact that phentolamine, an α-adrenoceptor blocker, prevented the effect of NA [61] indicated that its effect is mediated via α-adrenoceptors. When the noradrenergic input was somehow impaired, either by phentolamine [60], by inhibition of the effect of NA on cholinergic axon, or by 6-OH-dopamine pretreatment [14], or by ipsilateral locus coeruleus lesion the release of ACh from cortical slice of the rat was enhanced. The fact that the removal of noradrenergic input results in an enhanced rate of ACh release indicates that cholinergic neurons in the cortex are continuously controlled by NA released from remote noradrenergic varicosities. Unilateral locus coeruleus lesion resulted in an enhanced release of ACh from ipsilateral side, however, the release from contralateral side did not differ significantly from the value obtained in slices dissected from non-lesioned animals.

The electrophysiological findings that the administration of iontophoretic NA [11] and the stimulation of the locus coeruleus inhibits the neuronal firing in the cortex are in line with the neurochemical observations.

Since it is generally agreed that the neocortical noradrenergic innervation originates in the locus coeruleus in the pons and lesions of the nucleus results in a marked decrease of histochemically demonstrable axonal varicosities and of biochemically measurable NA, it is suggested that coerulo-cortical noradrenergic fibres exert a presynaptic inhibitory effect on the cortical cholinergic neurons.

It was shown that in rat cerebral cortex a high proportion of noradrenergic axon terminals is non-synaptic [15]: among 1835 noradrenergic terminals examined only 61 – less than 5% – exhibited junctional complexes of morphologically defined synaptic contacts as opposed to 50% of unlabelled boutons similarly sampled in the neighbouring neuropil [15].

It has been established that presynaptic inhibitory α_2-adrenoceptors exist on serotonergic nerve terminals of the rat [24, 34] and of the human neocortex [42]. These α_2-heteroreceptors are physiologically relevant, i.e. can be activated by endogenous NA released from neighbouring noradrenergic nerve endings [21].

Table 1 shows the subtypes of α_2-adrenoceptors involved in presynaptic inhibition of transmitter release. Activation of α_2-adrenoceptors results in an inhibition of Ca^{2+}-entry, increase of K^+ permeability and hyperpolarization [5].

Table 1

Subtypes of presynaptic α_2-adrenoceptors expressed on axon terminals whose stimulation results in an inhibition of transmitter release

		Subtype of α_2-adrenoceptors	References
Noradrenergic			
Hippocampus	(rat)	α_{2A}	29
Hypothalamus	(rat)	α_{2A}	28, 47
Cortex	(rat)	α_{2A}	34
	(human)	α_{2A}	41
Spinal cord	(rat)	α_{2A}	58
	(human)	α_{2A}	33
Ileum	(guinea-pig)	α_{2B}	7
Thymus	(rat)	α_{2B}	25
Spleen	(rat)	α_{2B}	17
Heart	(guinea-pig)	α_{2B}	Vizi, E. S., unpublished
Cholinergic			
Auerbach plexus	(guinea-pig)	α_{2A}	6, 7
Cortex		α_2	64
Serotonergic			
Synaptosome	(rat-brain)	α_{2A}	36, 26

The presynaptic interaction [70] between the noradrenergic, serotonergic and cholinergic system could explain how a selective NA reuptake blocker by increasing the biophase concentration of endogenous NA at α_2-adrenoceptors expressed on varicosity, may at the presynaptic level, reduce the function of the 5-HT or ACh system. The serotonergic neuron has been related to suicidal behavior [2]. Such an NA-induced decrease in the function of 5-HT system may prevail during the initial phase of treatment with a blocker of NA transporter that is assumed to be dangerous in terms of suicidal tendencies when, among other pathophysiological changes, the α_2-heteroreceptors are still up-regulated after a previous lack of the corresponding transmitter.

In summary, since the first suggestion, the physiological and pharmacological importance of nonsynaptic chemical signal transmission between neurons via presynaptic sites seems to achieve growing recognition.

While the $[Ca^{2+}]_0$-dependent release of transmitter can be presynaptically modulated, the carrier-mediated release of transmitter [56, 68] is not subjected to presynaptic modulation.

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COMPARISON OF THE ENDOGENOUS HEPTAPEPTIDE Met-ENKEPHALIN-Arg^6-Phe^7 BINDING IN AMPHIBIAN AND MAMMALIAN BRAIN*

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In previous communications [4, 38] we published that [^{3}H]Met-enkephalin-Arg^6-Phe^7 (MERF) binds to opioid (kappa$_2$ and delta) and sigma$_2$ sites in frog and rat brain membrane preparations, however no binding to kappa$_1$ sites could be established. In the present paper we compare the frog, rat and guinea pig brain membrane fractions with respect to their MERF binding data. No qualitative differences were found between the three species but specific binding of labelled MERF was maximal in frog brain and lowest in guinea pig brain, which corresponds to their kappa$_2$ opioid receptor distribution. The naloxone resistant binding was also present in all investigated species and varied from 25% in frog and guinea pig cerebrum, to 50% in rat cerebrum and cerebellum, but no naloxone inhibition was found in guinea pig cerebellum where no kappa$_2$ opioid receptors have been found. The presence of sigma$_2$-like receptor was demonstrated in each investigated membrane fraction with displacement experiments using (–)N-allylnormetazocine as competitor of tritiated MERF. It was shown that this site was responsible for 60–80% of [^{3}H]MERF binding. The remaining part of the naloxone resistant labelled MERF binding could be displaced only with endogenous opioid peptides as met-enkephalin, dynorphin and β-endorphin. The eventual physiological role of multiple MERF receptors is discussed.

Keywords: Met-enkephalin Arg^6-Phe^7 – radioligand binding – frog – rat – guinea pig

INTRODUCTION

Met-enkephalin-Arg^6-Phe^7 (MERF) is an endogenous peptide derived from Proenkephalin A and was first isolated from bovine adrenal medullary granules and from striatum [32]. The peptide occurs not only in the central nervous system of different mammals [30, 33, 10] but also in amphibian brain [15]. Among its pharmacological actions a naloxone reversible antinociceptive effect was observed in mice after intracerebroventricular administration [13].

Binding experiments have been performed using labelled EKC (ethylketocyclazocine), diprenorphine or etorphine displacing with unlabelled MERF in spinal cord

*Dedicated to Professor János Salánki for his 70th birthday.

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membrane preparations of guinea pig and rats [1, 11], but no direct measurements with labelled MERF have been reported. In these competition experiments the opioid binding site of MERF has been named "kappa$_2$" because the K_d value of MERF increased substantially in the presence of 5 μM DALE (D-Ala^2,Leu^5-enkephalin) in contrast to displacements with unlabelled dynorphin which was not DALE-sensitive. These competition experiments with unlabelled MERF were proved also in amphibian and mammalian brains with roughly similar data, although differences between the species were also established [2].

After the cloning of the main types of opioid receptors, delta, mu and kappa [14, 34, 20] the primary amino acid sequence of these proteins became known, but none of the putative subtypes (delta$_2$, mu$_2$, kappa$_2$) were cloned until now. Nevertheless, a new, opioid-like receptor called nociceptin or orphanin FQ receptor was discovered by molecular cloning [21], which proved to be surprisingly naloxone-insensitive although displaying 63–65% homology with the known opiate receptors. It showed a unique ligand specificity toward a new endogenous heptadecapeptide called nociceptin or orphanin FQ [21, 29], which had a somewhat similar amino acid composition to dynorphin$_{(1-17)}$, the endogenous ligand of the kappa opioid receptor, but displayed a nociceptive effect in low doses unlike the other opioid peptides.

Recently multiple opioid receptor-like genes were identified and compared in diverse vertebrate phyla [18]. No orphanin FQ receptor could be cloned from frog, but the kappa opiate receptor from this species *(Rana catesbiana)* proved to be in certain aspect similar to the orphanin FQ receptor having a negatively charged amino acid residue at position 28 (which is valine in all the mammalian kappa receptors), but is present in all known orphanin FQ receptors. Therefore it was of interest to compare the binding of the alledged kappa$_2$ ligand MERF in the three different species.

MATERIALS AND METHODS

Peptide synthesis and radiolabelling of MERF was performed as described earlier [4, 35, 38]. The following opioid peptides were applied: DAMGO, DALE, dynorphin$_{(1-13)}$, β-endorphin and DSLET (Bachem, Switzerland). DPDPE, nociceptin and Met-enkephalin were prepared in our laboratory by solid phase synthesis. Synthetic alkaloids used were: EKC, bremazocine (Sterling-Winthrop N.Y.), levorphanol, dextrorphan (Roche, N.J.), U-69,593 (Upjohn, MI), naloxone (Endo Labs N.Y.). Norbinaltorphimine was a gift from P.S. Portoghese (University of Minnesota, Minneapolis, MN). Naloxone-benzoylhydrazone was kindly provided by G.W. Pasternak (Cotzias Lab. SKCC, N.Y.), (+)- and (–)N-allyl-N-normetazocine (SKF 10,047) were a generous gift from NIDA, NC, naltrindole was a gift from S. Hosztafi (Alkaloida, Tiszavasvári), morphine was obtained from the pharmacy of the Medical University (Szeged). All other chemicals were obtained from Sigma Chemicals, the purity was of analytical grade.

Membrane preparations from frog *(Rana esculenta),* rat and guinea pig brain were performed according to Pasternak [24]. Radioligand binding studies were conducted in 50 mM Tris-HCl buffer, pH 7.4, supplemented with peptidase and proteinase inhibitors as previously used [4, 38] in order to prevent enzymatic degradation of [^{3}H]MERF during incubation. The final reaction volume was 1 ml and contained 300 μg protein. Incubation (0 °C, 40 min) was terminated by rapid filtration under vacuum followed by washings with 2×10 ml ice-cold Tris-HCl buffer through Whatman GF/C glass fiber filters using a Brandel M24R cell harvester. Radioactivity was counted in a toluene-based scintillation cocktail by a Wallac 1409 spectrophotometer. Nonspecific binding was defined as the bound radioactivity in the presence of 10 μM unlabelled MERF, when not otherwise stated. Specific binding of [^{3}H]MERF did not change significantly in the presence of 1 mg/ml BSA in the reaction mixture nor after using filters preincubated in 0.3% polyethyleneimine. All assays were performed in duplicate and repeated several times. Protein concentration was measured according to Bradford [6] using bovine serum albumine as standard.

Data analysis was made by nonlinear least square fitting programs. Homologous competition data were calculated by the LIGAND program considering models of one and two binding sites [23]. Heterologous competition experiments were analyzed by the GraFit program using the "four parameter logistic" fitting option for sigmoid displacement curves [17].

RESULTS

When equilibrium binding data of the labelled heptapeptide were compared in brain membrane preparations of the three species examined, the following rank order could be established for the maximal binding capacity of [^{3}H]MERF: frog > rat > guinea pig, however ligand affinity (K_d) values varied in the following way: frog > guinea pig > rat (Table 1).

Investigating the number of binding sites of [^{3}H]MERF multiple sites could be found with displacement methods, but using different opioid ligands as naloxone, levorphanol and dextrorphan it became evident that only about half of the binding

Table 1
Equilibrium binding parameters for [^{3}H] MERF binding in different tissues

	Frog	Rat	Guinea pig
K_d (nM)	3.6 ± 1.7	9.9 ± 1.3	8.6 ± 1.7
B_{max} fmol/mg protein	630 ± 28	503 ± 16	256 ± 22

Equilibrium parameters for [^{3}H]MERF specific binding at 1 nM labelled ligand concentration were determined by homologous competition experiments using twelve concentrations (10^{-12}–10^{-5} M) of unlabelled MERF. Data were evaluated by LIGAND analysis. Values represent the mean of ± S.E.M.

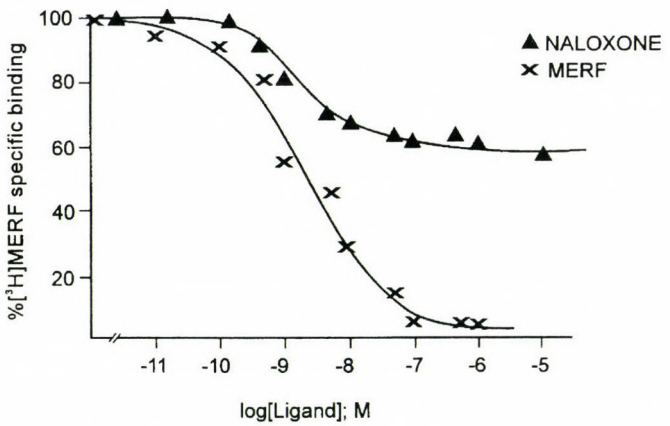

Fig. 1. Displacement of 1 nM [^{3}H]MERF binding to rat brain membrane preparation with various concentrations of unlabelled MERF and naloxone

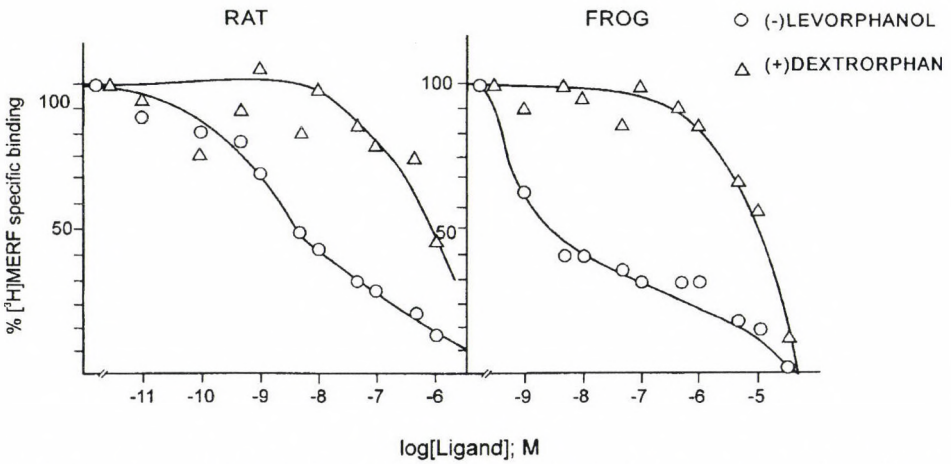

Fig. 2. Displacement of 1 nM [^{3}H]MERF binding to rat and frog brain membrane preparations with various concentrations of unlabelled levorphanol and dextrorphan

sites were displaced by these compounds (Figs 1, 2). The opioid binding site can be considered as high affinity site using benzomorphans and endogenous opioid peptides. The low affinity site is probably not of opioid character. This lower affinity site, which is in the micromolar region did not appear in the ligand saturation experiments, because the applied concentration of [^{3}H]MERF did not exceed 100 nM. None of the investigated species showed displacement of labelled MERF binding applying U-69,593 or U-50,484 in the nanomolar range (Fig. 4 and Table 2), which

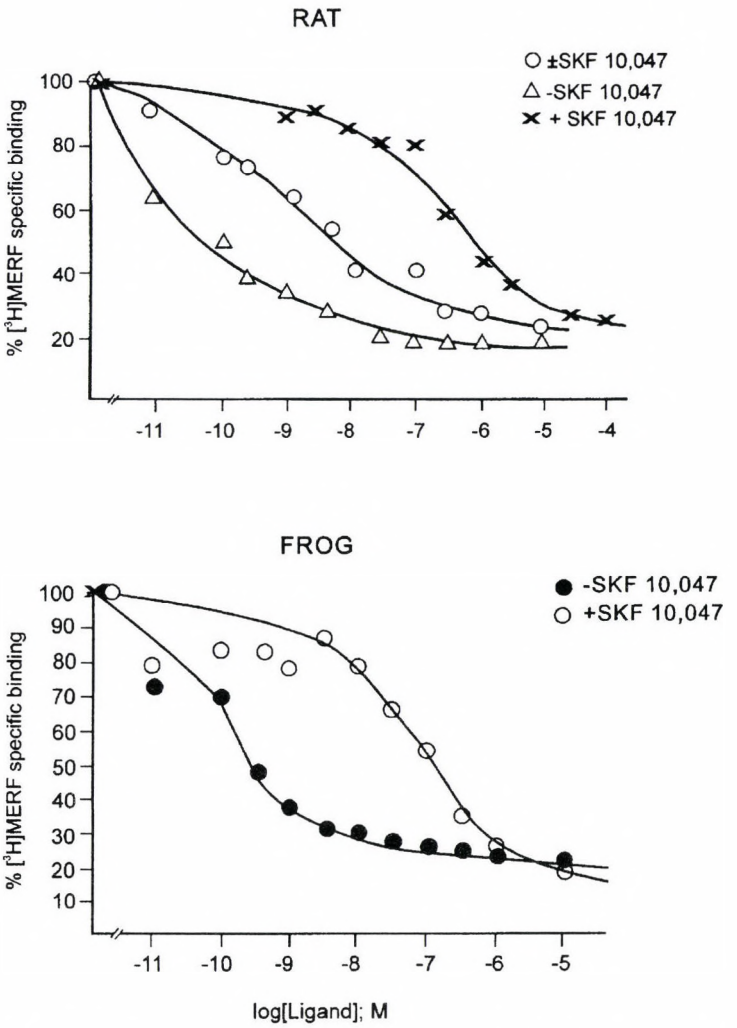

Fig. 3. Displacement of 1 nM [³H]MERF binding to rat and frog brain membrane preparations with various concentrations of unlabelled SKF 10,047 (N-allyl-normetazocine)

are considered as specific kappa$_1$ agonists, however nor-binaltorphimine and dynorphin which are not as specific for kappa$_1$ receptor as the former ligands, displayed higher affinities, although differences between the species were established (Table 2). Among the other unlabelled compounds used, delta agonist (Met-enkephalin) and antagonist (naltrindole) proved to be generally better competitors than mu opioid ligand (DAMGO), but the more specific the delta opioid agonist (DSLET, Table 2) is, the less affinity was observed using MERF as labelled ligand

Table 2
Inhibition of [^{3}H]MERF binding to frog, rat and guinea pig brain membranes by various ligands

Unlabelled ligand	Ki (nM)		
	frog	rat	guinea pig
Naloxone	120	1.4	1.9
Bremazocine	0.46	4.6	3.4
Levorphanol	2.57	4.8	3.1
Nor-binaltorphimine	7.8	78	63
Ethylketocyclazocine	44	16.4	50
(–)N-allyl-normetazocine	0.33	0.43	5
(+)N-allyl-normetazocine	130	1 721	2 660
Met-enkephalin	24	26	46
Dynorphin(1-13)	70	11.8	34
β-Endorphin	42	29	22
Naloxone-benzoylhydrazone	0.3	0.6	–
U-69,593	38 700	>7 000	>7 000
DAMGO	7 000	109	1 100
DSLET	560	212	199
Naltrindole	0.88	58	18.6

Brain membranes were incubated with 1 nM radioligand in the presence of various concentrations of opioids for 40 min at 0 °C. K_i values were determined by LIGAND analysis. Each value represents the mean of several analysis in which the coefficient of variation was below 0.4.

in the observed species. Naloxone benzoylhydrazone a nonselective opioid ligand at kappa and mu opioid receptors [27] had good affinity in the investigated species (Table 2).

Optically pure stereoisomers of benzomorphan distinguish between opioid and nonopioid sites. The benzomorphan (–)N-allyl-normetazocine (SKF-10,047) reveals high affinity for opioid sites, whereas the (+)enantiomer has good affinity to the sigma1 receptor site [28].

$Sigma_2$ sites are characterized by higher affinity for $sigma_2$ ligands than for $sigma_1$ and none of them were blocked by naloxone. Labelled MERF was displaced by (–)SKF-10,047 with high affinity in the three investigated species and (+)SKF-10,047 with very low affinity (Fig. 3, Table 2). Interestingly the high affinity site was only partially inhibited by naloxone and in the guinea pig cerebellum where no $kappa_2$ opioid receptors are present [40], naloxone did not inhibit at all MERF binding [5]. Hence the SKF-10,047 displacement of labelled MERF can be classified at least partially as $sigma_2$-like.

Endogenous peptides are fairly good competitors of labelled MERF binding: dynorphin$_{(1-13)}$ had highest affinity in rat brain membranes followed by guinea pig brain membranes, lowest affinity was measured in frog brain membranes (K_i: 11.8 nM > 34 nM > 70 nM). Using β-endorphin the rank order of MERF competition was somewhat similar (K_i 29 nM in rat 22 nM in guinea pig and 42nM in frog). Met-enkephalin affinity was alike in rat and frog brain membranes (K_i 26 resp. 24 nM), and somewhat less in guinea pig brain membrane fraction (46 nM). Nociceptin or orphanin FQ displaced also labelled MERF but K_i was above 100 nM in all investigated species (data not shown). Interestingly the displacement curve with the endogenous peptides were complete unlike those of naloxone competition (Fig. 5). Therefore we must suppose that the investigated endogenous peptides bind also to a nonopioid site.

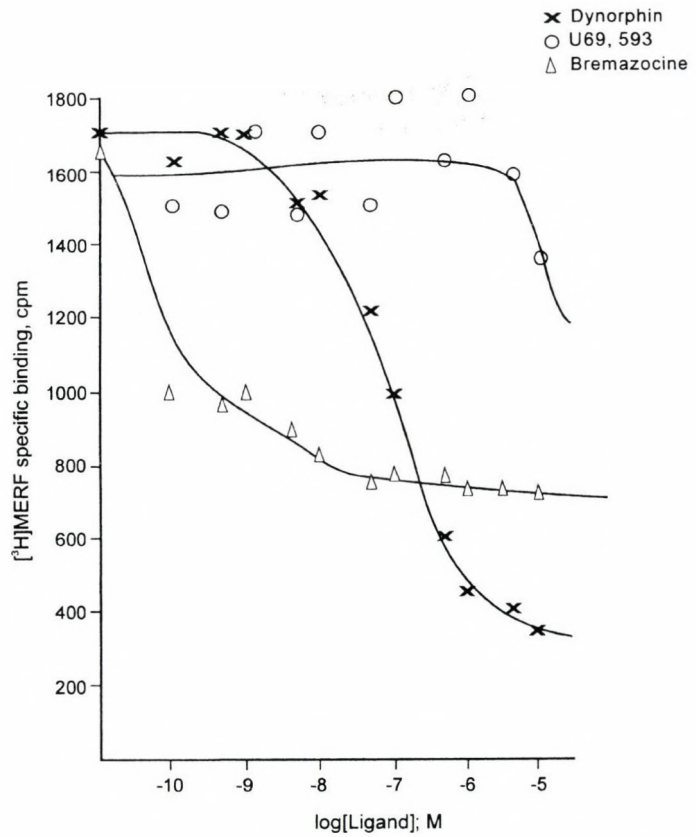

Fig. 4. Displacement of 1 nM [³H]MERF binding to frog brain membrane preparations with various concentrations of unlabelled U 69,593, dynorphin and bremazocine

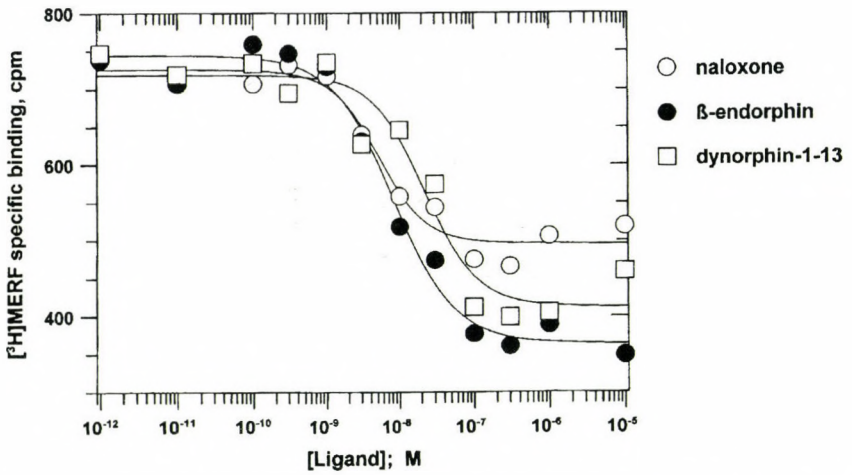

Fig. 5. Displacement of 1 nM [^{3}H]MERF binding to guinea pig brain membrane preparation with various concentrations of unlabelled naloxone, β-endorphin and dynorphin

DISCUSSION

The aim of this work was to compare the receptors responsible for binding of an endogenous peptide MERF in frog, rat and guinea pig brain membranes. We succeeded to establish that beside the well-known opioid receptors: mu, delta, kappa, also nonopioid receptors are involved in binding.

Among the binding data of MERF to opioid subtypes, kappa$_1$ agonists were the weakest competitors in all three species. This was not surprising in frog brain where we could not demonstrate earlier stereospecific binding with labelled [^{3}H]U-69,593 [3] and as it was published [18], the cloned frog kappa opioid receptor differed substantially from the mammalian subtype, resembling more to the nociceptin receptor, than any other kappa receptor. In contrast to these effects no subtype selectvity but relatively high affinity has been reported using subtype specific labelled agonists in cloned and expressed mu, delta and kappa opioid receptors from rat brain with unlabelled MERF as competitor [19]. The reason for this discrepancy is not yet clarified [39].

In frog, rat and even in guinea pig brain labelled MERF binding could be displaced only using μM concentrations of kappa$_1$ agonists, whereas benzomorphans (EKC > bremazocine > N-allyl-normetazocine), which are known to bind also to kappa$_2$ sites are effective in nanomolar and subnanomolar concentrations (Table 2). Among benzomorphans the most potent competitor was (–)N-allyl-normetazocine in frog and rat brain followed by guinea pig brain, however the (–)SKF-10,047 competition could be only partially attributed to kappa$_2$ opioid receptor binding of labelled MERF, because naloxone was not effective in inhibiting [^{3}H]MERF binding in

guinea pig cerebellum, where kappa$_2$ sites were absent [40], nevertheless (–)SKF-10,047 inhibited in a stereospecific manner MERF binding [5].The naloxone-resistant part of (–)N-allyl normetazocine can be classified as sigma$_2$-like site although it has a higher affinity to this receptor, than the originally described sigma$_2$ site [28]. The nonopioid binding sites of labelled MERF can be also distinguished by the competition curves of levorphanol and dextrorphan where differences between high affinity stereospecific binding is clearly distinguished from low affinity less stereospecific competition (see Fig. 2).

Nonopioid effects of endogenous peptides as dynorphin [31] and β-endorphin [12] has been described in the literature. The observed effects of dynorphin are mediated partly through kappa$_2$ and partly directly through NMDA receptors in guinea pig and rat brain [7, 8]. Direct specific binding of labelled dynorphin to cells expressing NMDA receptors was also recently established [26].

What is the physiological function of kappa$_2$ receptors, sigma$_2$ receptors and nonopioid effects of opioid peptides? The kappa$_2$ effects are claimed as "neuroprotecting" [16]. The function of sigma$_2$ receptor is poorly established but motor effects [37], anxiolytic activity [25] and potentiation of NMDA receptors [9] has also been reported.

In conclusion, we can say that all these receptors were present in the three species, but their structure is somewhat different, although tritiated MERF acted on all three kinds of receptors. Based on these binding experiments the physiological action of MERF will be investigated in the future.

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THE CHRONOINOTROPIC EFFECTS OF NEW REGULATORY INPUT TO THE HEART OF LAND PULMONATES*

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The postjunctional potentials and chronoinotropic reactions of the heart evoked by activation of multimodal neurons and/or left pallial nerve were investigated in three species of land pulmonates: *Achatina fulica* Ferrussac, *Helix lucorum* L., *Arianta arbustorum* L. Both spikes of giant homologous neurons (d-VLN, d-RPLN by *A. fulica,* command neurons of pneumostoma LPa3, RPa3 by *H. lucorum)* and stimulation of the peripheral end of the left pallial nerve evoked the similar biphasic inhibitory-excitatory junction potentials in the heart, in mantle muscles and in different parts of visceral complex. The positive chronoinotropic effects of this input in the hearts of whole-mount preparations were modified due to interaction with well-known neural cardioregulating network of the system of intestinal nerve.

Keywords: Multimodal identified neurons – heart regulation – neural networks – land mollusks – snail

INTRODUCTION

In experiments on new whole-mount preparations with intact neuroeffector connections it was found that the two largest neurones in the visceral and right parietal ganglia of the giant African snail *A. fulica*, d-VLN and d-RPLN, both of which have been well-investigated previously, have direct connections with the heart, mantle muscles and some visceral muscles [17]. These multimodal neurones evoke in all muscles the discrete biphasic inhibitory-excitatory junctional potentials (I-EJPs). The burst of spikes in d-VLN and d-RPLN are followed by strong contractions of many extracardial muscles and by inhibition of heart beats.

Comparison of basic morphofunctional characteristics has shown shared homology among the giant neurons d-VLN and d-RPLN of *A. fulica*, command neurons of pneumostoma LPa3, and RPa3 of *H. lucorum* [6, 10]. The probable existence of direct connections between the heart and pneumostomal command neurons has been

*Dedicated to Professor János Salánki for his 70th birthday.

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demonstrated [7, 14]. However, the cardioregulating function of these cells remained unclear, because the distinct chronoinotropic reactions during discharges of command neurones were not registered.

Giant neurons d-VLN and d-RPLN by *Achatina*, and LPa3 and RPa3 by *Helix*, do not send axons into intestinal nerve, the main cardioregulating input by molluscs. Both the morphological data and preliminary physiological experiments have shown that the main route of this neuronal system is the left pallial nerve. The connections between the heart and left pallial nerve by *Helix* were assumed for a long time, but the functional significance was unclear [11].

In this study we have simultaneously investigated both the electrical and the mechanical effects in the heart evoked by giant neurons and left pallial nerves in three land snails. The aim of this paper is to compare the neuron-effector interactions of homologous cells of phylogenetically related animals to understand the mechanisms of interactions of cardioregulating inputs through the intestinal and left pallial nerve.

MATERIALS AND METHODS

The experiments were carried out on adult giant African snails *Achatina fulica* Ferrussac, edible snail *Helix lucorum* L., and *Arianta arbustorum* L. (*Gastropoda, Pulmonata, Stylommatophora*). The two first species are used very widely in experimental biology. The elegant *A. arbustorum* L. (*Helicidae, Ariantinae*) represents, in our opinion, a promising new model for neurophysiological investigation. The giant African snails were cultivated under laboratory conditions, while other species were taken from natural populations.

Preparation

The animals were anaesthesized by succinylcholinechloride (0.1 ml of 1% solution per 10 g of full body weight) [2]. Further preparations were made in the modified physiological solutions for *Achatina* [5] and for *Helix* [16]. Tris-buffer in these solutions was substituted for HEPES, pH adjusted to 7.6 by NaOH. For *A. arbustorum* the *Helix*'s solution was used.

During preparation only one incision of the body wall from the head to the mantle edge was made. The crop and some muscles were carefully shifted with the small loops. A special fork from stainless-steel was inserted to pull apart the muscles surrounding the suboesophageal ganglia. The fork consisted of two lower (I and II, see Fig. 1A) teeth and one upper tooth (III).

The lower pair of teeth was situated under the parietovisceral ganglionic complex between the pedal nerves, and the upper tooth above the visceral nerves. The upper tooth could be raised and lowered and was used for fixation of the ganglionic complex. It was possible to turn the fork around on a longitudinal axis for the study of various zones of the dorsal surface of the ganglia.

After preparation the solution in the chamber was refreshed. In the absence of strong external disturbances it was possible to carry out the long-lasting intracellular recording in this whole-mount preparation.

The intracellular recordings from giant neurons were made with bevelled electrodes having a tip diameter about of 2–3 m (1–5 MOhm). A special electromechanical device to advance the microelectrodes for impaling the ganglionic sheeth was used [15].

Registration of electro- and mechanograms of muscles

For simultaneous registration of monophasic potentials and mechanical strength of the heart the combined transducer was used. The polished glass pipette connected to the suction electrode [9] was fixed on a mechanotransducer. This construction allowed us to register the electro- and mechanograms from the same point of the heart for an extended period (Fig. 1B).

For registration of extracellular electrograms of visceral and mantle muscles the bevelled glass pipettes about 1 cm long with 100–200 μ of tip diameter were used. The pipettes were connected to an amplifier by thin flexible tubes. These macroelectrodes were inserted by hand into muscles to a depth of approximately 1–3 mm.

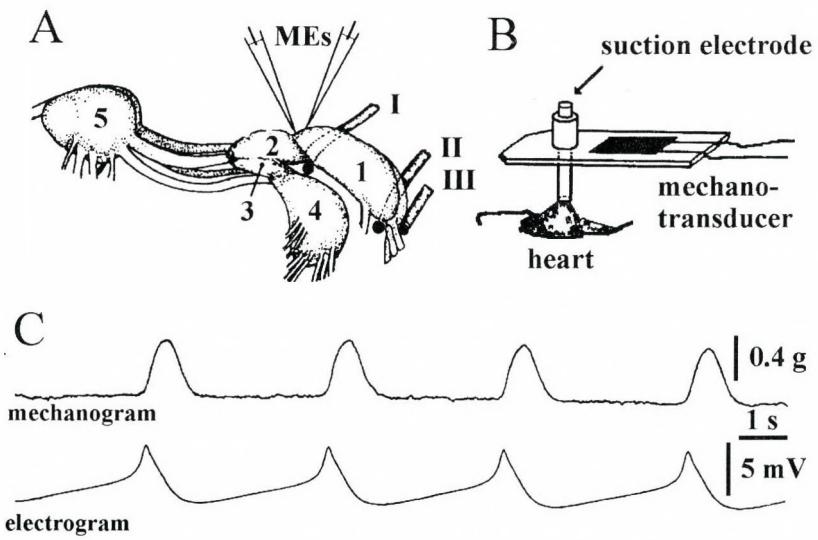

Fig. 1. **(A)** The ganglionic complex of *A. fulica* fixed on the fork, left side view. I, II – lower teeth, III – upper tooth; (1) – visceral ganglion, (2), (3), (4) and (5) – left parietal, pallial, pedal and cerebral ganglia, respectively; MEs – intracellular microelectrodes. **(B)** Suction electrode combined with mechanotransducer for the simultaneous registration of the electro- and mechanograms of the heart **(C)**

RESULTS

The method of combined registration of the electrical activity and mechanical strength of the heart appeared to be very useful. The pictures of the heart's electro- and mechanograms in all investigated species did not differ in quality: the myocardial action potentials (AP) have the the typical biphasic "spike-plateau" form at all points of the ventriculus or the atrium where the slow dyastolic depolarisation were registered (Fig. 1C). The delay between the spike peak and the strength peak varied between 0.3–0.7 s depending on contraction frequency.

For the traditional semi-intact preparations the periods of regular rhythm were replaced by the inhibitory pauses. It is known that the heart frequencies in *H. pomatia* and *A. fulica* are modulated by spontaneous change in spikes flow of the central heart inhibitory motoneurones [1, 13]. For the whole-mount preparations the heart rhythm was more stable just as in intact animals [18]. The slow dyastolic depolar-

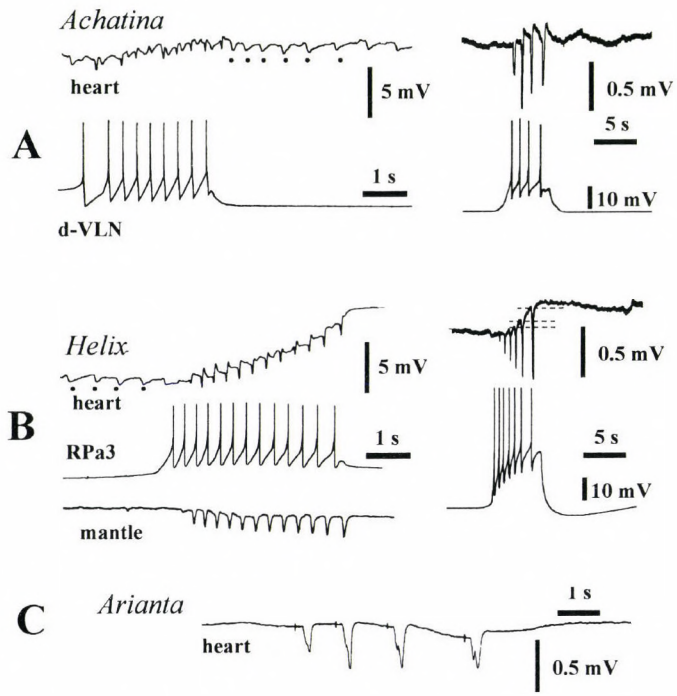

Fig. 2. Biphasic junction potentials (BJPs) in the heart of *Achatina f.* **(A),** in the heart and mantle muscle of *Helix sp.* **(B)** during bursts of action potentials in giant homologous neurones of right parietal ganglia d-RPLN and RPa3. The black circles mark some of peaks of inhibitory junction potentials (IJPs) evoking by activity of well-known cholinergic heart-inhibitory multifunctional neurones. Note at the differences in time constant of BJPs and IJPs. The time constant of myocardial syncytium is close to BJPs [19]. **(C)** Biphasic JPs in the heart of *Arianta a.* during electric stimulation of peripheral end of the left pallial nerve

ization in all points of the heart creates problems for the registration of small junctional potentials (JPs) during dyastolic pause. That is why these JPs were registered mainly in spontaneously stopped heart or during inhibitory pauses evoked by heart inhibitory motoneurones.

The combined method of registration of the heart's activity confirmed the direct proportionality between the amplitude of heart's AP and the strength of the heart's contraction [8]. Only at near-extreme points were more complex correlations between electric and mechanic peaks observed. Therefore, at times it was possible to register the contractions without electric waves, or small APs without contractions.

In whole-mount preparations of *A. fulica* the largest neurones on the dorsal surface of the suboesophageal ganglia, d-VLN and d-RPLN evoked discrete JPs in the myocardium and in the mantle and the muscles of the visceral sac [17]. Nearly identical JPs were registered in an analogous experimental situation in muscles of *H. pomatia* during stimulation of homologous nerve cells – command neurones of pneumostoma LPa2, RPa3, and in muscles of all three species during stimulation of the peripheral end of the left pallial nerve. The homologous command neurons of pneumostoma *Arianta* have not yet been found because the ganglionic complex of *A. arbustorum* is different from that of *Helix*.

The JPs consist of the first distinct negative phase (H-phase), followed by a relatively slow and small positive phase (D-phase). The second component of JPs, the D-phase, might be easily masked by fluctuation of membrane potential or by other JPs (Fig. 2). The duration of the H-phase is 3–4 times shorter than the duration of IJPs from the cholinergic heart motoneurones.

The most distinct JPs could be registered only during rhythmic activity of giant neurons or during repetitive stimulation of the nerve. The first JP after a long period of silence in a giant neurone (so-called "rest-JP") is often near the noise level. During repetitive discharges of neurones due to expressive facilitation of JPs the amplitude of the H-phase increased to 4–7 times depending of spike frequency. These changes of efficiency of synaptic transmission after rhythmic activity (potentiating effect) decrease with time constant near 150–200 s in all three snails. The previous measurements were made only for the H-phase of JPs, but it seems that the D-phase changes in the same manner.

By means of successive sections of visceral nerves it was found that the main part of axons of giant neurons both in *A. fulica* and *H. lucorum* go to the heart through the left pallial nerve. The minor part of terminals reached the heart in right pallial and/or anal nerve but has never been observed in intestinal nerve.

In all three snails the stimulation of the peripheral end of the left pallial nerve was accompanied by the same JPs as those during giant neurones discharges. After prolonged stimulation the desynchronization of individual JPs could be observed (Fig. 2C).

Nevertheless, on the whole the heart's response to activation of the giant neurones and left pallial nerve are more complex. Besides a series of biphasic JPs, hyperpolarisation with variable latency begins to be expressed in the heart. It is natural to propose that the reason for this hyperpolarisation is the summation of H-phases of bipha-

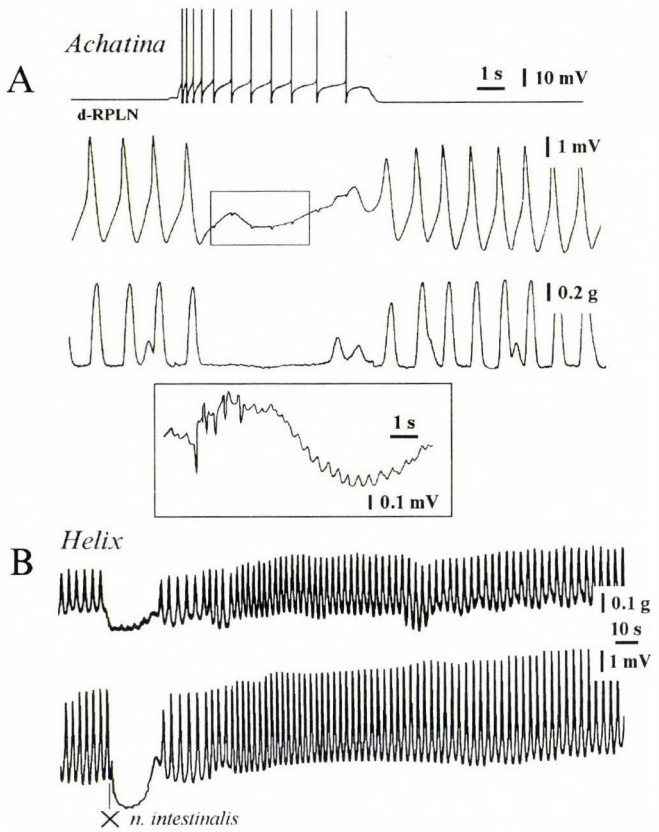

Fig. 3. **(A)** The effect of giant neurone of *A. fulica* on cardiac electro- and mechanograms. Inset: the biphasic junctional potentials (short) and inhibitory junctional potentials under large magnification. **(B)** Changes in the heart activity of *H. lucorum* after cutting of intestinal nerve (×)

sic JPs after discharges in the giant neurones. Amplified electrograms have shown that hyperpolarisation and inhibition of heart beats is the result of summation of long cholinergic IJPs (Fig. 3A).

To separate the cardioregulating activity from different inputs, the only route of the inhibitory cholinergic motoneurone, the intestinal nerve [13, 16], was cut. The cutting of intestinal nerve evokes the same complex effects in all three snails: (1) inhibitory reactions are dominant in the first few seconds due to summation of cholinergic IJPs; (2) three to ten seconds later the long-lasting activation of the heart contractions starts (Fig. 3B). The magnitude of systoles, the heart frequency, and basic tonus are increased. These positive effects continue for tens of minutes. Cutting of the nerve evokes strong activation of all cardioregulating inputs. To diminish this effect the preparations were cooled up to +2–3 °C before nerve cutting.

Destruction of intestinal nerve changed the chronoinotropic responses of the heart to the stimulation of the left pallial nerve and to activation of giant neurones in general. At this time only small positive effects could be registered in heart activity during bursts of spikes in giant neurones (Fig. 4, *Achatina*). In this example the amplitude increased by 25–30% and the frequency by 5%. In some preparations only a small increase in basic tonus of the heart were observed.

Intensive stimulation of the peripheral end of the left pallial nerve is more effective. After the first train of stimuli the heart achieves a new stable basic status. It looks like the heart has surpassed the upper limit of its chronoinotropic abilities, and repetitive stimulation of nerve causes no change any more now (Fig. 4, *Helix*).

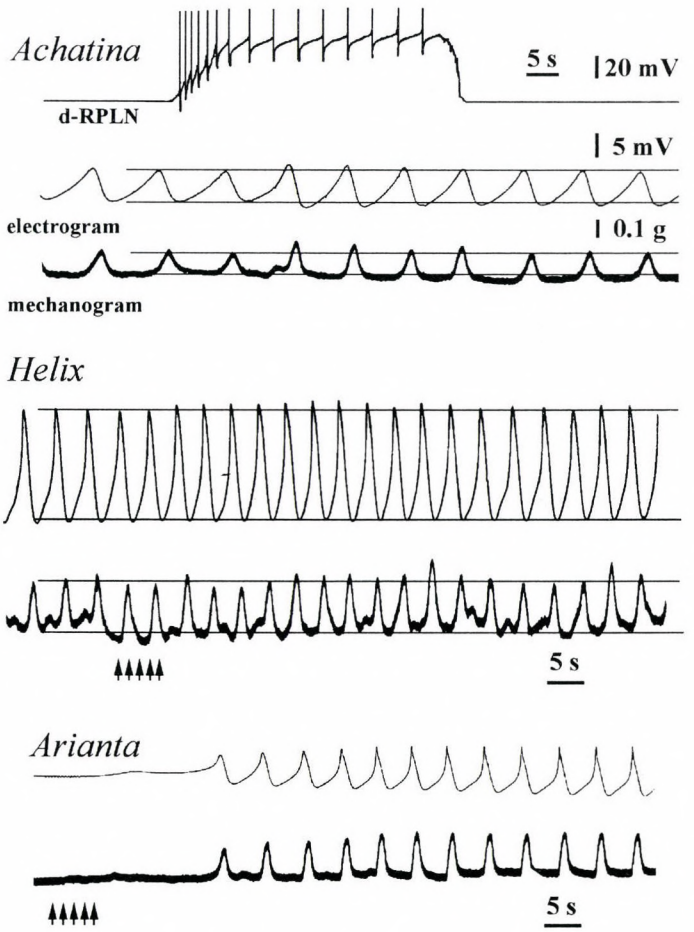

Fig. 4. The weak positive chronoinotropic effects at land pulmonates during activation of cardioregulating inputs of left pallial nerve system after cutting of intestinal nerve. The arrows indicate the stimulation of peripheral end of left pallial nerve (1.5 of thresholds, 10 imp/s)

Sometimes the repetitive activation of the nerve evokes the local arrhythmias in the heart (in the ventricular part). By visual control, it looks like two to three small bubbles in the ventricular wall contracting independently with similar frequences. The interference of these local contractions gives the second order waves on the electro- and mechanograms.

In the spontaneously stopped heart the short stimulation of the left pallial nerve activates rhythmic beatings. Increasing stimulus intensity decreases the latency of heart activation (Fig. 4, *Arianta*). The spontaneous contractions of the heart after stimulation of the left pallial nerve start without extrasystole.

DISCUSSION

The elaboration of the whole-mount preparation of land pulmonates confirms the existence of cardioregulating inputs primarily through the left pallial nerve in these snails. The electrical responses are very similar in all investigated species; they are the same in the heart and in other visceral muscles. The quantitative analyses of the facilitation and potentiation of H-phase of new JPs at *A. fulica* revealed direct connections of giant neurones, d-VLN and d-RPLN, with the heart and visceral muscles [19].

The ionic mechanisms of new JPs are still unknown. The H-phase is not blocked by atropine, d-tubocurarine, picrotoxine, succinylcholinchloride, strychnine, tetraethylammonium and is Cl^--independent [17]. The existence of two phases in the new JPs suggests that the availability of cotransmitters in giant cells is significant. It is most likely that peptide neurotransmitters are responsible for left pallial nerve effects. It is known that command neurones of pneumostoma by *Helix* are immunoreactive to FMRF-antibodies [3]. In homologous d-VLN and d-RPLN of *Achatina* this immunoreactivity were not found [4]. These homologous cells may contain various peptides from the related family.

Although it is clear that the new biphasic JPs are indisputably very stable and almost identical in all preparations from all three species, the chronoinotropic responses of the hearts are not so obvious. They are not reproduced every time at the same repetitive trains of stimuli. It appears that the chronoinotropic reactions themselves depend on the complex interactions of the cardioregulative structures belonging to systems of intestinal and left pallial nerves. The intraganglionic interaction of the giant neurones and other earlier identified motoneurones of the heart both in *A. fulica* and *H. lucorum* have not been found in isolated ganglia or in traditional semi-intact preparations [1, 16].

Activation of giant neurones or stimulation of peripheral end of left pallial nerve evokes increasing contraction or tonus in many visceral muscles [10]. Just all viscerae are innervated by endings of mechanosensory multifunctional cholinergic heart inhibitory neurones HI 1, HI 2 of *A. fulica* and $V21_{-1}$, $V21_{-2}$ of *H. lucorum* [1, 13]. These neurones may themselves provide the viscerocardial or cardiocardial one-neuronal reflexes [16].

We suppose that the potentiation of activity of cholinergic motoneurones during the discharges of command neurons may occur due to mechanism of viscerocardial reflexes: (1) the spikes in giant neurons d-VLN and d-RPLN (*A. fulica*), and LPa3 and RPa3 (*H. lucorum*) or stimulation of left pallial nerve evoke the contractions of different visceral muscles; (2) the muscular contractions and/or shifts of viscerae activate the mechanosensory endings of multifunctional heart inhibitory cells HI 1,2 (*A. fulica*), $V21_{-1}$, $V21_{-2}$ (*H. lucorum*); (3) the increased impulsation in these cells evoke the trains of effective cholinergic inhibitory JPs in myocardium; (4) the heart stops.

The cutting of intestinal nerve should reveal the purely positive chronoinotropic heart responses to the stimulation of the new input. In general, it confirmed the previous investigations of Ripplinger [11] about a small increasing in the tonus and systole amplitudes under the stimulation of the left pallial nerve in *H. pomatia*.

One of the main problems in neurobiology of invertebrates is "whether or not the behaviour of the neurones observed in isolated ganglia might be regarded as being identical with those in the intact animal" [12]. The use of the new whole-mount preparation allowed us to minimize the damage to neuro-effectory connections, to approximate investigations of the intact animals, and to get new data about neurones *in situ*.

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CONTENTS

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EDITORIAL

The present issue of the Acta Biologica Hungarica is dedicated to the celebration of the 70th birthday of Professor György Csaba, Professor of Semmelweis University of Medicine, founder and earlier Head of Department of Biology, former President for Society for Biology and member of numerous Hungarian and international scientific communities and journals.

All papers and contributions to this jubilee issue are pieces of laudation for a György Csaba who spent (and is spending presently, too, with outstanding activity) his life with teaching and science. The authors are from different countries of the world, from Japan to Germany and from Denmark to Russia. An other company of authors is from Hungary and are mostly his students and co-workers.

In his memories Professor Csaba modestly mentions, that he was a medical doctor among biologists and a biologist among medical doctors. I am convinced that he provided both at highest level. His scientific activity was always completed by the motivation to explain and teach the complex biological processes to a wide range of readers. Moreover, Professor Csaba's open-minded skill and his originality were successfully combined. His strong character and permanent curiosity for mechanism of life generated such a fruitful carrier. He has a primary role in the description of the hormonal imprinting in both uni- and multicellular organisms. His clear and continuously refreshing view on biology makes him possible to have a real phylogenetic concept on cellular events.

These papers are a kind of expressions of honor to Professor György Csaba; he initiated, generated and organized most of these works. I hope that this jubilee commemoration provides all readers a sense about the impact and life carrier of György Csaba.

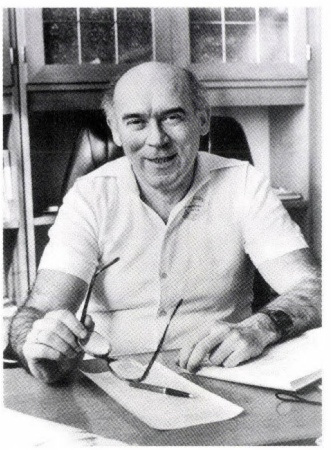
ANDRÁS FALUS

Akadémiai Kiadó, Budapest

Acta Biologica Hungarica 50(4), pp. 321–324 (1999)

LECTORI SALUTEM...

György CSABA



When I was invited by the Acta Biologica Hungarica to write about my life for this issue, dedicated to me, I found it very difficult. In a *memorial issue* dedicated to a researcher, somebody usually writes about a person, who is dead, and this is named obituary. This has an advantage over the autobiographical form written for a jubilee issue, as the personality can be excessively, perhaps uncritically praised since the praised person is not in the position to choose. As a result, somebody's failures and wrong-doings in life may be beautified after his death. However, an autobiography written by the subject himself has the unsurpassable advantage, that the writer and the praised person are the same and can enjoy the love of friends and co-workers, as he – though seventy years old – is still living. So, I will try to summarize fifty years of my scientific career in a nutshell.

I was a second year student of the Budapest Medical University (Semmelweis University of Medicine, at present) when my work started in the 2nd Department of Anatomy, Histology and Embryology. If I remember well, I serially sectioned the ciliary ganglion of a cat in order to study the ganglion cells. At the end of the year, my tutor presented a summary of this work at a Conference of the Hungarian Anatomists and Pathologists, in which I also participated. This was a critical point in my scientific future, as I realized that morphology is an important field, however, only when considered together with function, which takes the priority for me.

The department where I was working was headed by T. Huzella, who was an internationally renowned, ingenious histologist and embryologist. Unfortunately, at that time he was already seriously ill and died very soon thereafter. However, his disciples continued working in the department, inspired by his spirit. When I became a fourth year student, Imre Törő, a disciple of Huzella, a prominent embryologist was

appointed as the head of the department. At that time, being a university teacher and researcher in Hungary, was a heavy responsibility. After the Second World War, the university institutes were ruined and impoverished, and we were living in the shadow of the Stalin-governed Sovjet Union. There were not enough instruments and books, and for a long time there were no normal contacts with the western scientific world. The local biological science was dominated by the heterodoxies of Lepeshinskaya and Lisenko. However, we wanted to work and enjoyed the possibility of working. As a third-year student, I gave twelve hours of anatomy practice a week for first-year students, prepared the specimens and worked in the lab until late at night. When I became a fourth-year student, I had a laboratory of my own with student co-workers and when finishing my university studies, three original papers on the neural control of the reticulo-endothelial system were published (in Russian and Hungarian journals, of course).

After completing my university studies in 1953, I was doing a Ph.D.-like study for three years at the same place, then called Department of Histology and Embryology. Törő recommended the investigation of the biology of xenotransplants, which I pursued. This work resulted in the creation of the three-phase theory of heterotransplants and in the anti-antiserum method for prolonging the life of xenotransplants. However, papers about the former were published only in German, and those of the latter in Hungarian Acta-s (not available internationally at that time), so they vanished, as did most of the publications written by Hungarian researchers of that time. Nevertheless, these transplantation studies led to the agar-binding reaction (for the diagnosis of cancer), to a new principle in treating malignant proliferation, and to the discovery of two anti-tumour agents. These results aroused international interest, however, because they were produced by an "outsider", provoked such a storm of indignation at home by the experts not involved in the moral or (possible or presumed) financial benefits, that I abandoned such type of clinic-nearing experiments for a life. However, I was never able (and never wanted) to tear myself away from medicine, which always influenced all of my further activity in biological research.

The explanation of the agar-binding reaction focused attention on mast cells, the presence, accumulation and degranulation of which around malignant tumours was discovered by us. So, for a long time the study of mast cells was the subject of our research. We demonstrated the presence and development of mast cells in the lymphatic organs, first of all in the thymus, the presence of their developmental forms in the blood, and the endocrine regulation of mast cell formation. Concerning the latter, we also described the effect of the interrelation between the pineal body, thyroid gland and the thymus. These observations introduced the thymus (not solely as a mast-cell-producing organ) and, later the pineal body (the immunological role of which was first demonstrated by us) into our field of research. This pointed the way to the general iodine uptake capacity of the gut and other endodermal organs. Thus, the years of roaming finished.

In 1971 Törő retired and the Department was divided into two parts: a 2nd Department of Anatomy and the Department of Biology, which was headed by me. This basically changed the possibilities and direction of our works. The original

department taught anatomy, histology and embryology, as well as biology. That part of the staff which taught biology was delegated to my department after the split, and these researchers used different experimental animals, namely Tetrahymena, Planaria, snails, frogs, hens, rats and others, in addition to using different methods.

I have always believed that the correct recognition of a situation, and honoring human rights in all scientific endeavours will ultimately be rewarded. As ours was a small department without valuable instruments (simple laboratory microscopes were the most expensive tools obtained from the old department), I wanted to concentrate our scientific powers on one new subject, in which all of the experimental animals, methods and scientific experience could be utilized and which would satisfy my endocrinological interest. This subject was the phylogeny of hormone receptors. I recommended this subject to my old and new co-workers and they accepted it. Already, the first experiments with Tetrahymena justified this choice: the unicellular ciliated protozoan reacted to hormones of higher-ranking metazoa and, they were even able to select between closely related hormones. These results appeared in an international journal in 1973, the first occasion on which such type of reaction was published. After that, the mostly specific reactions to vertebrate hormones of invertebrates were demonstrated and published. Today, the reaction of invertebrates (unicellulars included) to vertebrate hormones is textbook-data.

The unicellular Tetrahymena did not only react to the hormones, but also remembered its first encounter with them. In addition, hundreds of subsequent progeny generations remembered the first interaction and could bind the hormone or respond to it in a different way. This phenomenon was named hormonal imprinting and, after publication, was also demonstrated by others. Based on these experiments, till now the receptors, signal transduction pathways and second messengers of unicellular organisms (similar to those of the higher-ranking animals) have been elucidated by many scientific groups (ours included). I personally believe that hormonal imprinting is a basic step in the selection of molecules which were suitable for bearing signals (hormones, neurotransmitters, etc.) and therefore a crucial process in hormone and receptor evolution. In addition, the receptor studies on Tetrahymena laid the foundation for clarifying, despite relatively primitive working conditions, the fundamentals of signal transduction.

I mentioned above that I have never forgotten medicine and I consider myself a doctor, who is teaching and investigating biological mechanisms. Considering Haeckel's rule, I supposed that in addition to phylogeny, hormonal imprinting also has an importance during ontogeny. This was the reason why, after recognizing hormonal imprinting in Tetrahymena, we began to study this phenomenon in rats. Neonatal rats were given the hormone and we studied the long-lasting effects in adult animals. Already in 1976 the first publication on the role of hormonal imprinting during mammalian ontogeny appeared. After that, parallel experiments were running in Tetrahymena and in mammals. It became clear that in mammals not only the physiological (target) hormones participate in perinatal imprinting of the developing receptor (which is essential for normal maturation of the receptor and later for the normal response to the appropriate hormone of the receptor bearing cell) but

molecules similar to the hormones (hormone analogues, members of the same hormone family, drugs or environmental pollutants) can also cause faulty imprinting with lifelong effects. As hormonal imprinting is not a time-dependent, but developmental stage-dependent phenomenon, it can be provoked not only perinatally, but in some organs at puberty, and in cytogenic organs throughout life. In our chemically severely contaminated world this causes numerous problems and by knowing the effect of faulty hormonal imprinting many diseases and extreme-normal variants can be explained and thus avoided. Perinatal hormonal imprinting is also the subject of research by many teams now.

Summarizing my scientific career, maybe I had some new ideas. I did not hesitate to study and experimentally test their validity, and the investigations were extended for contributing towards formulating general laws. However, I could not always penetrate into the depth of one or another problem as this could have detained the generalization of the results. I was not a real doctor, as I was involved in biological research and I was not a real biologist as I always remained a doctor. Nevertheless, I always loved the borderland of biology and medicine, both as a teacher and a researcher. My virtues and failures also arose from my character and conditions. Our country is a small one, with limited possibilities for research. When I was younger, political conditions did not allow my working and training abroad. When I was older, my character permitted me to leave my home and motherland only for very short periods. So, my results represent the works of a Hungarian researcher, who was living and researching at home, taking the opportunities offered by a small, yet talented country.

I do not know whether the work I did in the last fifty years is significant or trifling. It seems to be significant, considering the amount of work accomplished and insignificant, considering the huge building of biological science in which the conglomerate of our work is only a brick. The building of science is continually demolished by time after time and only a few intact bricks are reused for the construction of the new building. Future will determine whether my life's work constitutes intact bricks or cement (sometimes I would be satisfied with the latter), or nothing at all. However, independent of this, exploring the secrets of nature has became an organic part of my life and I should like to continue this work till the exhaustion of my powers.

There is no successful experimental work in modern biology without the participation of co-workers, whose physical and intellectual accomplishment is manifested in the results, and whose durable sympathy and friendship creates the atmosphere in which the results are produced. Parts of these findings are presented in this issue, together with papers of some of my friends, whose work relates well with ours. I am rather honoured by this jubilee issue of Acta Biologica Hungarica and I would like to thank the board of editors, my disciple-co-workers and my friends, who are connected to me by the mutual spirit of scientific inquiry.

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STUDY OF HISTAMINE EFFECTS ON PHAGOCYTOSIS AND ENZYME SECRETION OF *TETRAHYMENA PYRIFORMIS**

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1. The biogenic amine histamine develops effects not only in mammalian cells and tissues but in ciliated unicellular *Tetrahymena* as well. In addition to binding and internalization of labelled histamine, low concentrations can stimulate the phagocytosis of cells in inorganic salt solution.
2. In inorganic solution *Tetrahymena* cells secrete acid hydrolases to the medium. High concentration of histamine (10 mM) decreases the secretion of three investigated acid hydrolases in a different manner. We think that in this process the primary determinant is the alkaline character of histamine.
3. The effect of histamine on phagocytosis differs from the effect on secretion since the low, physiological concentration of histamine stimulates phagocytosis, the higher concentrations inhibit it. In the background of these effects possibly the hormone character is dominant. It is supported by the fact that histamine antagonists influence the process differently.

Keywords: Tetrahymena – histamine – acid hydrolases – phagocytosis

INTRODUCTION

Many species of the ciliated unicellular *Tetrahymena* genus are used in biochemical, genetic and cell physiological studies. It is well known for a long time that during culture growth *Tetrahymena* cells secrete lysosomal enzymes to the medium or to the inorganic salt solution, too [1, 2, 18, 27]. Secretion of lysosomal enzymes is the characteristic feature of not only *Tetrahymena* genus, but other unicellulars, fungi or mammalian cells also release acid hydrolases to their environment [21, 22]. Many research groups have studied the mechanism of this process in *Tetrahymena* and the heterogeneity of the secretion was demonstrated [2]. Some results presented a constitutive secretion [25], that is influenced by Ca^{++} [23]. Some of the enzymes have been identified, their structure determined and compared to intracellular lysosomal enzymes and to the similar enzymes of other species [1, 2, 16].

* Dedicated to Professor György Csaba for his 70th birthday.

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Some of the weak bases like chloroquine and NH_4Cl increase lysosomal pH [20] and have influence on lysosomal digestion [8, 12]. It was shown that chloroquine inhibits the electron transport system also in *Tetrahymena* [3]. Histamine, a biogenic amine having significant regulatory role in several physiological and pathological processes [17] is from a chemical point of view also a weak base. Histamine resulted lysosomal membrane fragility in endothelial cells [27] and inhibited the zymosan induced β-glucuronidase release from granulocytes [4, 15]. In contrast with other weak bases like chloroquine and NH_4Cl that resulted vacuolisation of different cells [9, 19], histamine did not induce such effect in macrophages [19].

The binding of ^3H-histamine, the internalization and the appearance in food vacuoles were detectable in *Tetrahymena* [5]. Our previous results showed that low histamine concentration (1 μM) stimulates phagocytic activity of these cells while H_2 receptor antagonist methylamine reduced it in inorganic salt solution [6]. This effect is selective and dose-dependent [7] and found after histamine pretreatment (imprinting), too [7]. The binding of histamine to *Tetrahymena* was inhibited by histamine antagonists [13]. There was no significant change of lysosomal enzyme secretion to organic culture medium after histamine treatment [14].

In the present experiment we studied the influence of histamine (*i*) on acid hydrolases secretion to inorganic solution and (*ii*) on phagocytotic activity and the relation between these effects.

MATERIALS AND METHODS

Cultures of *Tetrahymena pyriformis* GL strain were grown in PPYFe organic medium (1% proteose peptone, 0.1% bacto-yeast extract (Difco, USA), 36 μM $FeCl_3$ (Reanal, Budapest) at 28 °C with continuous shaking. At the beginning of experiments 300 cells/ml were inoculated into 100 ml Erlenmayer flasks containing 10–10 ml PPYFe medium. Following 48 hours of growth, in early stationary phase of culture enzyme and phagocytic activity was measured in control and histamine treated cells.

Histamine treatment

The cells were washed twice in Losina-Losinsky solution (LL). After 30 minutes of adaptation the cells were treated for 30 min with histamine (Histamine dichloride, Reanal, Budapest) solved in LL, pH = 7.0. The applied concentrations were 10 μM, 100 μM, 1 mM and 10 mM. During the 30 minutes of treatment neither the density of cells nor their motility was changed.

Study of phagocytosis

The treated and control cells in LL solution were feed with Chinese ink for 10 minutes. Then the cells were fixed in 4% formaline (in PBS), washed and spread on

slide, dried and examined for the number of ink containing vacules. Phagocyte coefficient (PC) was calculated by relating the counts found in treated cells to control as 1. The ink containing vacuoles were count in 100 cells in each group.

Determination of enzyme activity

Total enzyme activities were determined from the PPYFe cultures and control and histamine treated cells kept in inorganic salt solution (LL). In addition to total activity acid hydrolase activities of the cells were also measured. Cells were separated by centrifugation (500 g) then the supernatant, medium was filtered on 12 μm pore sized filter (Kipszer, Paraplan, Budapest). After freezing-melting exposure the cells were homogenized on low rotation speed. Prior to measurements TritonX-100 detergent was added to the samples in 0.1% final concenration. To determine the activity of acid phosphatase, N-acetyl-β-hexoseaminidase (hereafter hexoseaminidase) and β-glucosidase 0.5 mM 4-methyl-umbiliferon derivates (Koch-Light, England, 100 μl/sample) were added in their adequte buffer. These were: 50 mM acetate buffer (pH = 4.5) for acid phosphatase, 50 mM citrate buffer (pH = 4.5) for hexoseaminidase, and 200 mM acetate buffer (pH = 5.5) for β-glucosidase [21, 22]. One ml final volume of incubating solution contained 10–200 μl cell homogenizate or medium (total protein 5–10 μg). Incubation was carried out at 28 °C for required time (5–45 min), then the reaction was stopped by addition of 2 ml 1M glycine/NaOH (pH = 10.5). To measure the fluorescence of samples Cliniflour 85M fluorimeter (Izinta, Budapest) was used (ex = 365 nm, em = 448 nm) [21, 22]. The activity of enzymes is given in U/mg protein (U = 4-methyl-umbilliferon nM/min). Protein content of cells and medium was measured by Coomassie-blue technique. From the total and cellular activities the activity of medium was calculated and the data are given in percentage.

RESULTS

Enzyme activities in PPYFe medium and LL solution cultures

The distribution of the studied acid hydrolases between cells and medium in PPYFe organic medium cultures is different. More acid phosphatase (APH) activity was measured in the cells then in the medium. On the other hand, this ratio is just the opposite in the case of the other two enzymes hexoseaminidase (H) and β-glucosidase (GL): more activity was found in the medium then in the cells (Fig. 1).

When cells of early stationary phase of growth were placed into inorganic salt solution, they continue to secrete all the studied enzymes. After one hour in LL, more enzyme activity was measured in cells than in the medium, but the difference between the two activities was greater in the case of acid phosphatase (Fig. 1).

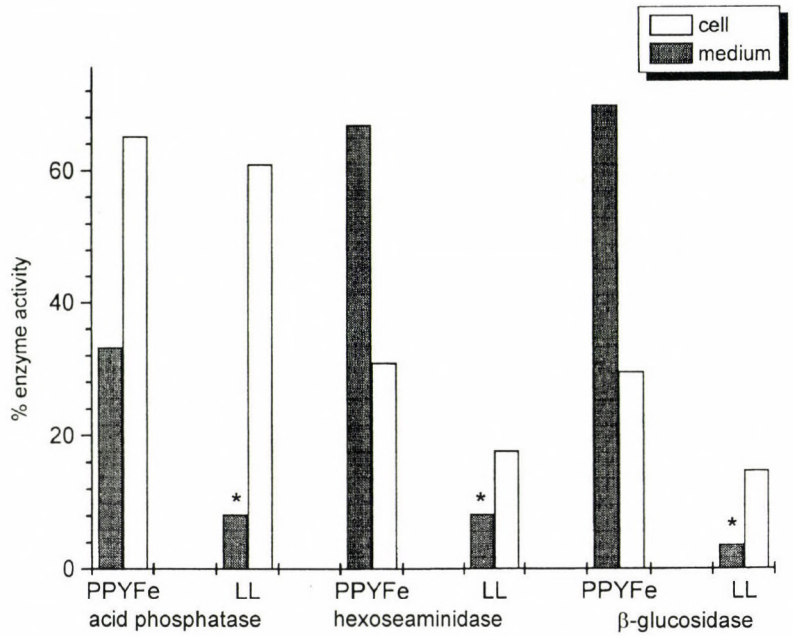

Fig. 1. Activity of acid hydrolases. For PPYFe and LL see Materials and Methods. Significance is indicated by asterisk ($p < 0.01$)

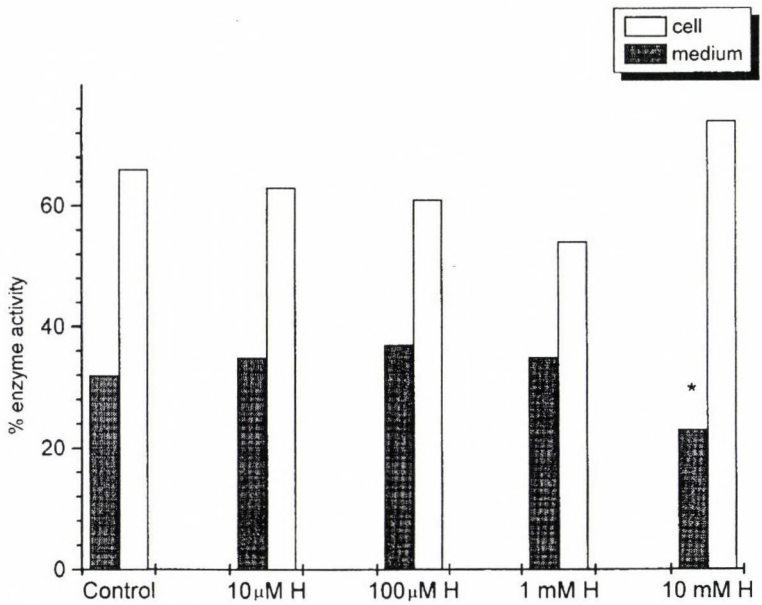

Fig. 2. Hexoseaminidase activity in the cells and medium. H = histamine treatment. Significance is indicated by asterisk ($p < 0.01$)

Effect of histamine on acid hydrolase secretion

No uniform change of activity and distribution of acid hydrolases induced by histamine treatment was found. Low concentrations (10 μM, 100 μM, 1 mM) do not alter the total activity or the distribution of hexoseaminidase compared to LL controls. The highest applied concentration (10 mM) of histamine significantly reduced the total activity, especially the medium activity. This change of distribution is shown in Fig. 2. The enzyme activity decreased from 33% to 24%.

β-glucosidase activity after histamine treatment is changed in a similar way, but the decrease of total activity is not significant (Table 1). Considering the distribution the enzyme activity measured in the medium, it declined to half of control, from 18% to 8% (Fig. 3).

The total activily of acid phosphatase was not changed, but a significant decrease was detected in the medium following the highest concentration histamine treatment (Table 1 and Fig. 4).

Table 1

Effect of histamine on the acid hydrolases activity (U/mg protein) in *Tetrahymena pyriformis*

	Total activity	Medium activity
	of hexoseaminidase	
LL solution	38.95 ± 2.08	12.84 ± 1.93
100 μM histamine	33.81 ± 3.79	12.74 ± 0.87
1 mM histamine	33.49 ± 5.37	12.10 ± 4.05
10 mM histamine	30.15 ± 6.24*	7.37 ± 1.98*
	Total activity	Medium activity
	of acid phosphatase	
LL solution	186.99 ± 13.16	12.84 ± 1.93
100 M histamine	195.11 ± 43.67	13.79 ± 1.51
1 mM histamine	173.78 ± 16.74	15.55 ± 5.84
10 mM histamine	156.55 ± 30.34	4.81 ± 2.64*
	Total activity	Medium activity
	of β-glucosidase	
LL solution	2.51 ± 0.50	0.61 ± 0.23
1 mM histamine	2.61 ± 0.11	0.44 ± 0.18
10 mM histamine	2.36 ± 0.51	0.18 ± 0.09*

* $p < 0.05$.

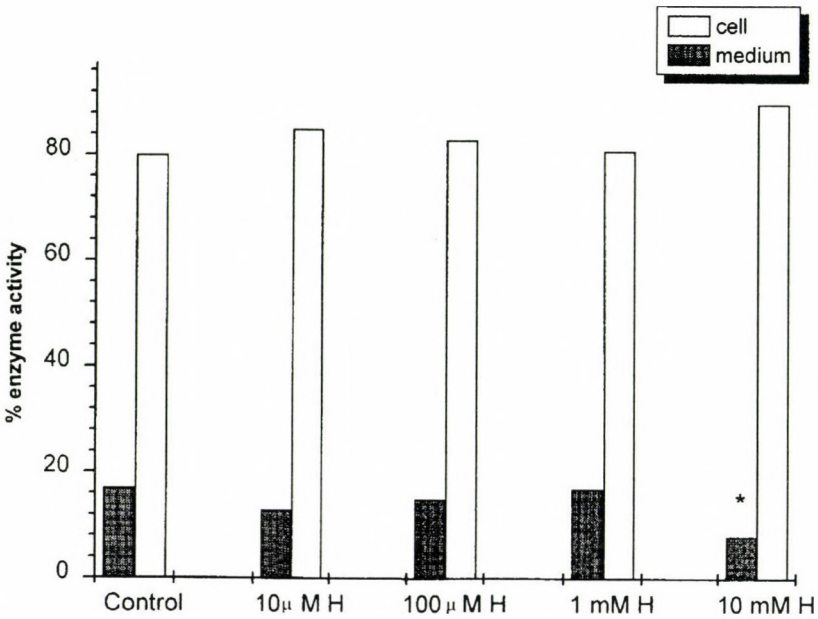

Fig. 3. β-glucosidase activity in the cells and medium. H = histamine treatment. Significance is indicated by asterisk ($p < 0.01$)

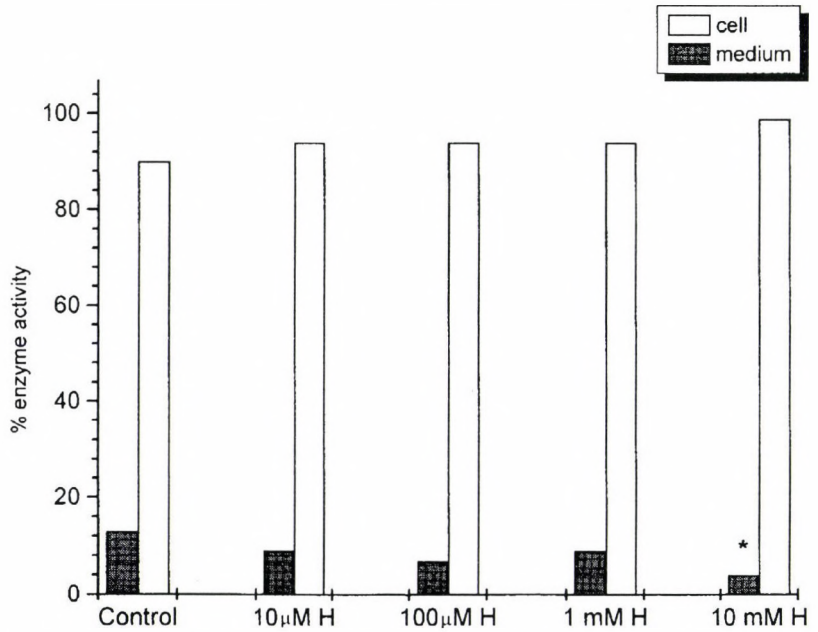

Fig. 4. Acid phosphatase activity in the cells and medium. H = histamine treatment. Significance is indicated by asterisk ($p < 0.01$)

Study of phagocytosis

Figure 5 shows the P. C. values, that represent the phagocytotic activity of experimental goups. The histamine effect is concentration-dependent. The lowest concentration has no effect on phagocytosis, while 100 μM and 1 mM histamine slightly increased the phagocytotic activity. The highest concentration (10 mM) strongly inhibited phagocytosis of the cells, P.C. value was reduced to the third of LL control.

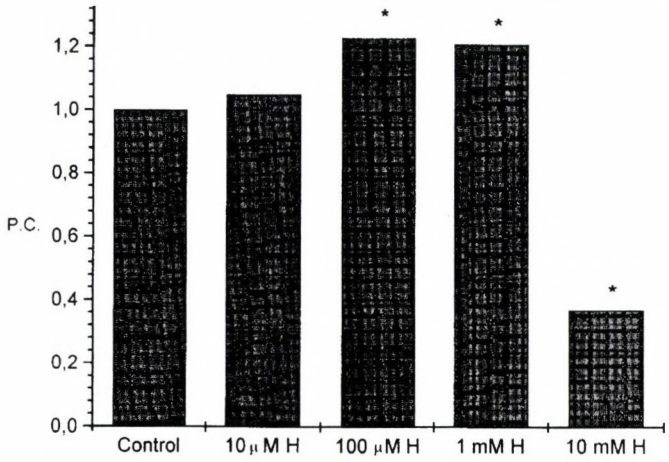

Fig. 5. Phagocytic coefficient (P.C.) values of histamine (H) treated *Tetrahymena* related to the control as 1. Significance is indicated by asterisk ($p < 0.01$)

DISCUSSION

Banno et al. [2] has described that the activity of different intracellular and extracellular acid hydrolases did not change parallel with the age of Tetrahymena pyriformis W culture. The activity of hexoseaminidase secreted into the medium increases with the cell number linearly, the intracellular activity does not alter after entering the stationery phase of growth. On the other hand, intracellular activity of acid phosphatase is higher than the extracellular prior to the stationery phase. In our actual model *Tetrahymena pyriformis* GL, we detected a higher activity of hexoseaminidase and β-glucosidase in the medium than in the cells, while acid phosphatase has a reverse distribution in 48 hour culture.

First Müller [18] showed that starving *Tetrahymena pyriformis* released its lysosomal, acid hydrolases into the inorganic salt medium. Banno et al. [2] found that these enzyme belonged to three groups on the basis of their secretory kinetics. Acid phosphatase and β-glucosidase are slowly secreted but hexoseaminidase is a rapid secreted enzyme. The total of most rapidly released hexoseaminidase appeared in the culture medium within two hours after their synthesis. It might be related to the het-

erogeneity of secretion that there is a difference in the intra- and extracellular distribution of studied enzymes when the cells were placed into LL solution for 1 hour. However, our results show that secretion of acid phosphatase and β-glucosidase having the same secretion kinetics was changed differently.

The weak bases like chloroquine used against malaria infection or NH_4Cl increase lysosomal pH and by this way the activity of hydrolytic enzymes [12, 20]. In *Tetrahymena* these compounds inhibit the secretion of acid hydrolases [2]. The alkaline histamine was found to inhibit the release of β-glucuronidase in different types of cells [11, 15]. The secretion of this enzyme is regulated, non-constitutive. In Tetrahymena high concentration of histamine (10 mM) like NH_4Cl decreased the release into the inorganic medium of all the three measured enzymes medium. So we found inhibitory effect of basic histamine on constitutive secretion.

Electron microscopic autoradiography of ^3H-histamine demonstrated that histamine enters Tetrahymena and appears in the food vacuoles (lysosomes) [5]. Similarly to chloroquine and NH_4Cl [2] it increased intralysosomal pH and altered the secretion of acid hydrolases in different degree. It had less effect on rapidly secreted hexoseaminidase and slowly secreted β-glucosidase, but it was effective on another slowly secreted enzyme, namely acid phosphatase. Different inhibition was probably resulted partly by the pH-dependent membrane binding of these enzymes. The same lysosomal enzyme e.g. acid phoshatase has membrane bound and intraluminar free forms having slightly modified structures [10]. Their pH sensitivity is different.

Comparing our data with the results of Banno et al. [2], histamine effect on enzyme secretion resembles to NH_4Cl effects, however, its inhibition upon acid phosphatase release is stronger. So histamine like other weak bases causes a decline in lysosomal secretion, revealed by the increase of intralysosomal pH.

Beside the elevation of lysosomal pH, other effects of histamine might be responsible for the inhibition of secretion. Histamine as an important general mediator has different receptors, H_1, H_2 and H_3 and through second messenger systems influence mammalian cells [17]. In *Tetrahymena* we found a receptor level effect of histamine on phagocytosis [7]. By changing the intracellular Ca^{++} level it can influence the activity of several proteins and that was demonstrated in *Tetrahymena,* the constitutive secretion [24] of lysosomal enzymes. But considering that the inhibitory effect was found only in high concentration of histamine, similarly to NH_4Cl, we have to suppose that the alkaline character of this molecule is primarily responsible for the decline of enzyme release.

A surprising result of our experiment was that beside the inhibited secretion no increase of intracellular enzyme level was detected, as it was expected. It refers to that there is a decrease in enzyme synthesis and the production is paralelly correlated to the lower level of secretion.

Our previous experiments demonstrated that in *Tetrahymena* phagocytosis was stimulated by physiological concentration of histamine [6, 7], but the high doses (10 mM) significantly decreased it. These results referred to a receptor level effect.

Taken together, it is presumable that histamine might have two, different types of actions on these cells. In one of them the hormone character (low concentration) of the molecule is responsible for the phagocytotic effect. In the other effect histamine having alkaline character (high concentration) by increasing the pH of lysosomes influence the secretion of lysosomal acid hydrolases.

ACKNOWLEDMENT

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INVESTIGATION ON THE FORMATION OF THE HORMONE RECEPTION MECHANISM*

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The formation of the hormone receptor mechanism is part of the embryonic development. The perinatal age seems to be critical for the maturation of this system, i.e. the development of the proper hormone selectivity, the number of the receptors, their sensitivity to the adequate hormone. Our team tried to get a closer view on this developmental period examining different parameters in different model systems. We were able to show that during the maturation of the hormonal system similar responses could be elicited by the later specific hormones or by the non-specific ones but structurally similar compounds. Also it became evident that one single hormonal treatment, applied during in the perinatal age, could influence the responsiveness to hormones of the adult animals.

Keywords: Hormonal overlap – ontogeny of hormone sensitivity – allylestrenol – glucocorticoids – liver regeneration

INTRODUCTION

Specific target organs are not sensitive to their adequate hormone before a given stage of the development [32, 39, 40]. The formation of the hormone reception mechanism and the sensitivity to hormones characteristic to adulthood require a specific period which depends on the species, sex and maturation level of the animal. The formation of the hormone reception mechanism – called maturation – is a long-lasting process, starting in the last third of the intrauterine life and taking end at the beginning of the extrauterine life. Its most critical period seems to be the early neonatal age. Conditions characteristic to adulthood are being developed during this maturation time.

Quantitative aspects of this period (time, hormone concentration, number of the hormone receptors, changes in the strength of the hormonal effect) were investigated by some teams [2, 14, 15, 32, 38, 39, 41]. Considering that changes in the structure, composition and immunological properties of the cell membrane are important factors of the complex process of the embryonic development [26], it has been proposed that besides these quantitative aspects the hormone selectivity might be altered as well during the maturation of the hormone reception mechanism.

* Dedicated to Professor György Csaba for his 70th birthday.

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EXAMINATION OF THE DEVELOPMENT OF THE HORMONE SELECTIVITY

The α-subunit of the glycoprotein type follicle stimulating hormone is identical with that of the thyroid stimulating hormone, their specificity in adulthood is maintained by their β-subunit, which, however, is slightly different in these hormones. Their low level specific cross-binding capacity in adulthood is probably due to their similar structure. Based on this, gonadotrop and thyroid stimulating hormones were chosen to run our experiments about the maturation of hormone selectivity. The overlapping effects of these hormones on the opposite target organ – on the gonads and thyroid glands – were first studied at different phylogenic and ontogenic levels in frog, rooster and rat.

The weight of the testis, the diameter of the seminiferous tubules and the number of the cells in these tubules increased in newly hatched roosters on the effect of both FSH and TSH [8, 21]. TSH – in contrast to FSH – had a definite effect on the connective tissue between the tubules. The ultrastructure of Sertoli and Leydig cells was altered by both hormones: the amount of the endoplasmic reticulum increased just like the rate of the formation of the lumen, i.e. the development of the testis was induced [24]. TSH had stronger effect on the testis than FSH in certain cases. FSH had weaker, still significant effect than the adequate hormone on the target organ of TSH [17]. The effect of TSH on the testis is still strong in five-week-old roosters but weaker than the effect of FSH. The morphological parameters of the thyroid glands were not influenced by FSH at this age, on the other hand, serum thyroxin level was increased by it [18]. The overlap was smaller both in neonatal and five-week-old rats than in roosters. The overlapping was extended in adult frogs [9, 10, 19].

The overlapping effect of TSH and FSH on each other's target cells must be the result of indirect effects as well. Based on our results, we suggest that direct effects, i.e. overlap at receptor level play important role in the effect of TSH on the testis and in the effect of FSH on the thyroid glands. All these observations support our hypothesis that hormone reception mechanisms are not completely specific in the early periods of the embryonic development.

MATURATION OF THE RECEPTION MECHANISM

Studying the literature it is also striking that several hormone serum concentrations reach a short-lasting peak during the perinatal age, i.e. during the development of the hormone reception mechanism [1, 3, 4, 30, 31, 33, 37]. It is also well known that in adult animals the hormone sensitivity and the number of the hormone receptors in self and non-self (heterolog) target cells are influenced by the serum hormone concentrations. This mechanism, regulating the hormone receptor concentration does not work during the period of the formation of the hormone receptor mechanism [39].

It seems probable that the endocrine system, one of the most important regulatory factors of the internal environment must also undergo an adjustment to "self" during the embryonic development. We assumed that high amounts of hormones appear-

ing at given times during the ontogeny play role in the adjustment of the endocrine system: it may influence or direct further maturation of the hormone reception mechanism. The very first encounter of the maturing system with the hormone has a definite importance for the subsequent events. This would mean some kind of accomodation of the whole system and of the developing receptors when still being plastic toward the first qualitative and quantitative stimulus. This trigger determines the formation of a specific hormone reception mechanism and the pattern of hormone receptors characteristic to adulthood. Upon the terminology used by Lorenz in etology, this feature has been called hormonal imprinting [5, 6].

Our hypothesis suggests that the maturation of the reception mechanism is a critical phase of ontogeny, since it can be altered by aphysiological effects such as aphysiological hormone concentrations or the binding of inadequate hormones and hormone analogues. Abnormal hormonal imprinting will lead to more or less abnormal development which will result in an abnormal selectivity of the reception mechanism and in an abnormal affinity (responsiveness) to the specific hormone of the cells in the adult organism.

Since the development of the hormone reception mechanism can be influenced in a critical period before and after birth, we examined the possible changes in the number of receptors, in the kinetics of hormone binding and in the specificity of the hormonal effects in adult animals after early postnatal hormone treatments. We were able to demonstrate the existence of hormonal imprinting with several hormones in several systems – most of the time with the help of receptor kinetic techniques as well.

In our first group of studies FSH and TSH, the glycoprotein type hormones used for the study of the overlapping effect, were administered to the animals [11, 18, 20, 23]. In roosters a single FSH treatment after hatching had a long-lasting effect regarding several parameters both in the testis and the thyroid glands. In roosters, the treatment used after hatching in the case of both hormones increased the effect of a second treatment at the age of five weeks on the testis. Analyzing the histology of the thyroid glands it was found that the effect of TSH was weaker in the group pretreated after hatching with hormone (FSH, TSH) than in the control group (pretreated with physiological solution). This may be explained by the damaging effect of the pretreatment. Similar results were found at the age of six weeks after neonatal pretreatments in rats, both in females and males.

Diethylstilbestrol (DES) was used in the fifties as a medicine in the maintenance of endangered pregnancy. Changes in the microsomal enzyme system of the liver and high frequency vaginal cancer have been reported in puberty or adulthood, as later consequences of the treatments.

In response to these observations the use of DES has been prohibited. Not much later an other gestagen, the progesterone-analogue allylestrenol was introduced. Its effect on the developing embryo was thought to be negligible. For years it was used without limits. Based on the results of our previous studies, we decided to examine the late effects of perinatal treatments of both allylestrenol and DES, respectively.

In roosters the weight of the testis and the diameter of the seminiferous tubules decreased by one allylestrenol administration after hatching. On the other hand, in

chicken the weight of the ovaries, the diameter of the follicles and the number of the granulosa cells increased [36]. In the uterus of adult rats the number of estrogen receptors decreased to halt as the result of one neonatal allylestrenol administration [37]. Certain changes were also observed in their estrus cycle: one perinatal allylestrenol treatment significantly increased the length of the estrus phase. Since the length of the whole cycle did not change, the ratio of the estrogen/gestagen phase shifted [28]. The progesterone and testosterone serum levels in adult rats were significantly increased by one pre- or neonatal allylestrenol treatment [35]. The inducibility of some microsomal enzymes in the liver was also altered by a single neonatal allylestrenol treatment [16]. The sexual behavior of female adult rats was strongly influenced by one neo- or prenatal allylestrenol administration: depending on the parameters, the sexual activity of the animals decreased 50 to 75%. The number of glucocorticoid receptors (GcR) also decreased in the thymus of adult rats as the result of a single neonatal allylestrenol treatment [27].

The constantly increasing number of the aromatic hydrocarbons is one of the biggest environmental problems in our days, considering that practically all living organisms are exposed to it.

We were interested to study the possible influence of one neonatal allylestrenol treatment on the effects of benzo(a)pyrene treatments in adulthood. The increased adult serum testosterone level in neonatally allylestrenol treated male rats further increased after exposition to benzo(a)pyrene in adulthood [34]. Exposition to benzo(a)pyrene in puberty (in five-week-old rats) decreased steadily the sexual activity in adult animals [12]. The number of estrogen receptors in uterus decreased as a result of perinatal benzo(a)pyrene exposition, similarly to the results after allylestrenol treatment [13].

It is well known that vitamin D has a steroid-like structure and its receptor belongs to the steroid receptor superfamily. The main target organ of this compound is the bone system that is in close relationship with hormonal regulatory mechanisms. This gave the idea to follow some studies about the possible influence of perinatally introduced D_3 on the process of mineralization. The body weight of adult female rats significantly decreased as a result of one neonatal D_3 treatment, just like the body mineral density (BMD) and the body mineral concentration (BMC) in adult males. Dexamethasone treatment in adulthood resulted in the reduction of the body weight and in the elevation of BMD and BMC in both sexes [29].

EFFECTS INFLUENCING THE HORMONE RECEPTION MECHANISM DURING LIVER REGENERATION

We supposed that the adjustment of the hormonal system depends greatly on the stage of the development, more than on the age. This means that although it happens mostly during the perinatal phase of the development, it can occur on different differentiating cells of the body as well. The best subjects to follow studies on this purpose are either cells that have kept their developmental potential and play role in the

physiological regeneration (e.g. hemopoetic cells) or cells that take role in the reparative regeneration.

The method of partial hepatectomy was first described by Higgins and Anderson in 1931 [25]. The left side and the medial lobe of the four lobes contained by the rat liver (they represent about 70% of the weight of the whole liver) can be relatively easily removed in medial laparatomy under ether anesthesia. Both macroscopic and microscopic structures of the liver reestablish within three weeks after the operation. Taking the regeneration of the liver as a model of the embryonic development from some point of view, this system seemed to be suitable for studying the hormone reception mechanism and its influencing effects.

In our liver regeneration experiments we examined the late effects of glucocorticoid pretreatments in the critical stage of the ontogeny on the glucocorticoid receptor expression in rat model system, in the 8th week following one neonatal pretreatment. Since the immunological aspects of the effects of glucocorticoids are also well known, we extended our studies on the glucocorticoid receptor expression during the liver regeneration process influenced by one neonatal endotoxin pretreatment. Our first results (under publication) show that both dexamethasone and endotoxin pretreatments induced significant changes in the receptor content of the adult liver of intact animals. The ratio of liver cells containing detectable glucocorticoid receptors in their nucleus was elevated by one single neonatal dexamethasone administration, meanwhile this ratio was decreased by a single endotoxin treatment. During the regeneration process following partial hepatectomy in pretreated animals, the ratio of liver cells containing nuclear receptors reached the maximum peak at different times, compared to the control animals. The maximum value shifted to earlier after dexamethasone pretreatment and to later after endotoxin pretreatment, compared to the controls. Changes in the receptor expression during the process of regeneration were monitored by molecular biological method, the Northern-blot technique.

Based on our experiments mentioned above, we can conclude that the development of hormone selectivity is a relatively long-lasting process during a given stage of the ontogeny. During this period of time, similar responses can be elicited by the later specific hormones and the nonspecific but structurally similar hormones (compounds). This so-called overlapping effect depends not only on the stage of the ontogeny but also on the level of the phylogeny. Examining different parameters in different model systems we were able to show that one single hormonal treatment in the perinatal age can influence the responsiveness to hormones of the adult animals.

The hormone responsiveness of an organism can be influenced by hormonal effects during certain periods of ontogeny. This recognition may have a high impact on clinical medical science. Considering that the pollution is more and more increasing and that pregnant women often undergo medical therapy – frequently for protecting their pregnancy – we should not ignore the effects of inadequate hormones or hormone-like chemicals in embryos which may influence the hormone sensitivity in later times.

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NON-CONVENTIONAL LOCATIONS OF HORMONE RECEPTORS (BINDING SITES). A REVIEW*

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Recent findings on the noncanonical positions of some well-known extracellular mediators and their receptors are reviewed. Peptide hormones (insulin) and/or their binding sites (cell membrane insulin receptor, nuclear insulin receptor); steroid hormones (corticosteroids and estrogens) and their putative membrane receptors are in the scope of this paper. The possible roles of these unusually located receptors in the intracellular signal propagation and physiological responses are also discussed.

Keywords: Hormones – insulin – steroids – receptor location

INTRODUCTION

In university textbooks and introductory courses into cellular biology, the location of binding sites for intercellular mediators are divided into two main groups: the plasma membrane receptors and intracellular receptors. Peptide hormones, factors, cytokines and amino acid derivative hormones – except the thyroxin family – are regarded to bind to cell surface receptors. Isoprenoids, including steroid hormones, vitamin D and retinoic acid derivatives, and thyroxin group act via intracellular – cytosolic or nuclear – receptors. In reality there is no strict correlation between the chemical nature of the mediator and the place of their binding sites. In the past decades, evidence has been provided that steroid hormones have plasma membrane binding sites, and by this way they really generated metabolic changes, acting as intercellular signals in the cytoplasm. On the other hand, more and more papers appeared on the intracellular or even peri- or intranuclear localization of polypeptide ligands – mainly insulin and angiogenic peptides – or their binding sites.

* Dedicated to Professor György Csaba for his 70th birthday.

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Abbreviations: ABC – antibody coupled; DHEA – dehydro-epiandrosterone; GR – glucocorticoid receptor; IGF – insulin like growth factor; iGR – intracellular GR; mGR – membrane bound/resident GR; RB – retinoblastoma protein; SC – sedimentation coefficient; SPM – synaptic plasma membrane.

In this review we focus on the unusual localization of mediators and/or their binding sites, but because the relevant biological response is a strict requirement for mediators, as well as for receptor function, we will refer shortly the possible roles of these non-conventionally positioned signal molecules and binding sites.

The pathological receptor protein mistargeting is out of the scope of this paper.

The location of receptors may change during the membrane isolation. It possible to loose the receptors during this procedure, or the cytosolic receptors may associate with the membranes [39, 69]. Using immunocytochemistry, the specificity, the cross reactivity and the accidental epitope masking may be questionable. For this reason it is very satisfactory that most of the researchers applied both morphological (autoradiography, immunocytochemistry) and biochemical (binding assay, kinetic analysis) methods in their studies. The authors used different terms to name the binding site, for example receptor, membrane binding sites, but they generally concluded, that the characterized binding site fulfilled the receptor criteria.

PEPTIDE HORMONES: INSULIN

The story of insulin internalization research is very instructive, but controversial. First it was thought that the membrane receptor-insulin complex is translocated to the nucleus [58, 65]. Later insulin, but not the plasma membrane insulin receptor was found to be accumulated in the nucleus [46, 64, 66]. Quite recently a nuclear localization signal has been found in the cell membrane insulin receptor (alpha-subunit) [61]. Up to now this story has not been closed.

Insulin translocation

In 1977, the nuclear envelope was reported to be the major site of insulin binding in rat liver nuclei [64]. The binding sites for insulin in the nuclear envelope were characterized with high affinity and hormone specificity; and they were found having characteristics differing from those on plasma membranes.

Later immuno-electron microscopic results showed that intact insulin, but not the insulin receptor, accumulated in the nuclei of insulin treated H-35 (rat hepatoma) and 3T3 (fibroblast) cells [66]. Ultrastructural and biochemical methods demonstrated that insulin associated with the nuclear matrix of H-35 rat hepatoma cell nuclei. The ^{125}I-insulin was apparently intact, except it could be found in a higher molecular mass complex, probably with nuclear matrix proteins [70]. Nuclear accumulation of insulin in H-35 rat hepatoma cells was energy independent, time and temperature dependent, and apparently was not saturable at insulin concentration which resulted in full receptor occupancy. Insulin could be internalized by both receptor-mediated and fluid-phase endocytosis. It was suggested internalized insulin was probably released from endosomes into the cytosol where the modulation of insulin degrading enzyme activity, by controlling the translocation of insulin to the cell nucleus, could play a crucial role in insulin's regulation of gene expression [40].

In the '90s it was found that insulin stimulated the nuclear accumulation of insulin receptors by threefold at 5 min. Phosphotyrosine content associated with the nuclear insulin receptor was also increased. These nuclear receptors differed from the membrane-associated insulin receptor in the solubility in Triton X-100. A nuclear protein ($M \approx 220$ kD) different from insulin receptor it substrate protein 1 (IRS-1) was tyrosine phosphorylated, and insulin further stimulated this process. From the surface labeling of insulin receptor it was concluded that insulin-induced accumulation of nuclear insulin receptor cannot be accounted for by internalization of surface membrane receptors [46].

In addition, at the same time a putative RB (retinoblastoma oncoprotein) binding site (LXCXE) on the insulin β-chain (and related motifs in IGF-I and IGF-II) and a suggestion that this insulin fragment may inhibit the transcriptional repressor activity of RB [62] were published.

Open questions: Is it not possible, that the nuclear insulin receptor is a complex of cell membrane insulin receptor and a nuclear matrix protein, or it forms a heterodimer with another transcription factor [46, 70]? In this complex the epitopes of cell membrane insulin receptors may be masked, and they cannot be detected by immunochemistry [66] and the solubility may be altered, too [46]. This question may be answered by Radulescu's theory [59, 62] mentioned in the next chapter.

Insulin receptor translocation

Using a biologically active photosensitive insulin derivative and the photolabelling of insulin receptor, the translocation of occupied surface insulin receptor to the nuclei of isolated rat hepatocytes was demonstrated. The translocation was time- and temperature-dependent. Accumulation and possible degradation of insulin receptors in lysosomes involved only a small percentage (1.6%) of the receptors internalized. The nuclear translocation of occupied cell surface insulin was receptors may be a mechanism which mediates long-term insulin effects [58].

Similar phenomenon was observed in 3T3-L1 adipocytes using ferritin-labeled insulin and electron microscopy or biochemical methods. Adipocytes accumulated potentially significant amounts (3%) of insulin in nuclei by a cell membrane insulin receptor-mediated process. In the nucleus usually the labeled insulin was found near nuclear pores and associated with the peripheral heterochromatin [65].

More recently, a bipartite nuclear localization signal, several zinc finger-like motifs and an RGG box have been found on insulin receptor (alpha-subunit). These findings have intriguing implications with regard to a presumable role of the insulin receptor (alpha-subunit) as a gene regulatory molecule [61]. Insulin together with insulin receptor may form a putative gene regulatory complex with RB [60, 62].

A nuclear localization signal in insulin-like growth factor-binding protein-3 (IGFBP-3) was also published [59] and the nuclei of a lung cancer cell line were immunostained by IGFBP-3 antibodies [45].

Insulin is not an exceptional polypeptide, regarding the nuclear translocation. Angiogenic peptides (e.g. angiogenin, aFGF) also translocate to the nucleus after endocytosis, suggesting that nuclear translocation of angiogenin and other angiogenic molecules is a critical step in the process of angiogenesis [31, 49, 50, 52, 73].

Amino acid derivative hormones and the unicellular Tetrahymena

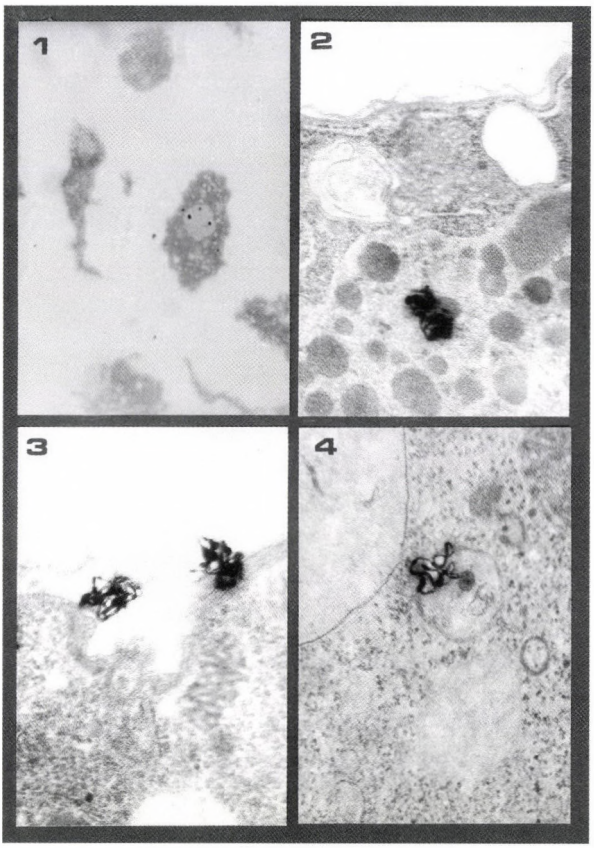
The nonconventional location of insulin or insulin binding sites were investigated not only in the usual vertebrate models, but interesting evolutionary aspects have been also raised.

The membrane of the unicellular *Tetrahymena* contains certain structures, which are able to bind the hormones of higher vertebrates and to initiate a response to the hormone as a result of the binding reaction [4, 5]. Primary interaction with a hormone gives rise to hormonal imprinting. The protozoa are able to store the imprint of hormone molecules as "memory" and to transmit it to their offspring generations [6]. It was demonstrated experimentally that the hormone receptors in general, including those of protozoa in particular, developed from primitive food receptors [47]. Therefore, the protozoa may take up and use as nutrient the amino acid and polypeptide hormones of higher vertebrates. Since the vertebrate target cells have polypeptide hormone receptors on their nuclear membrane and may contain such hormones also on intracelllar localization [72], it was postulated that hormone molecules could also enter the nucleus of the unicellular organisms. Presence of amino acid hormones, such as histamine, adrenaline, diiodothyronine and serotonin, had been in fact demonstrated within the nucleus in experimental conditions [19, 20, 21, 22]. We examined the binding, possible location and fate of radiolabeled insulin in *Tetrahymena,* with special regard to the behavior of cells pre-exposed and not pre-exposed to non-labeled "cold" insulin. The cilia of *Tetrahymena* can be grouped on the basis of their localization into oral and non-oral [44]. The investigations aimed to compare these two types of cilia by studying hormone receptors after hormonal imprinting, using light and electron microscopy. Gold-labeled insulin is bound first of all to the cilia of the oral field of *Tetrahymena.* The first encounter with insulin (hormonal imprinting) increased the binding capacity even after 24 h and made it more sensitive for appearance a week after, within a minute of giving insulin-gold [32]. The binding was specific for insulin, since polyethylene glycol (PEG)-gold was not bound at all. The results called attention to the binding and the increasing role of hormonal imprinting, and particularly to the marked role of the oral region in this binding. The roles of mucocyst extrusion and specific binding by receptors were also discussed [33].

The experimental results were confirmed, the unicellular *Tetrahymena* first internalized, than partly released the labeled insulin. Insulin-pretreated *Tetrahymena* cells behaved differently from non-pretreated cells, in that they retained a greater part of internalized insulin in the cytoplasm. Additional exposure to excessive non-labeled (cold) insulin caused a decrease in intracellular labeled insulin retention. Internalized

insulin also entered the nucleus of *Tetrahymena* (Figs 1–2), where it was found association with heterochromatin [31], resembling the location found in adipocytes [65].

Our study was the first to show isolated nuclei from *Tetrahymena* was able to bind FITC-labeled insulin, and pretreatment with different concentrations of unlabelled insulin accounted for a decrease in FITC-insulin binding of the nuclear membrane [41]. Insulin pretreatment increased the specificity of insulin binding which became even more pronounced in the distant offspring generations of the imprinted cells.



Figs 1–4. Light (Fig. 1) and electron microscopic (Figs 2–4) autoradiographs suggest the nuclear location of ^{125}I-insulin in the nucleus (Figs 1–2) and the membrane binding sites for ^{3}H-steroids (Figs 3–4) in *Tetrahymena.* Insulin was associated with the heterocromatin in the nucleus (Fig. 2). The steroid binding sites may be located in the plasma membrane (Fig. 3) near to the ciliar trough, which is a known site of pinocytosis; and due to the internalization in vesicular membrane (Fig. 4). The bulk culture of *Tetrahymena pyriformis* GL cells were tretaed with ^{125}I-insulin for 30 min (Fig. 1) or 15 min. (Fig. 2). After fixation the cells were processed for routine sectioning and autoradiography (Figs 1–4). The imprinted cells (3 days, 10^{-8} M dexamethasone) was incubated with ^{3}H-dexamethasone for 1 h (Fig. 3), or non-imprinted cells with ^{3}H-estradiol for 1 h (Fig. 4). ×660, ×42.000, ×58.000, ×57.000

"Virgin" (non-imprinted) *Tetrahymena* cells showed a greater specificity of insulin binding at the nuclear than the plasma membrane level. This difference became equalized under the influence of imprinting with exogenous insulin which increased the specificity of binding at the plasma membrane level [10, 12].

A physiological stress, starvation was studied as a regulator of insulin binding and imprinting in *Tetrahymena* and it had different effects on the plasma membrane and nuclear insulin binding sites [42]. Other experiments emphasized the increased sensitivity of binding sites of nuclear envelope and the role of it in the development of imprinting [11].

Simultaneously, chloroquine can inhibit the insulin binding in the nucleus itself, but not in the plasma membrane. There is a dose dependent increase of binding of labeled insulin to the nuclear envelope. The control-related binding of nuclei pretreated with insulin or chloroquine is similar and has a direct ratio, independently of the applied concentrations [43].

Hormonal influence on a unicellular is in certain cases measurable in terms of a specific response. For example, insulin, glucagon and adrenaline act on the glucose metabolism of unicellulars [15, 16], and histamine and serotonin act on phagocytic activity [14] similar to their action in higher organisms. The morphogenetic action of thyroxin and its precursors is realized as mitogenic action at the level of *Tetrahymena* [17]. Since, however, the target mechanisms of certain hormones are lacking at the unicellular level, the effects of these are assessed by certain alterations induced in more general mechanisms.

The representatives of the main hormone groups, polypetide insulin, steroid prednisolone and amino acid diiodothyronine act on the transcription of *Tetrahymena,* too. Insulin reduces the RNA synthesis, while the others enhances, with different extent in the imprinted (with the same hormone) and non-imprinted cells [30].

Also the protein synthesis is essential for the formation of new receptors which evolve as a result of the contact with the hormone. The *Tetrahymena* has receptors for amino acids and the contact with amino acids promotes the strengthening of these receptors [7]. Insulin imprinting given to the unicellular *Tetrahymena* considerably increased the uptake and intracellular storage of amino acids even many generations after the actual contact with the hormone. On the other hand, both the first and the second contacts with insulin increase the rate of the excretion of the stored amino acids. On the basis of the results obtained it seems to be possible that both protein synthesis and exocytosis of the *Tetrahymena* change as an effect of imprinting, either in general or specifically due to the formation of new hormone receptors [29].

STEROID HORMONES

Estrogens

The membrane association of an estrogen binding site has been proposed very early in hepatocytes [56, 57] and uterine endometrium [54, 55]. It was revealed that the membrane bound estradiol modified the cellular metabolism in hepatocytes [56].

Presence of specific plasma membrane binding sites for 17β-estradiol in human pre-osteoclastic cell line was demonstrated, using a cell-impermeable and fluorescent estrogen conjugate [24]. In addition the effect of the stimulation of this receptor was screened and found a prompt and significant increase in cellular pH; dose dependent elevation in both cAMP and cGMP levels; and a rapid increase in intracellular calcium ion concentration. These results suggest, that not only the nuclear estradiol receptor, but the membrane-bound, signal generating binding-site for estradiol may also influence the bone-remodeling.

Corticosteroids

The reversible corticosterone binding to, and mediated transport through the isolated membrane of rat hepatocytes are independent parts of a strongly linked process [1, 2]. The tritiated corticosterone binding to purified plasma membrane fraction of rat liver is specific, reversible, temperature dependent and saturable. The high affinity binding sites (K_d = 11 nM) are different from the glucocorticoid, progesterone nuclear receptors and the Na^+, K^+-ATPase digitalis receptor. A presence of enzymes on the plasma membrane that metabolize corticosterones has been disclosed. The binding of steroid to the plasma membrane receptor could cause Ca^{++} release into the cytosol. [61]. Ultrastructural evidence is also presented for the presence of specific membrane bound glucocorticoid recognition and binding site on AtT 20 (mouse pituitary tumor) cells and the microvilli rich region of isolated rat hepatocytes [67].

After *in vivo* administration of tritiated dexamethasone, a very potent synthetic glucocorticoid, 0.35% of the radioactivity recovered is bound on plasma membrane fraction of rat liver. Dexamethasone also bound *in vitro* specifically to plasma membranes, and this was comparable to binding to cytosol. Using polyclonal antibody to glucocorticoid receptor and indirect immunofluorescence technique, an intense decoration of the plasma membrane was observed. The location of the receptors on plasma membranes could be of potential importance for their interaction with agents (mitogens, growth factors) initially acting on the cell membrane, regulating subsequent cell proliferation and growth at the level of cell nucleus [39].

Specific and high affinity (K_d = 0.5 nM) tritiated corticosterone binding sites were localized by autoradiography in the synaptic plasma membrane (SPM) of an amphibian brain, in the neuropil, outside of the regions of perikarya. Because the affinities of corticoids for this binding site were linearly related to their potencies in rapidly suppressing male reproductive behavior, it is assumed, that this corticosteroid receptor of the brain membranes could participate in the regulation of behavior [53]. Membrane bound glucocorticoid binding sites can be found not only in amphibian, but also in mammalian brain [25, 26]. The radioligand binding assay showed that tritiated corticosterone could bind to GCMB specifically in rat brain and this GCMB is significantly different from the cytosolic glucocorticoid receptor. The selectivity of GCMB proved to be very wide [38]. The silver-grains marking GR-immunoreactive sites associated with the plasma membrane and coated and regular vesicles [48]. ^{3}H-

corticosterone binding sites were detected throughout in the calf adrenal cortex by autoradiography. Corticosterone binding was found to be time dependent, saturable and reversible. This high affinity (K_d = 7.7 nM) plasma membrane binding site proved to be selective for corticosterone and progesterone derivatives, and different from nuclear glucocorticoid, mineralocorticoid, estrogen and progestin receptors [3].

The ontogenetic aspect of the glucocorticoid binding to synaptic plasma membrane from rat brain were investigated using tritiated triamcinolone acetonid. The increase of membrane binding sites during postnatal brain development provides additional evidence that this binding has a physiological significance in brain function [68]. Dexamethasone has an excitatory effect on neuronal activity in the medial vestibular nucleus of cat, and this effect is mediated by membrane glucocorticoid receptors. This finding may explain the anti-vertigo effect of glucocorticoids used sometimes for the treatment in certain human disorders, such as Meuniere's disease [74].

A glucocorticoid receptor like antigen was detected in the plasma membrane of the mouse S-49 lymphoma cells, after some clinical observations that the intracellular glucocorticoid receptor number was occasionally not in correlation with the steroid responsiveness of lymphomas. Evidence was presented that mGR may play a role in mediating the lymphocytolytic effects of glucocorticoids. The patching and capping of membrane resident glucocorticoid receptor (mGR) was observed by fluoresceinated monoclonal [34] and colloidal-gold conjugated antibody [36, 37]. Membrane bound GR was demonstrated with an antibody specific for intracellular GR (iGR), in human leukemic cells (CCRF-CEM). This mGR proved to be larger (M = 145 kD, SC > 7 S) than the iGR (M = 94 kD, SC = 4 S) [35]. A mathematical analysis of glucocorticoid distribution in thymocytes revealed two topographically different cell receptor systems: intracellular and membrane bound glucocorticoid receptors [23].

Up to now not only the presence and role of membrane-bound glucocorticoid receptors (mGRs) have been investigated, but the regulation of the number of this receptors, too. Influence of the cell cycle was studied on the expression and function of the membrane-resident glucocorticoid receptor in human leukemic cell line (CCRF-CEM) and in a subline. All three of the used synchronization procedures demonstrated that the mGR expression was cell cycle regulated and it is the highest from the late S-phase to the G2/M transition. In this period the cells were most sensitive to glucocorticoid in apoptosis assays [63].

Open questions: Are the steroid membrane binding sites receptors or transporter molecules (20, 20a)? What is the function of membrane glucocorticoid receptors in the hepatocytes? In case of glucocorticoids and mineralocorticoids the isolation and characterization of the putative membrane bound receptors have proven elusive [27].

Association and effect of steroids in Tetrahymena

Steroid hormones also act on *Tetrahymena* [8, 18] and induce in it the formation of steroid binding sites after preexposure [28]. Steroid hormones (estradiol, dexamethasone, DHEA, triamcinolon) were taken up by *Tetrahymena* (Figs 3–4), and

appeared intracellularly in both cytoplasmic and intranuclear localizations [9, 28]. Treatment of unicellular *Tetrahymena* with 10^{-8} M prednisolone gave rise to both early and late ultrastructural changes, which were partly similar to, partly different from those elicited in the target cells of higher organisms [8]. The dexamethasone induced glucocorticoid receptor of *Tetrahymena* differs from the receptor of higher vertebrates, in as much as it is able specifically to bind DHEA, but it does not interact with other steroids [13].

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THE ENVIRONMENTAL POLLUTANT AROMATIC HYDROCARBON, BENZPYRENE HAS DELETERIOUS EFFECT ON HORMONE RECEPTOR DEVELOPMENT*

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In this review, works to clear the effect of perinatal and pubertal benzpyrene imprinting to the steroid hormone receptors of adult animals are summarized. There is a comparison between the perinatal and adolescent effects and between the effects on the receptors of males and females. On the basis of the experiments benzpyrene is a general and dangerous imprinter which can influence durably the development of different steroid receptors given directly to the animals studied (perinatally) of given to the nursing mothers or influencing the offspring generations of the perinatally treated parents or grandparents. The results call attention to the outstanding deleterious effects of benzpyrene pollution.

Keywords: Benzpyrene – hormonal imprinting – steroid hormones – steroid receptors – transgenerational effects

INTRODUCTION

As early as 1984 [10] we started to investigate the possibly deleterious hormonal imprinting effects of environmental pollution on organs at receptorial level. That time there a considerable wealth of evidence has accumulated calling attention to the danger caused the living world by chemicals [23, 30, 31].

Although the hormone receptors are genetically encoded stuctures, they display certain plasticity and alternability in some stages of their maturation. In these critical periods external influences may have a lasting impact on their complete maturation [6, 7, 9].

A decivise event of maturation is the phenomenom called hormonal imprinting [8, 12, 14, 22]. Depletion or excess of the adequate hormone in the critical period accounts for abnormal development of receptors. Above all, structurally related but functionally different hormones or environmental molecules which are able to bind to the immature hormone receptors may bias receptor maturation [12, 14, 18].

* Dedicated to Professor György Csaba for his 70th birthday.

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Evidence has been accummulated that neonatal synthetic steroid dexamethasone treatment caused about an 50% decrease of the binding capacity of adult thymic glucocorticoid receptor concentration [24], synthetic sexual hormone derivatives (diethylstilbestrol, allylestrenol) accounted for 50–65% decrease in uterine estradiol receptor number after a single neonatal exposure [11]. That is why we exposed our investigations on thymic glucocorticoid and uterine estrogen receptor characters studying the possible alterations caused by the external environmental pollutant benzpyrene. This review paper is a summary of our receptor-binding experiments carried out with aromatic hydrocarbon (benzpyrene) treatment and a comparison of the results.

MATERIALS AND METHODS

We performed all of our experiments according to the accepted receptor binding assay method. Organs examined were collected, stored in liquid nitrogene until use. One measurement was done on soluble receptor preparation from 4–5 pooled organs.

Experimental animals (Wistar rats of our closed breed-P generation) were treated with benzpyrene (Fluka, Buchs, Switzerland) in the concentration of 2 μg/g body weight. Newborn was regarded within the first 24 hours after birth, adolescents were the 6-week-old animals, and the adults in their 3rd month after birth.

Measurements were repeated 3–6 times depending on the standard deviation of the final data of assays computed.

Preparation of cytosol fraction for receptor assay

All procedures were performed at melting ice temperature. Organs examined were homogenized in TS buffer (0.01 M Tris-HCl with 1.5 mM EDTA pH 7.4, freshly supplemented with 2 mM dithiotreitol and 20 mM molybdate) 1.5 ml/net wt using 2×4 strokes of a motor-driven glass-teflon Potter homogenizer. Homogenates were centrifuged at 100,000 g for 60 minutes at 4 °C , the supernatants (cytosolic fraction) were used for the receptor assays. Protein content was estimated by the Coomassie blue method.

Saturation analysis

Increasing concentration of the ligand 1,2,4 ^{3}H dexamethasone (Amersham), in glucocorticoid receptor assays or 2,4,6,7 ^{3}H estradiol (Izinta, Budapest), in estradiol receptor assays (0.1–20 nM) were incubated with the cytosol (500 μg protein of thymic and 300 μg protein of uterine receptor fraction) in polypropylene tubes in a 0.1 ml total volume at 0 °C for 18 h for total binding (T). To estimates nonspecific binding (NS) there was another set of tubes containing a 100-fold excess of the unla-

belled ligand for each radioligand concentration. All assays were performed in duplicates. The reaction was terminated by adding 200 µl 0.5% dextran-coated charcoal suspended in assay buffer, then pelleting the unbound steroid by centrifugation at 1500 g for 10 minutes. Aliquots (200 µl) of the supernatants were transferred into scintillation cocktail and counted in a Beckman apparatus (30% efficiency). Specific binding was regarded as S = T–NS.

Competition analysis

Cytosolic fractions (500 µg protein from thymi and 300 µg protein from uteri per tubes) were incubated with the labelled ligand in the concentration determined in the saturation analysis (generally 10 or 5 nM) in the presense of increasing concentration of the unlabelled ligand examined. Condition of incubation, termination of the reaction and counting were identical to those of saturation analysis.

Analysis of results

Analysis of the results were carried out by the computer program called EBDA LIGAND [26]. Statistical data of the final parameters were calculated by the computer program DATAANALYIS, v.1.0 (analysis of variance, simple F test comparison).

RESULTS AND DISCUSSION

We examined systematically the receptor state of thymi and uteri of experimental animals exposed to benzpyrene in fetal, newborn or adolescent periods. Moreover, it was also investigated, whether breastmilk can mediate chemical imprinting of the offspring if mothers are exposed to benzpyrene during lactation. We also had some experiments to get information on the possibly transgenerational effects.

Our summarized data of the receptor binding assays present K_d (M) values: the affinity of the receptor to, and B_{max} (M) values: the maximal receptor density for the ligand examined. The results are shown in Tables 1, 2, 3, 4.

Steroid hormones and steroid structures can influence each other's effect in adult age even at receptorial level [5, 27]. Aromatic hydrocarbons e.g. dioxin (TCDD) modulates receptors for glucocorticoids and estrogen [3, 4, 13, 19, 25, 32]. Receptors of the aromatic hydrocarbons (e.g. TCDD or benzpyrene) do not belong to the steroid-thyroid receptor superfamily [28] but the structure of these aromatic hydrocarbons is similar to that of steroid hormones, so they can disturb the normal development and function of steroid receptors [1, 2].

The results demonstrate that alteration in binding sites density of receptors examined is closely related to the immature stage (foetus or newborn) or the state of dif-

Table 1
Thymic glucocorticoid receptor character in adulthood altered by benzpyrene treatment in different ages

Age at treatment	Sex of animals	K_d (nM)	B_{max} (nM)
Fetal 15th day [16]	mixed	2.08	2.19**
Control		2.20	3.30
Fetal 19th day [15]	males	2.28	2.90*
Control		2.06	3.18
Newborn [10]	mixed	10.6	6.69**
Control		10.6	7.99
3-week-old [10]	mixed	9.88	5.26**
Control		10.0	7.99
6-week-old [15]	males	7.2	2.30*
	females	7.0	2.31*
Control	mixed	7.2	3.19
Sucking offspring maternally	males	4.03	3.14*
treated during lactation [19]	females	7.2	3.19
Control		3.61	4.10
Sucking offspring maternally treated during lactation	females	2.44	3.35*
Control		2.82	4.05

Significances: * $p < 0.05$; ** $p < 0.01$; references in parenthesis.

Table 2
Uterine estrogen receptor character in adulthood altered by benzpyrene treatment in different ages

Age at treatment	K_d (nM)	B_{max} (nM)
Newborn [17]	0.46	0.45**
Control	0.48	0.62
Adolescent (6-week-old) [21]	0.56	0.35**
Control	0.92	0.6

Significances: ** $p < 0.01$; references in parenthesis.

ferentiation (cytogenic organs, developing cells). The density of receptors is emphasized in this case, as receptor affinity was not influenced at all. This is in accordance with hormonal imprinting studies of other intracellular receptor bounded molecules [12, 14]. The affinity of receptors seems to be strictly regulated by genes and first of all the maximum evokable number of receptors (density) can be influenced by the imprinting. However, this effect of benzpyrene is so intensive that the minimal quan-

Table 3
Transgenerational effect of benzpyrene; treatment when newborn

Generation	Sex of animals	Organ	K_d (nM)	B_{max} (nM)
F-1 [20]	males	thymus	3.85	3.04**
Control			3.90	4.56
F-1	females	thymus	6.69	0.88*
Control			6.16	1.54
F-2	males	thymus	3.46	3.57*
Control			4.44	4.41
F-2	females	thymus	3.83	3.48
Control			4.44	3.15
F-4	males	thymus	3.20	2.44
Control			2.29	2.58
F-1	females	uterus	1.77	0.97
Control			1.44	1.01

Significances: *$p < 0.05$; **$p < 0.01$; reference in parenthesis.

Table 4
Transgenerational effect of benzpyrene treatment in adolescent age (6-week-old)

Generation	Sex of animals	Organ	K_d (nM)	B_{max} (nM)
F-1 [21]	females	uterus	0.38	1.19*
Control			0.32	0.68

Significances: *$p < 0.05$; **$p < 0.01$; reference in parenthesis.

tity, which could be passed into the breastmilk also falsely imprinted the glucocorticoid receptors.

It seems to be very interesting that in the case of pubertal treatment the females' glucocorticoid receptors are sensitive to the imprinting in contrast to the males' receptors. This may be explained by differences in the microsomal enzyme activity (and metabolism) of the two genders [14, 15]. There is also a difference in the transgenerational effect of the two sexes, however, here in favour of the males.

The uterine estrogen receptors equally were sensitive to the neonatal and pubertal imprinting which means that the imprinting effect of the benzpyrene is not only intensive, but also generalized.

Aromatic hydrocarbons polluting the environment are present in varying amounts depending on the frequency of sources and can be incorporated by pregnants, nursing mothers or newborns and adolescent youngs. Considering that the harmful effects can last up to the third generation (F_2 in males) and knowing that many molecules similar to benzpyrene are around us as environmental pollutants we have draw attention on public care problems in the future.

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THE EFFECT OF β-CHLORO D-ALANINE AND L-CYCLOSERINE ON THE SERINE, PHOSPHORUS AND PALMITIC ACID UPTAKE AND METABOLISM OF *TETRAHYMENA* LIPIDS*

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The serine palmitoyltransferase inhibitors β-chloro-D-alanine and L-cycloserine resulted in the uptake and metabolism of ^{3}H-serine, ^{3}H-palmitic acid and ^{32}P significant alterations in the unicellular *Tetrahymena pyriformis* GL as compared to the untreated cells. In contrast with the higher eukariotic cells, by these treatments – except 5 mM L-cycloserine – the ceramide formation were not inhibited in *Tetrahymena.* L-cycloserine inhibited the conversion of phosphatidylserine (PS) to phosphatidylethanolamine (PE) by decarboxylation, and the conversion of PE to phosphatidylcoline (PC) by methylation. The shorter L-cycloserine treatments caused lower, and the longer treatments higher label in glycerophospholipids. β-chloro-D-alanine resulted in the glycerophospholids higher lipid precursor incorporation both in the shorter and longer treatments. Presumably β-chloro-D-alanine treatments inhibit the transaminase activity, and the higher concentration (5 and 10 mM) proved to be toxic for *Tetrahymena.* We found differences between the metabolism of serine and palmitic acid labeled lipids in the β-chloro-D-alanine and L-cycloserine treated groups. This phenomenon is probably due to a difference in the uptake of phospholipid head group component serine and hydrophobic tail precursor palmitic acid: the incorporation of palmitic acid in *Tetrahymena* is extremely quick, on the other hand, the uptake of serine is slower, a clear time dependence was measured.

Keywords: Serine palmitoyltransferase – β-chloro-D-alanine – L-cycloserine – *Tetrahymena* – phospholipids

INTRODUCTION

The unicellular *Tetrahymena pyriformis* similarly to the cells of higher level organisms, contains the second messenger systems which are able to transmit information induced by environmental signals e.g. hormones toward the effector. The cAMP [3]; cGMP [23]; guanylate cyclase-dependent Ca^{2+} – calmodulin [19]; phosphatidylinositol [11]; glycosyl phosphatidylinositol [12]; protein kinase C (PKC) – like systems [7] have been demonstrated in this organism. In the signaling, very important phos-

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pholipases (PLA_1 [7]; PLA_2 [16]; PLD [20]) were also demonstrated in *Tetrahymena.* These results shed light on the importance of the lipid second messengers – besides another messengers – in the signaling pathways of *Tetrahymena.*

The amino acid serine is not only a precursor for protein synthesis, but plays a significant role in the synthesis of both sphingolipids and glycerophospholipids (Fig. 1).

In glycerophospholipid biosynthesis, serine incorporated directly into the head group of phosphatidylserine (PS). Metabolism of phospholipid head groups converts these molecules into different phospholipids: phosphatidylethanolamine (PE) can be formed by decarboxylation of PS; phosphatidylcholine (PC) can be formed by methylation of PE.

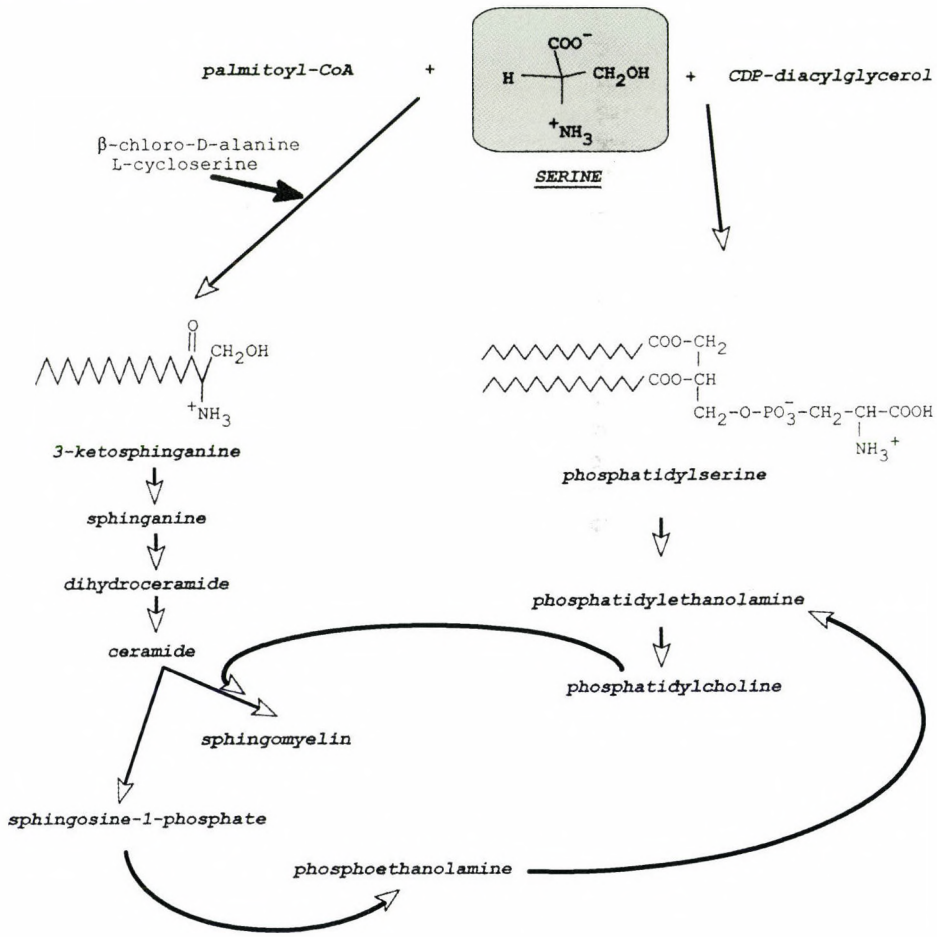

Fig. 1. The role of serine in the synthesis of sphingolipids and glycerophospholipids. Connection between the sphingo- and glycerophospholipid systems → Inhibition of serine palmitoyltransferase by β-chloro-D-alanine and L-cycloserine

During the synthesis of sphingolipids the initial reaction in the formation of long chain bases (LCB) is the condensation of serine with a fatty acyl coenzyme A (commonly palmitoyl-CoA). Ceramides are comprised of a LCB and a fatty acid. Sphingomyelin and gangliosides are formed by the addition of various head groups to ceramides.

These systems are in metabolic connection: breakdown of sphingosine-1-phosphate yields phosphoethanolamine, which is a possible precursor for the PE synthesis; the PC after the reaction with ceramide yields sphingomyelin.

The sphingolipids and glycerophospholipids – besides the function in the membrane structure – provide many signaling molecules. For instance PLD catalyses the hydrolysis of PC to phosphatidic acid (PA), which can be further converted to 1,2-diacylglycerol (DAG) [1]: both PA and DAG have been shown to act as second messengers. The sphingomyelin pathway has been implicated as a major signaling mechanism mediating the action of a number of extracellular agents (such as TNF-α, Fas ligands) causing the activation of sphingomyelinases that cleave membrane sphingomyelin resulting in the formation of ceramide [10].

It has become clear that these lipid messengers are playing important signaling role also in *Tetrahymena pyriformis.* A lot of treatments alter the synthesis and metabolism of phospholipids (PC; PE; PS; PA, PI etc.), as among others TNF α [22]; indomethacin [17]; local anaesthetics and phenothiazines [15]; phorbol esters [14]. The ceramide analogs (e.g. cell permeable C_2 ceramide) caused detectable alterations on the actin skeleton of *Tetrahymena,* and inhibited the normal function of contractile vacuoles [18]. Making change in the phospholipid metabolism spectacular alterations were manifested in signaling [e.g. 13].

Much of the current knowledge on metabolism of serine containing lipids has been derived from studies with compounds specifically inhibiting defined steps in the synthesis of these phospho- and sphingolipids. Very useful tools for this purpose are β-chloro-D-alanine and L-cycloserine: they inhibit the activity of serine palmitoyltransferase, the rate limiting enzyme of sphingolipid synthesis [5, 25, 28].

To gain an insight into the mechanism of *de novo* sphingolipid and phospholipid synthesis, and connection between these lipid classes in *Tetrahymena,* in the present study ^{3}H-serine, ^{32}P and ^{3}H-palmitic acid uptake and metabolism in presence and absence of β-chloro-D-alanine and L-cycloserine were investigated.

MATERIALS AND METHODS

Materials

[9,10-^{3}H] palmitic acid (specific activity 2 TBq/mM) and L-[3-^{3}H] serine (specific activity 962 GBq/mM) were obtained from Amersham (Buckingshamshire, UK). ^{32}P Na-orthophosphate (specific activity 76 GBq/mM) was purchased from Izinta (Budapest, Hungary). β-chloro-D-alanine, L-cycloserine, tryptone and lipid chromatographic standards (PI, PIP, PIP_2, PE, PS, PC, PA, DAG, ceramide) were pur-

chased from Sigma (St. Louis, MO, USA). Silica gel G TLC aluminum sheets were obtained from Merck (Darmstadt, Germany). Yeast extract was purchased from Oxoid (Unipath Ltd., Basingstoke, Hampshire, UK). All other chemicals used were of analytical grade available from commercial sources.

Tetrahymena *cultures*

In the experiments *Tetrahymena pyriformis* GL strain was cultured at 28 °C in 0.1% yeast extract containing 1% tryptone medium. The cultures in the mid-exponential growth phase (~5×10^5 cells/ml) were used. The cells were washed with fresh culture medium and harvested by centrifugation at ~400 g for 3 min at room temperature, and were adjusted to ~5×105 cells/ml with fresh culture medium.

Assay of ^{3}H-*serine and* ^{3}H-*palmitic acid incorporation into the phospholipids and ceramide of* Tetrahymena

Tetrahymena cultures (3 ml) were treated with 1 mM β-chloro D-alanine; or 1 and 5 mM L-cycloserine and after 5 and 60 min 1 μCi/ml ^{3}H-serine or ^{3}H-palmitic acid were added to the cells for 60 min in the continued presence of β-chloro D-alanine or L-cycloserine. Untreated cultures served as controls. After treatments the lipid content was extracted by the method of Bligh and Dyer [2]. The lipids were separated on oxalate pretreated silica gel G TLC aluminum sheets. The solvent was (by vol.) chloroform : methanol : H_2O = 65 : 35 : 2.5. Phospholipids and ceramide were identified by parallel run of authentic standards of PI, PIP, PIP_2, PS, PC, PE, PA, DAG and ceramide. After development, 0.5 centimeter strips were cutted into scintillation vials and the radioactivity was determined by liquid scintillation counter.

Assay of ^{32}P *incorporation into* Tetrahymena *phospholipids*

In case of L-cycloserine treatments also the incorporation of ^{32}P incorporation was tested. To the *Tetrahymena* cultures after 60 min from the beginning of L-cycloserine treatments 0.6 MBq/ml ^{32}P Na-orthophosphate were added for 60 min. The lipid extraction and analysis were done after the methods described above.

Statistical treatment of data

The experimental data represent the means of quadruplicate experiments. Student's *t*-test was used for calculations, with $p < 0.05$ accepted as the level of statistical significance.

RESULTS

The Rf values of different lipids on the chromatographic sheets by using chloroform : methanol : H_2O = 65 : 35 : 2.5 solvent were:

PC = 0.05; PS = 0.13; PI = 0.2; PA = 0.34; PE = 0.55; DAG = 0.88; Cer = 0.92.

Considerable amounts of the radiolabel from ^{3}H-serine and ^{3}H-palmitic acid were incorporated into *Tetrahymena* lipids.

Treatment of the cultures with β-chloro D-alanine dramatically alters the amount of incorporated ^{3}H-serine, and the ^{3}H-palmitic acid incorporation was also influenced significantly (Table 1). The incorporation of serine after 5 min of the beginning of β-chloro-D-alanine treatment was significantly higher in the examined lipids – except PS – than in the control ones. This increase was much stronger if the ^{3}H-serine incubation was followed after 60 min the beginning of β-chloro-D-alanine treatments.

Table 1

Incorporation of ^{3}H-serine and ^{3}H-palmitic acid into the β-chloro D-alanine treated *Tetrahymena* phospholipids and ceramide

	Control				5 min treatments				60 min treatments			
	^{3}H serine		^{3}H palmitic acid		^{3}H serine		^{3}H palmitic acid		^{3}H serine		^{3}H palmitic acid	
	DPM	%	DPM	%	DPM	%	DPM	%	DPM	%	DPM	%
PS	315.6 ±25	100	4242.7 ±124	100	335.6 ±18	106.3	5207.6 ±153*	122.7	1335.0 ±49*	423.0	6184.3 ±78*	145.7
PC	483.6 ±31	100	3266.9 ±88	100	625.3 ±34*	129.3	3170.9 ±95	97.08	5639.0 ±67*	1166.0	3473.4 ±50	106.35
PE	983.0 ±43	100	16419 ±440	100	1237.6 ±55*	125.9	15496 ±563	94.38	2118.0 ±30*	215.4	20780 ±611*	126.56
Cer	1602.0 ±78	100	2663.0 ±70	100	1831.6 ±38	114.3	5071.2 ±93*	190.45	4928.0 ±73*	307.6	3617.7 ±67*	135.85

The lipids were isolated from 3 ml 5×10^5 cells/ml *Tetrahymena* cultures. The radioactivity (DPM) was measured with liquid scintillation counter. The data represent means (±SD) of four independent experiments. Values are expressed also as percent of control (as 100%). * = $p < 0.01$ to the control.

In the case of ^{3}H-palmitic acid incorporation the β-chloro-D-alanine treatments did not result in a spectacular alterations of radioactivity: after 5 min the radioactivity in the PC and PE decreased, whilst in the PS and ceramide elevated. After 60 min in the examined lipids – except ceramide – the radioactivity an additional elevation were measured.

Table 2
The incorporation of ^{3}H-serine into the L-cycloserine treated *Tetrahymena* lipids

	Control	5 min treatments		60 min treatments	
		1 mM	5 mM	1 mM	5 mM
PS	110 ± 9 100%	163 ± 14* 148.1%	200 ± 16*^{a} 181.8%	169 ± 9* 153.6%	152 ± 11*^{b} 138.18%
PC	939 ± 53 100%	281 ± 19* 29.9%	187 ± 11*^{a} 19.9%	841 ± 54^b 89.6%	601 ± 44*^{ab} 64.0%
PE	538 ± 32 100%	265 ± 18* 49.25%	315 ± 29* 58.5%	433 ± 32*^{b} 80.48%	294 ± 24*^{a} 54.64%
Cer	329 ± 16 100%	482 ± 32* 146.5%	232 ± 16*^{a} 70.51%	1317 ± 320*^{b} 400.3%	203 ± 16*^{a} 61.7%

The radioactivity (DPM) was measured by liquid scintillation counter.

The data represent means (±SD) of four independent experiments, and the values are expressed also percent of control, as 100%. * $p < 0.01$ to the control; $^a = p < 0.01$ to the 1 mM treated; $^b = p < 0.01$ to the 5 min treated.

Table 3
Incorporation of ^{3}H-palmitic acid into the L-cycloserine treated *Tetrahymena* lipids

	Control	5 min treatments		60 min treatments	
		1 mM	5 mM	1 mM	5 mM
PS	7002 ± 68 100%	5921 ± 166* 84.5%	5886 ± 344* 84.06%	10111 ± 420*^{b} 144.4%	11326 ± 375*^{b} 166.08%
PC	1523 ± 62 100%	1174 ± 95* 77.0%	797 ± 37*^{a} 52.33%	542 ± 24*^{b} 35.58%	2871 ± 96*^{ab} 188.5%
PE	19225 ± 220 100%	14423 ± 185* 75.02%	3736 ± 136*^{a} 19.43%	7800 ± 237*^{b} 40.57%	19057 ± 320^{ab} 99.12%
Cer	4274 ± 125 100%	4886 ± 97 114.31%	4140 ± 103^a 96.86%	5000 ± 278 116.9%	4800 ± 277 112.3%

The radioactivity (DPM) was measured by liquid scintillation counter.

The data represent means (±SD) of four independent experiments, and the values are expressed also percent of control, as 100%.* $p < 0.01$ to the control; $^a = p < 0.01$ to the 1 mM treated; $^b = p < 0.01$ to the 5 min treated.

The 5 and 10 mM β-chloro D-alanine treatments proved to be toxic: the speed of movement was very slow, and the occurrence of rounded and dead cells was relatively high (data not shown).

L-cycloserine caused different alterations in the incorporation of ^{3}H-serine related to β-chloro D-alanine treatments. Apart from PS (1 and 5 mM, both 5 and 60 min

Table 4
Incorporation of ^{32}P into the L-cycloserine treated *Tetrahymena* phospholipids

	Control	1 mM	5 mM
PS	4543 ± 157 100%	2921 ± 84* 64.29%	676 ± 37*[a] 14.88%
PC	13749 ± 667 100%	7331 ± 221* 53.32%	14924 ± 540[a] 108.54%
PE	24800 ± 883 100%	11839 ± 559* 47.73%	2025 ± 73*[a] 8.16%

The radioactivity (DPM) was measured by liquid scintillation counter.
The data represent means (±SD) of three independent experiments, and the values are expressed also percent of control, as 100%. * $p < 0.01$ to the control; [a] = $p < 0.01$ to the 1 mM treated.

treatments) and Cer (1 mM, both 5 and 60 min) the incorporation of ^{3}H-serine occurred at only low levels. Accordingly in *Tetrahymena* only the 5 mM L-cycloserine treatments inhibit the initial condensation reaction in LCB formation, which is indicated by low level of Cer labeling (Table 2).

The incorporation of ^{3}H-palmitic acid was influenced significantly by L-cycloserine: on the whole the radioactivity of lipids decreased in the case of 5 min treatments (except Cer in the 1 mM treated cultures) and in the 60 min treatments the radioactivity increased (except PE and PC in the 1 mM treated cells) (Table 3).

L-cycloserine treatments resulted in the incorporation of ^{32}P a very spectacular decrease – ^{32}P treatments it were applied after 60 min of the beginning of L-cycloserine treatments (Table 4).

DISCUSSION

The incorporation of serine (and also the another phospholipid head group component phosphorus) into the phospholipids of *Tetrahymena* shows a time dependence, while the incorporation of palmitic acid is extremely quick: no significant alterations were found between the 5 and 60 min incubations [21]. Compared with other cell types, the uptake of LCB precursors may appear different. In intact LM cells for example ^{14}C-serine was taken up by the cells and rapidly reached steady-state concentrations similar to those of the medium. Palmitic acid was also taken up rapidly and increased LCB synthesis in a concentration-dependent manner [26]. It appears that *Paramecium* take up and metabolize exogenous lipids much faster than amino acids [8]. It is conceivable that the differences between the lipid precursor uptake results in the different metabolism and different sensitivity on the treatments compared to various cell types.

Serine palmitoyltransferase (SPT) (EC 2.3.1.50) is the key enzyme in sphingolipid biosynthesis. It catalizes the pyridoxal-5'-phosphate-dependent condensation of L-serine and palmitoyl-CoA to 3-ketosphinganine. β-chloro-alanine inhibits the de novo synthesis of 3-keto-sphinganine, the activity of SPT. β-chloro-alanine is known to inhibit other pyridoxal-5'-phosphate-dependent enzymes, as aminotransferases, but in the case of CHO cells 5 mM β-chloro-alanine treatment was not inhibited the activity of alanine and aspartate transaminases. In these cells, the effects of β-chloro-alanine appeared to occur with little perturbation of other cell functions: therefore total lipid biosynthesis from acetic acid was not decreased [25].

Tetrahymena cultures in a complete synthetic nutrient medium show high growth rate. This medium contains among others both essential and nonessential amino acids and pyridoxal HCl. If nonessential amino acids were excluded from the nutrient medium, the cells multiply extremely slow in the absence of carbohydrate [27]. The possibility is raised that in this phenomenon the transaminase activity plays an important role, and this activity is pyridoxal-dependent. The disadvantageous effect of β-chloro-alanine for *Tetrahymena* may be a consequence of inhibition of transaminases. The toxic effect of 5 mM β-chloro-alanine in *Tetrahymena* (which concentration did not inhibit the activity of these enzymes in CHO cells) corroborate this possibility, and refers to the differences between mammalian (CHO) and *Tetrahymena* cells.

In the literature, available data are contradictory: Florin-Christensen et al. [5] found the β-chloro-D-alanine to be efficacious on the inhibition of LCB formation; while Medlock and Merrill [25] found inhibitory activity only in the case of L-variant.

In our present experiments β-chloro-D-alanine caused spectacular alterations in the incorporation of serine: applied the ^{3}H-serine treatments after 60 min of the beginning of β-chloro-alanine treatments in PC; PE; PS and ceramide, significantly higher radiolabel was detected than in the controls, and also the shorter β-chloro-D-alanine treatments elevated the radioactivity in these lipids (Table 1).

β-chloro-D-alanine treatments resulted in the ^{3}H-palmitic acid incorporation similar alterations, except in the PC and PE in the 5 min groups, where values similar to the controls were measured.

At present we do not know the reason of these results. In all probability there are differences in inhibition/stimulation between the head group component serine and the hydrophobic tail component palmitic acid uptake and metabolism. In the palmitic acid-labeled lipids the decarboxilation of PC, and methylation of PE are inhibited, but in the case of ^{3}H-serine labeled lipids this phenomenon is unrecognizable. A further unexplainable phenomenon is the higher radioactivity in ceramide, extracted from β-chloro-alanine treated *Tetrahymena,* compared to the controls. β-chloro-alanine inhibits ceramide formation and serine incorporation into this lipid in all other cell types tested so far. Although in *Tetrahymena* the presence of sphingolipids are well documented [e.g. 9], the presence of enzymes catalizing the metabolic steps in sphingolipid metabolism are not widely known.

Similarly to the β-chloro-alanine, L-cycloserine inhibits the activity of SPT [28]. In *Pneumocystis carinii* L-cycloserine was a potent inhibitor of serine incorporation into LCB, but less potent of incorporation into PE, PC and proteins [5]. The rate-regulatory enzyme for PC synthesis is CTP : cholinephosphate cytidylyltransferase (CT). L-cycloserine was shown to stimulate CT activity by 74% and increase disaturated PC in alveolar lavage by 52% [24]. In *Giardia intestinalis* L-cycloserine inhibited the transport and metabolism of L-alanine, as did a number of amino acids including glycine, serine and threonine [4]. These results indicate that the alterations in the uptake and metabolism of lipid precursors are explainable in different manner, and there are differences between certain cell types.

In the case of L-cycloserine treatments the inhibition of PS → PE conversion by decarboxylation and PE → PC by methylation are clearly recognizable in the ^{3}H-serine treated *Tetrahymena* cells (Table 2). In this treatments the ceramide labeling was also decreased. Another head group component, phosphorus caused similar results in phospholipid metabolism (except PC in the 5 mM L-cycloserine treated groups) (Table 4). Also in the case of L-cycloserine treatments differences were detected between the serine and palmitic acid-labeled cultures. The labeling of ceramide also in these groups was higher (Table 3). However, the phospholipids showed similar metabolic pattern to the ^{3}H-serine labeled ones (except PC in the 5 mM treated groups after 60 min).

The rates of uptake and metabolism of exogenous materials in the case of free-living cells is very complex, involving several factors including mechanisms operative in the cell, what they can and cannot synthetize, the availability of the substrates of enzyme pathways in the cells or in the environment (culture medium), the alterations in the environmental conditions, etc. In the case of cells living in multicellular organisms, where the surroundings are precisely determined, these conditions are presumably not so varying, hence in these cells the determined conditions make more independent the metabolic pathways as compared to the free-living cells, where these metabolic pathways alter very sensitively by the effect of environmental changes. In the course of these alterations in the free-living cells the connections between different pathways (e.g. the sphingolipid and gycerophospholipid) influence each other more vigorously than in the cells of multicellulars. The unicellulars are complete individual; they are able to carry out each life phenomenon characteristic for complete multicellular organisms, thus their reactions frequently differ from the reactions of cells derived from multicellulars after treatments. Also the results of the present experiments indicate that in the case of a certain treatment it is very difficult to state a causal relation between the treatment and the results.

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CHEMOTAXIS: THE PROPER PHYSIOLOGICAL RESPONSE TO EVALUATE PHYLOGENY OF SIGNAL MOLECULES*

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In this review we summarize our results gained on the investigations focused to characterize ligand and signaling mechanisms required for the chemotaxis in the unicellular model *Tetrahymena.* Our data show that short chain signal molecules (amino acids, oligopeptides) are distinguished upon their physicochemical characteristics – lipophylicity, residual volumes and statistical distribution of side-chain distances (e.g. in proline containing dipeptides), while the vertebrate hormones have also specific attractant or repellent effects in the model (FSH vs. TSH). Hormonal imprinting developed by pretreatments has also special, signal molecule dependent effect (histamine vs. serotonin). It is shown that "chemotactic selection" of cells, by the new probe developed by us is a suitable tool to provide subpopulations possessing enhanced chemotactic receptor–effector mechanisms with respect to the selector signal molecules (IL-8, TNF-α).

Keywords: Chemotaxis – signalling – hormonal imprinting – chemotactic selection – *Tetrahymena*

INTRODUCTION

The ability to recognize and select different molecules had a significant role in the early evolution of molecular development in living organisms. Because the detection of chemical signals was essential to both uni- and multicellular organisms, the development of ligands and their proper receptors represented an intercellular communication dependent and molecule-dependent event. According to Lenhoff's theory, molecular selection played a key role in this process [47]. Although the number of candidate molecules (e.g. short and long peptides) was high, most of them were consumed simply as nourishment, and had no special physiological effect on the target cells. Only those molecules "selected" as efficient signal molecules which were able to induce metabolic or other pathways. This mechanism is rather complex, involving physicochemical characteristics (stereo- and electrochemical properties) as well as

* Dedicated to Professor György Csaba for his 70th birthday.

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structural matching between ligand and receptor which influence selection. The present day ligand–receptor complex interactions are thought to be the results of the above mentioned processes [9, 11].

A wide rage of different experiments proved the feasibility of the unicellular ciliated *Tetrahymena pyriformis* GL as a good model for evaluation and characterization of the phylogeny of signaling [10]. Homologies were described on different levels: these cells possess binding sites/receptors in the surface membrane for vertebrate type hormones e.g. insulin [8], ACTH [20], ET-1 [32]; presence and adequate functioning of second messenger systems like cAMP [18], cGMP [29], IP3 [26] and Ca^{2+}-calmodulin [28] show also homologies with the higher ranked models and essential metabolic activities like carbohydrate metabolism, are modulated also similarly as in the higher ranked animals [30]. The complexity of the unicellular signaling is more underlined since on the basis of pioneering works of Csaba [19] several endogenous vertebrate hormones were described e.g. insulin [49], relaxin [58], ACTH [48], ET-1 [32] in *Tetrahymena.* The biological role of these molecules is still obscure, possibly they have a role in autocrine and paracrine regulation or they represent phylogenetic ancestors of the vertebrate isoforms.

One of the most "physiological" response of these motile model cells is the chemotaxis. Although these cells have different swimming behaviours (e.g. swimming, creeping, sliding, coiling) [54, 55] their frequent trajectory swimming makes possible to detect the required range of concentrations for the chemotactic response. Previous data showed that similarly to bacterial tumbling, in this movement there are also "stop" phases, due to the ciliary reversal. This interrupted way of swimming makes possible to develop a short-term memory by consecutive shifts from adaptation into deadaptation phase [46]. Scanning of environment via the above-mentioned motions makes possible to detect a variety of different chemoattractant or chemorepellent signal molecules. Inorganic salts [61], amino acids [3, 50], short and longer peptides [44] were reported as specific chemotactic factors to *Tetrahymena,* the effectiveness of these molecules is dependent both on the molecular structure and concentration. The high selectivity and sensitivity of these cells to chemoattractant or chemorepellent substances explains our decision, to consider chemotactic response as an index to evaluate the character of signal molecules or effect of different treatments on the basis of the chemotactic responsiveness of the cells.

In the last decade most of our projects focused on the characterization of phylogeny of signal molecules. Our goal was to describe the preferred and proper molecular structures possessing chemotactic character to answer a general problem: what makes signal molecule suitable for signaling in chemotaxis? In the majority of these works axenic cultures were investigated in the logarithmic phase of growth, but the effect of inorganic environment was also tested. The chemotactic activity of cells was determined in capillary assays [45] modified by us [40]. In the study of chemotactic selection the optimal chemotactic concentrations were applied according to the former concentration course experiments.

We approached the general problem from three main aspects: the ligand; receptors (binding sites) and pretreatments; receptors (binding sites) and selection.

On the basis of the above mentioned facts our present work attempts to summarize our results in four fileds of problems:

1. Do amino acids and short chain peptides have characteristic, signal-molecule like chemotactic effects on the phylogenetically lower level eukaryote *Tetrahymena?*
2. Do vertebrate hormones influence also chemotactic effects at this level of phylogeny?
3. Does hormonal imprinting have any molecule dependent influence on chemotactic response in the cellular progeny?
4. Will cellular progeny of signal molecule selected subpopulations via chemotaxis have a higher response to the selector substance?

CHEMOTACTIC EFFECTS OF AMINO ACIDS AND SHORT CHAIN PEPTIDES

Among other organic substances [7] amino acids and short chain peptides are the primary candidates to be consumed as nourishment [13]. In contrast the "simple" usage of these molecules, it was demonstrated in prokaryotes that amino acids e.g. Asp or di- and tripeptides have the ability to induce chemotactic responses and some significant receptors are well characterized [6, 53]. The protein circuit based on phosphorylation-dephosphorylation of "Che" proteins, mediating chemotactic stimuli from the receptor to the effector system is almost the only completely known biochemical information process in intracellular signaling [2, 52]. On the other hand, bacteria are not only migratory units of the environment, but some of the bacterial short peptides e.g. formyl Met-Leu-Phe are potent chemoattractants of vertebrate granulocytes [57]. All these findings were not surprising as both amino acids and oligopeptides have characteristic physicochemical characteristics, they are different in the size of side chains, hydrophobicity or solvent accessible areas. The high variance of these and other factors explains why we focused a significant part of our experiments onto this group of molecules. A selection of physicochemically divergent five amino acids representing structures with non-polar aliphatic R groups (Pro); molecules with positively (His) or negatively (Glu) charged R groups; polar amino acids with uncharged R groups (Met) or with an aromatic character (Phe) were studied, on the basis of their chemotactic potency. In these works the significance of environmental conditions and the optimal concentrations were evaluated since previous data of the literature were focused only to the higher concentration range (10^{-6} –10^{-3} M), and no environmental effect was analysed in parallel assays [3, 50]. Chemotaxis was tested in normal axenic culture medium and in Losina Losinsky solution, the physiological starving medium of *Tetrahymena* containing only inorganic ions. Our results showed in concert with previous data of literature [62] (Fig. 1) that *Tetrahymena* is really a very good and selective chemotactic sensor-organism. These introductory results showed us that there are strong chemorepellent molecules e.g. the aromatic Phe where neither the applied concentrations nor the environment could not modify this character.

The applied *concentration* was also decisive in other cases, e.g. Met could induce significant chemotactic effects in the low concentration regardless the composition of the environment. Similar characters were observed in the case of Pro, however, in this type of amino acid the higher concentrations made the chemotaxis reciprocal in the different media: in the normal medium Pro could work as a chemoattractant but in the inorganic environment its character turned into a strong repellent. The opposite tendency was observed in the case of Glu bearing negatively charged R groups. This molecule, or chemotaxis induced by it seems to be very sensitive to the *environment,* as both in low and high concentrations of Glu works as a strong repellent in the medium, however, in Losina solution it works as a chemoattractant. Histidine showed also high sensitivity to the composition of the environment. This amino acid could induce the most intensive chemotactic response, but only in medium and in the low concentration study.

Since the results reviewed above confirmed our basic concept that these relatively small organic molecules are still able to work not only as simple food substances but as efficient signal molecules in our *Tetrahymena* model. In the next step chemotactic effect of "mirror" variants of proline containing dipeptides was analysed. Building this special component as reference unit into peptides was promising for several reasons. Structure of proline differs sharply from that of other amino acids in that its side chain is bonded to the nitrogen as well as to the α-carbon of the central compound. This amino acid is very rare in alpha helical structures, as intramolecular

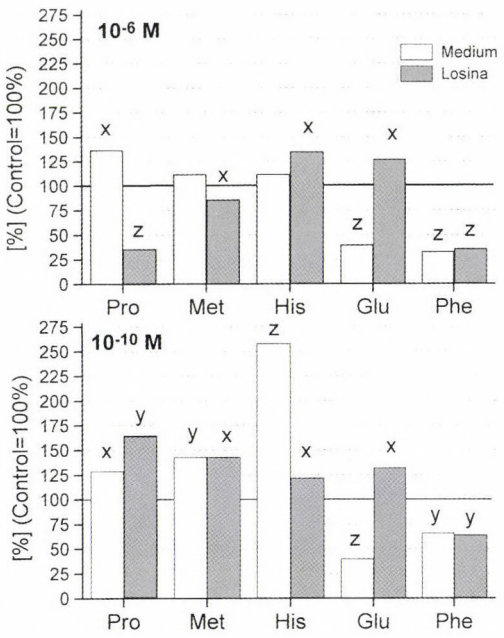

Fig. 1. Chemotactic effect of amino acids applied in 10^{-6} M and 10^{-10} M concentrations to *Tetrahymena* cells (x – $p < 0.05$; y – $p < 0.01$; z – $p < 0.001$)

rotations of N-Cα bound are limited by the rigid pyrrolidine ring, on the other hand, this is the responsible amino acid for reverse turns in proteins [43].

In these experiments [42] Pro was on the carboxy- or aminoterminal part of the molecules. Our results showed that presence of Pro makes the majority of the 12 tested substances chemoattractant. In the case of carboxy-terminal Pro some physicochemical characteristics of the partner amino acid like the residual volume or lypophilicity of the molecule had a good correlation with the chemoattractant character of the molecule (Fig. 2), in this way the small aminoterminal amino acids or the low lipophilicity was preferred like Gly-Pro and Ala-Pro, while chemotactic behaviour of Val-Pro – due to other, still obscure reasons – did not follow the trends described above. It is worth to mention that optimal separation distances between two sidechains in the dipeptides seem to be essential in the case of dipeptides (Fig. 3) which fact varifies our previous hypothesis that a well defined, optimal matching e.g. Gly-Pro and Ala-Pro is required between the ligand and its receptor for maximal chemotactic responses, while significant shifts or diversities of this profile (Phe-Pro or Val-Pro) results inadequate stimulation. All these data strengthen our concept that – although in the background there are complex interactions –, changes in the intramolecular polarity might be responsible for the modification of the chemotactic character in relatively small organic compounds.

In further experiments more mirror variants of dipeptides and some tripeptides composed by Ala, Ser, Leu and Gly were also tested. In these model peptides the

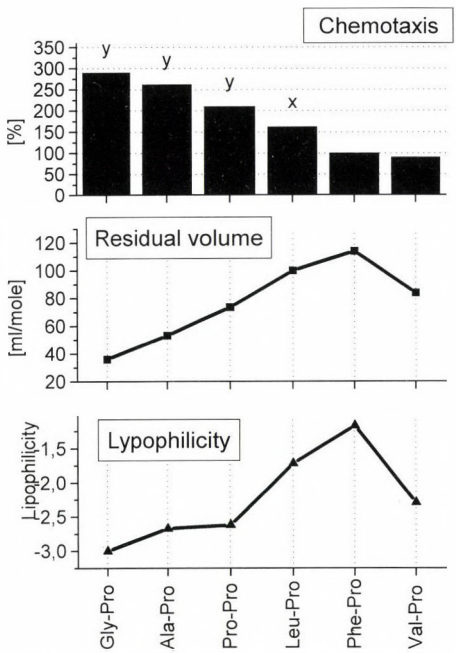

Fig. 2. Chemotaxis induced with dipeptides containing carboxy-terminal Pro. Relations of residual volumes or lipophilicity to the chemotactic activities elicited

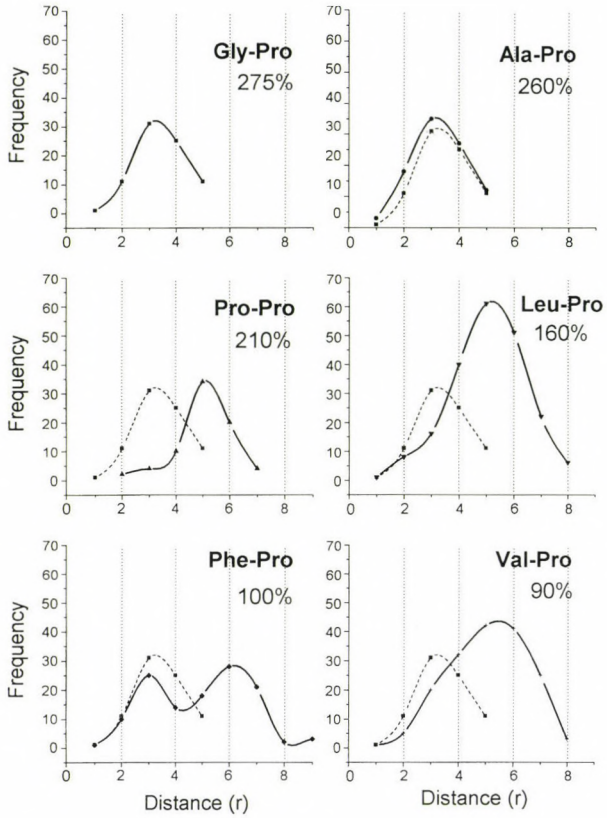

Fig. 3. Distribution of separation distances between the two sidechains of six Pro containing dipeptides. Percents represent the chemotactic activities of dipeptides compared to the identical controls

unique characteristics of proline, e.g. pKa values reaching the limiting ranges, were not expressed. Chemotactic assays with these molecules showed clearly that the size of amino acid has slight effect on the chemotactic behaviour of the molecule. The Leu-Leu dipeptide composed by big and the Ala-Ala dipeptide composed by small amino acids could induce chemotaxis on the same scale, while di- or tripeptides of the also small amino acid Gly were chemorepellent (Fig. 4). Other results suggested that the hydrophobic character is advantaged on the carboxy-terminal part of the molecule (Ser-Ala, Ser-Phe), however, there might be correlation between the hydrophobicity and pKa values of both the carboxyterminal and aminoterminal parts in identical peptides (Ser-Phe, Ala-Ser) with a preference for the higher values on both terminus (Fig. 4b). It was concluded from evaluation and comparison of data gained by tripeptides that increasing the length of the peptide chain with a single amino acid could result significant changes on the level of biological responses, but it is not regular. In the case of tripeptides, the modification of the Gly-Gly-Gly into

Ala-Gly-Gly results a more potent molecule (with a weak hydrophobic aminoterminal part), but the intramolecular modification of the molecule resulting Ala-Pro-Gly, has almost no further effect, while its mirror variant Gly-Pro-Ala proved to be the most chemoattractant among the peptides tested (Fig. 4a). This change of the molecular character is hard to be explained, nevertheless we have to remember that the activity of this molecule resembles its precursor dipeptide Gly-Pro and in this relation it is not neglectable the relatively small and close to neutral Ala probably has no potency to modify the strong chemoattractant character of Gly-Pro.

The problem of longer peptide chains mentioned above was tested more with three types of model oligopeptides composed by 1 to 5 units of the same amino acids (Gly,

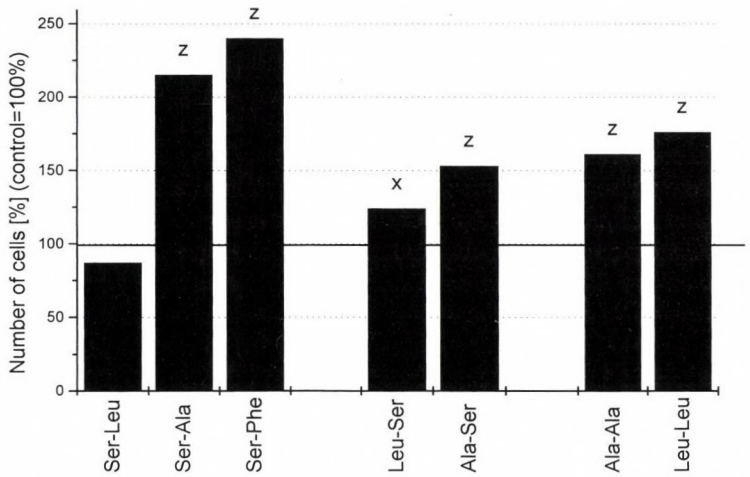

Fig. 4a. Chemotactic potency of 10^{-6} M di- and tripeptides composed by Gly, Ala and Pro

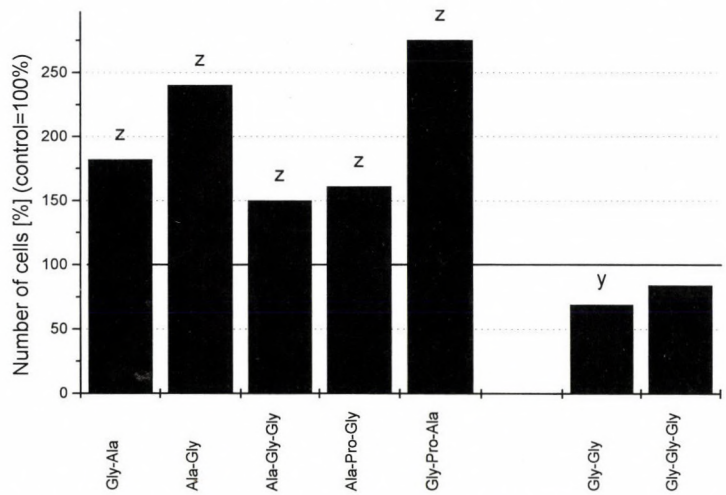

Fig. 4b. Chemotactic potency of 10^{-6} M di- and tripeptides composed by Ser, Leu and Ala

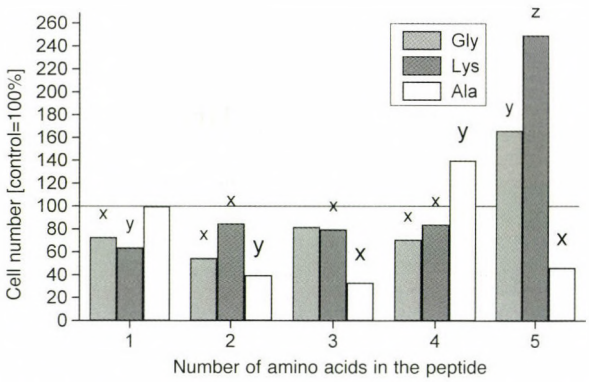

Fig. 5. Chemotactic activity of 10^{-6} M Gly, Lys and Ala and their oligopeptides composed by the same amino acid in *Tetrahymena*

Ala, Lys), in inorganic environment [39]. The chemotactic effect of these peptides (Fig. 5) showed that only the polar, hydrophilic oligopeptides, composed by 5 Gly or 5 Lys could work as potent chemoattractants, the shorter chains were chemorepellent or neutral. Comparison of the dipeptide results – gained in normal medium – with these data, underlined again the individual sensitivity of amino acids or peptides to the environment [17]. In these conditions 2-Ala (Ala-Ala) could not express its previously described chemoattractant character, however, the 4-Ala proved to be optimal with its significant chemoattractant character.

In summary of the experiments mentioned above, it can be concluded that these short molecules have the selective ability to work as chemoattractants or chemorepellents in our model system. Our results support the almost 20 year old findings of Tanabe et al. [62] that hydrophobicity is the determinant in chemotaxis as it has the proper depolarizing effect to the *Tetrahymena* membrane. On the basis of our investigations we suggest to comprise into previous theory that some other characteristics of the molecules like pKa values of the carboxyterminal amino acids or the intramolecular hydrophilic-hydrophobic axis seems to be essential, optimal matching of these factors – and other still unknown effects – is required for the induction of chemotactic process. The way of induction is not known but interaction with these molecules or incorporation of them into some compartments of the cell fulfills Leick's hypothesis [44], that an anterior location of small vesicles with a hydrophobic microenvironment in their membrane is important for the directed migration during chemotaxis.

CHEMOTACTIC EFFECTS OF VERTEBRATE HORMONES

On the basis of the specific chemotactic effects of amino acids and model peptides it was worth to evaluate the signal character of such larger oligo- or polypeptide hormones which possess well-defined biological effects in vertebrates. Detection of

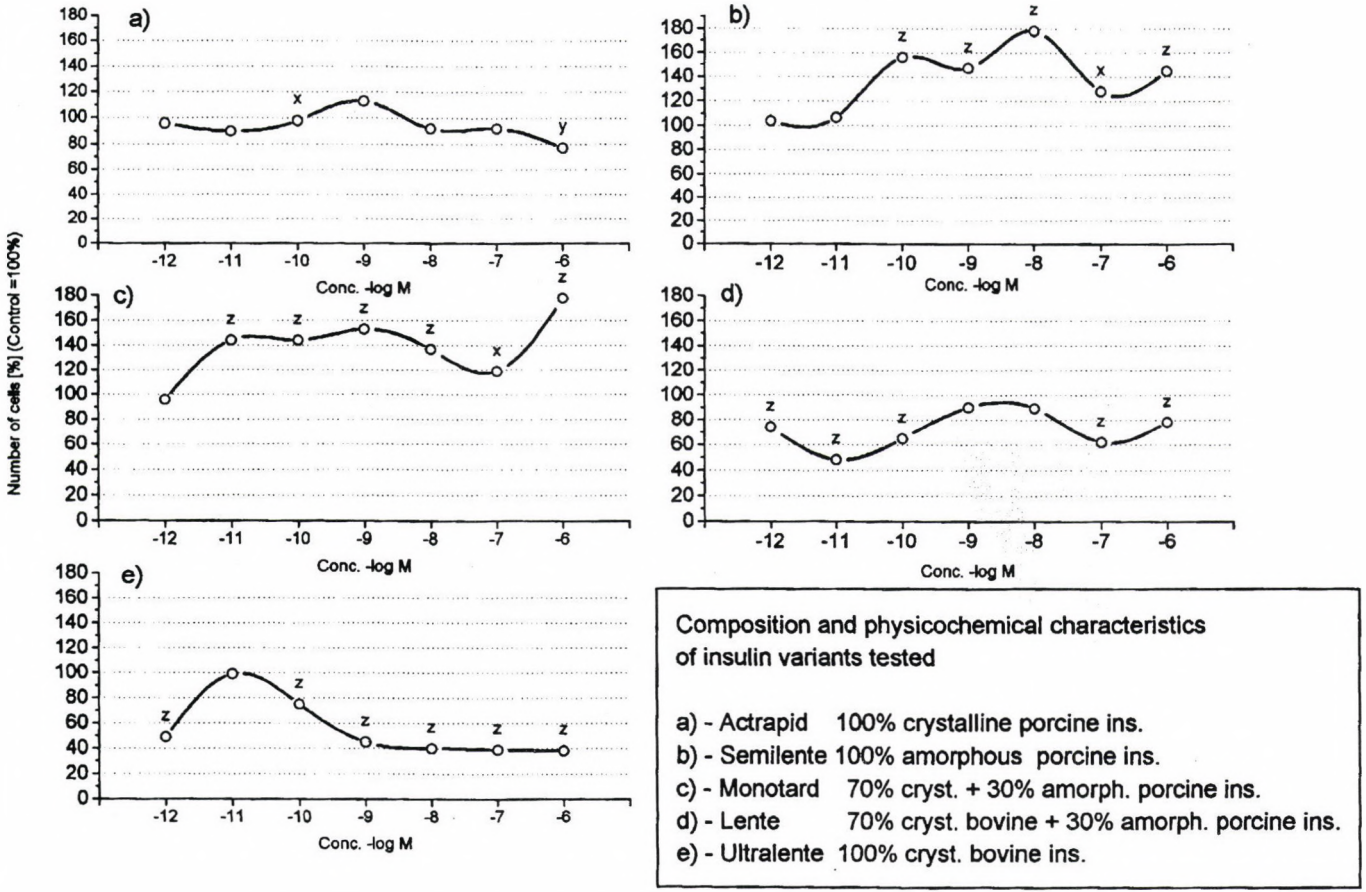

Fig. 6. Concentration course of chemotaxis induced with variants of insulin in *Tetrahymena*

their chemotactic nature in *Tetrahymena* was promising, even when they are not chemotactic in the higher ranks of phylogeny, as some of these hormones were already detected as endogenous substances of this ciliate.

Investigations of peptides present in different fluids of vertebrates showed that several growth factors proved to be effective chemoattractant to normal and malignant cells [65], some of them e.g. PDGF were also reported as chemoattractants for *Tetrahymena* [4].The frequently studied hormone, insulin belongs into this group as its chemotactic character and the requirement of selective induction of its receptor mediated signaling was demonstrated in CHO cells [64]. All the facts mentioned supported our purpose to test whether the system of vertebrate chemoreception works in ciliates. At first concentration dependence of chemotaxis induced by bovine and porcine isoforms was tested [14]. Results of these experiments point to that *Tetrahymena* is also able to distinguish relatively slight differences in insulin (Fig. 6) (they differ in their A chains as in the bovine form Ala and Val, in the porcine form Thr and Ile are present in the identical and functionally important positions) and prefers the porcine isoforms, while bovine insulins have repellent effects. Its nanomolar effectiveness has also good correlation to the chemotactic activity of porcine insulin to human T lymphocytes [5]. Over the above-mentioned observations, the chemoreception of our model seemed to be also sensitive to physicochem-

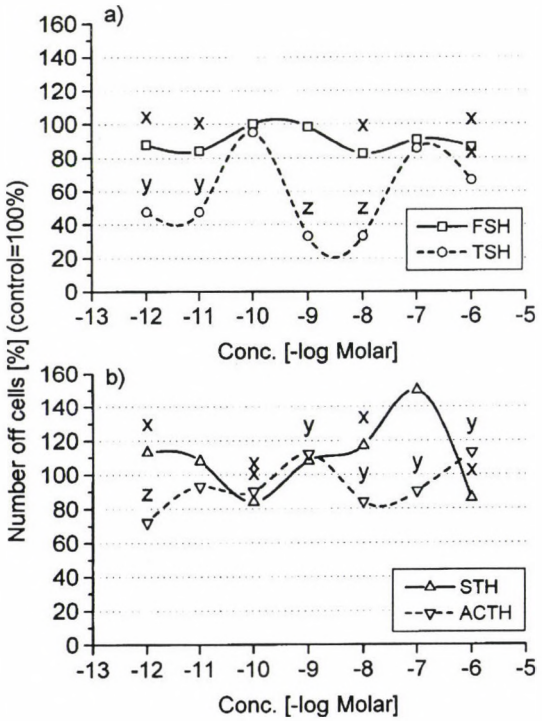

Fig. 7. Concentration course of chemotaxis induced with four characteristic hormones of the hypophysis

ical characters of the applied insulins as amorphous insulins were more effective than the crystalline isoforms.

Hormones of the hypothalamo-hypophyseal system were also good models in chemotaxis studies [33]. Although their molecular mass is ranging from 1000 to 40,000 and their size makes them ideal food substances, the size itself and differences in it had almost no influence on the chemotaxis which indicates to that only appropriate sequences or parts of the molecules are responsible for their action in this ciliate. Concentration course studies proved (Fig. 7) that two structurally homologous glycoprotein hormones TSH and FSH – their α subunits are identical and the β subunits have also homologous sequences – have diverse effects: the repellent effect in TSH was much more expressed than in FSH. Chemoattractant character was observed in the case of other two hormones STH and ACTH (Fig. 7). STH had a significant chemoattractant effect at 10^{-7} M but it was effective in the 10^{-9}–10^{-8} M range too, while ACTH had only one and weak peak at 10^{-9} M. Although the primary effects of these hormones is not to induce chemotaxis in vertebrates it is worth to note that serum concentrations of the two hormones are in a close, 10^{-11}–10^{-10} M range, which foreshadows the presence of a phylogenetical continuity of these signaling systems.

The selectivity of chemotaxis was also proved by two other members of the family mentioned above (Fig. 8). The sequence of the two nanopeptides vasopressin and oxytocin vary in position 3 (Phe-Vas.; Ile-Ox.) and position 8 (Arg-Vas.; Leu-Ox.) but these differences are so significant that these hormones have not only diverse physiological effects in mammals, but the chemotactic potency – repellent effect – of vasopressin in the low concentration range (10^{-12}–10^{-9} M) significantly differ from oxytocin. The chemorepellent effect itself in *Tetrahymena* is surprising as oxytocin was already referred to be chemoattarctant to other highly motile cells, the mouse spermatozoa [59], however, the presence of oligopeptides in the ovarian granulosa

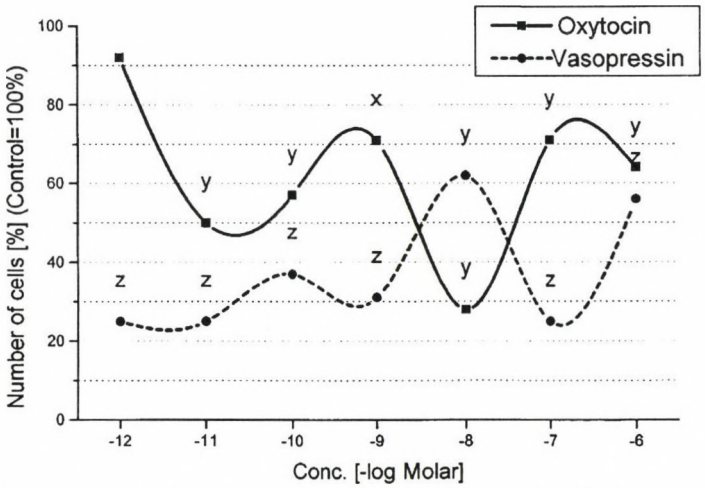

Fig. 8. Concentration course of two nanopeptides, oxytocin and vasopressin

cells and the fluid around, explains the attractant character. In addition, this the difference between the effects of the two relative peptides should be discussed. It is important to note that oxytocin is phylogenetically more ancestral [22] than vasopressin and it is presumable that because of this ancestral character of oxytocin it is considered as less "foreign" molecule than the strong repellent vasopressin. The more intensive induction of contractile vacuoles by oxytocin was also deduced from the above mentioned phylogenetical difference of the two molecules [48].

Finally we review the effects of two hormones thought to be specific to the circulatory system: endothelin-1 (ET-1), a representative of potent mediators of vasoconstriction in mammals and atrial natriuretic peptide (ANP), which complex physiological effects are portrayed as induction of natriuresis, inhibition of aldosterone, vasopressin and renin secretion and relaxation of smooth muscles. Both hormones are polypeptides, the effective form of ET-1 is composed by 21 amino acids, while the length of ANP varies from 21 to 73 amino acids in various vertebrate tissues. Though some references pointed to the chemotactic effect of ET-1 and ANP in PMN [21, 63] and monocytes [1]; our chemotaxis assays proved for the first time in the literature that these hormones can act selectively at the level of protozoa (Fig. 9). Comparison of the two hormones showed that, although *Tetrahymena* considers both hormones as chemoattractants even at very low concentrattions (10^{-15} M), the ANP-response is more significant. Here we have to remind of that ET-1 like endogenous substances were also detected in *Tetrahymena* which provides the possibility of auto- and paracrine regulatory mechanisms in contrast the "foreign" and therefore more inducing ANP. Problem of phylogenetical continuity is again propounded as we can find again overlapping ranges of the effective concentrations and the serum levels of these hormones even in human.

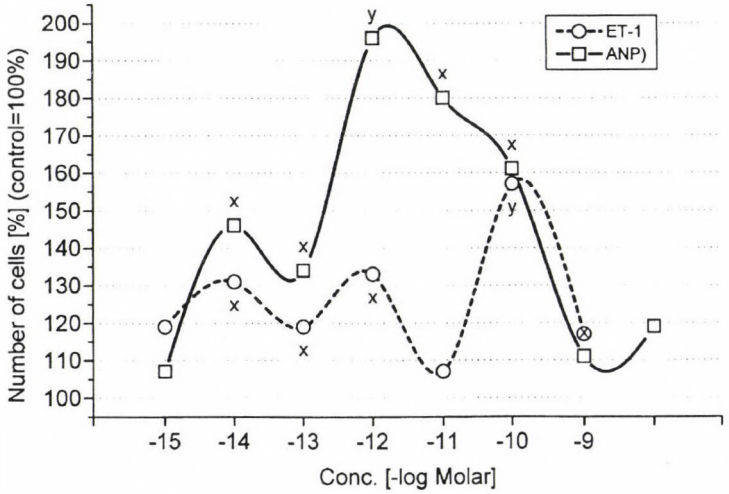

Fig. 9. Concentration course study of two cardiovascular peptides – endothelin-1 (ET-1) and atrial natriuretic peptide (ANP) in *Tetrahymena*

The conclusion of our results reviewed in this part show that not only small molecules – amino acids or short oligopeptides – have the potency to induce chemotaxis in our model cell. Even relatively small structural divergences e.g. insulin variants, TSH-FSH, oxytocin-vasopressin are selectively detected via chemotactic responses. These results point to that the *Tetrahymena* model is still a proper object for the ligand – receptor studies focused to characterize the optimal – big or small – signal molecule for chemotaxis or interactions between chemotactic signal molecules and the target cell.

RELATION OF HORMONAL IMPRINTING AND CHEMOTACTIC RESPONSE IN THE CELLULAR OFFSPINGS

Following characterization the main types of ligands required for chemotactic responses, essential part of our work was to get information about dynamics of the inducible mechanisms. In this case, to study the development of hormonal imprinting was very advantageous. Hormonal imprinting develops by pretreatments of cells or whole organisms, being in sensitive period, with signal molecules [12] (Fig. 10). The result of the pretreatments is a "tuned" cell or organism which has a altered responsiveness to the "imprintor" – the signal molecule treated with. In many cases this means that a special memory develops in the "imprinted" cells and at a second encounter with the imprintor molecule the cell or the offsprings of it usually express a changed, often an enhanced response for hundreds of generations [36]. There are several levels of which we can influence this process: the membrane and its receptors [41, 51], the second messenger mechanisms [24] and other enzyme systems [27]

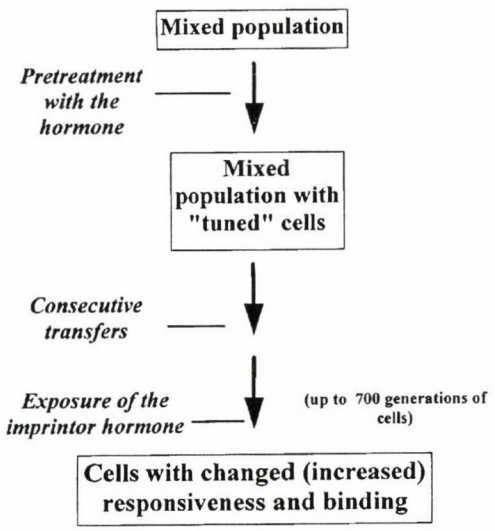

Fig. 10. Development of hormonal imprinting – general scheme

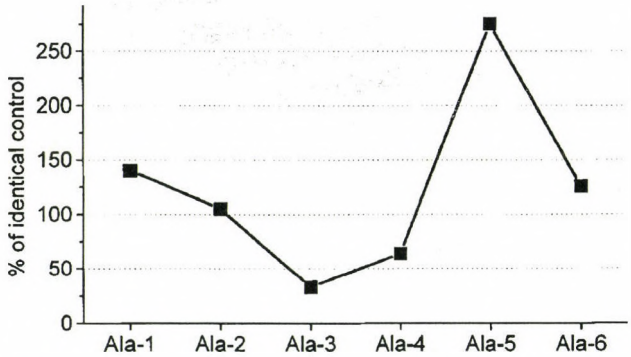

Fig. 11. Effects of hormonal imprinting with 10^{-10} M Ala and its oligopeptides on the chemotaxis to the imprintor Ala variants in *Tetrahymena*

or some nuclear mechanisms [38], all were proved to be responsible for development of the mechanism, but the size or other chemical characters of the acting ligand [31], the applied concentrations [16] the time courses and down-regulation [15] also influence the process, however, the whole pathway of saving the character of the first signal is still obscure.

In this relation study of chemotaxis in imprinted and in control *Tetrahymena* cultures provides a more complex understanding the ligand-receptor interactions of chemical signaling. At first the nonhormone Ala amino acid and its oligopeptides were studied [17] (Fig. 11). Following the pretreatments with these substances the cells were tested again in the 70th offspring generation. Results suggest that the length of chain is important in the imprinting, as the 5 alanine unit containing oligopeptide proved to be an excellent imprintor and chemoattractant, however, there is no consistent relation between the length and its imprintor capacity. Among others, four hormones – ACTH, insulin, histamine and serotonin –, were also tested as imprintors [37] (Fig. 12) and their ability to induce long-lasting memory was clearly different. Insulin and ACTH, the two moderately large hormones (Mol. weights: insulin – 5700, ACTH – 4500) worked in a diverse way, pretreatment with ACTH resulted a significantly enhanced chemotactic response, the insulin pretreated cells expressed also higher chemotactic activity then the absolute (C/C) or relative controls (INS/C), but this activity was lower than the response to insulin at the first encounter. The contradiction between the results cited above and our binding site studies points to that the high number of binding sites for insulin following pretreatments [25] is not unambiguous guarantee of enhanced physiological response e.g. chemotaxis in a ciliate.

Chemotactic potencies of the two structurally related biogenic amines – serotonin and histamine – were also different following pretreatments (Fig. 12). Although histamine and insulin differ not only in molecular characters but their signaling mechanisms are also diverse, the profiles of their chemotactic responses following the pretreatments are similar, the first encounter was more successful then the second. These

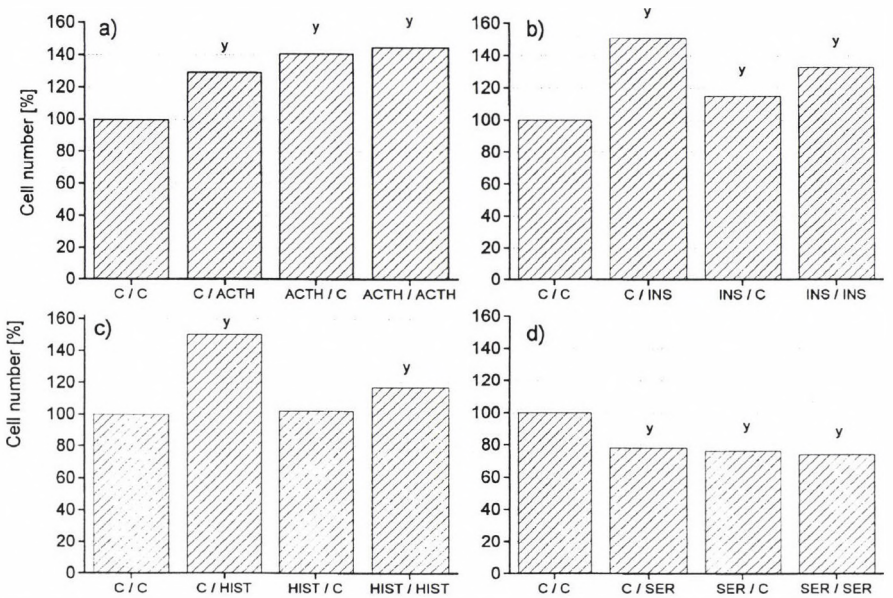

Fig. 12. Hormonal imprinting with four hormones (a – 10^{-9} M ACTH; b – 10^{-10} M insulin; c – 10^{-10} M histamine; d – 10^{-8} M serotonin)

experiments proved again our observation concerning the repellent effect of aromatic ring as serotonin, which molecule is different from histamine in its aromatic ring has adverse, chemorepellent character, and pretreatments with serotonin expressed the signal receiver mechanism to this effect.

EFFECT OF CHEMOTACTIC SELECTION ON THE RESPONSIVENESS OF SUBPOPULATIONS

Besides hormonal imprinting a new technique the "chemotactic selection" was developed by us [34] (Fig. 13). Since the hormonal imprinting is acting on mixed populations bearing receptors in different functional phases, there might be factors, (e.g. being in different phases of the cell cycle) which can influence the sensitivity of cells. Therefore our goal was to get "homogeneous" subpopulations concerning their responsiveness (receptors). In the basic hypothesis the chemotactic response is used as a receptor mediated process and positive responder cells are considered as a subpopulation possessing the appropriate receptor of chemotaxis and/or its signaling mechanism in a higher volume. Application of this technique made possible to evaluate different signal molecules whether the chemotactic responses induced by them is a dynamic, short-term character of the cells or it is a long-lasting and permanent quality of the subpopulation.

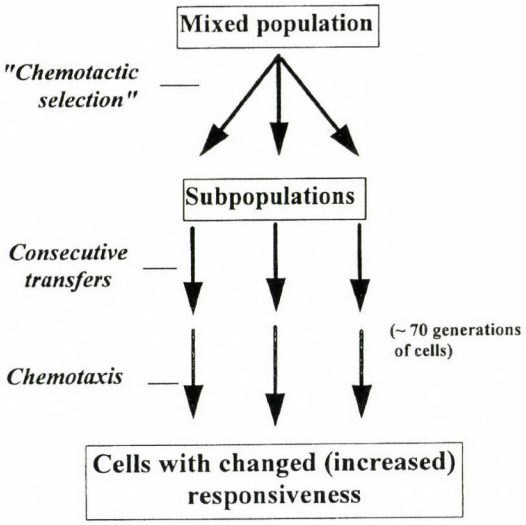

Fig. 13. Chemotactic selection – general scheme

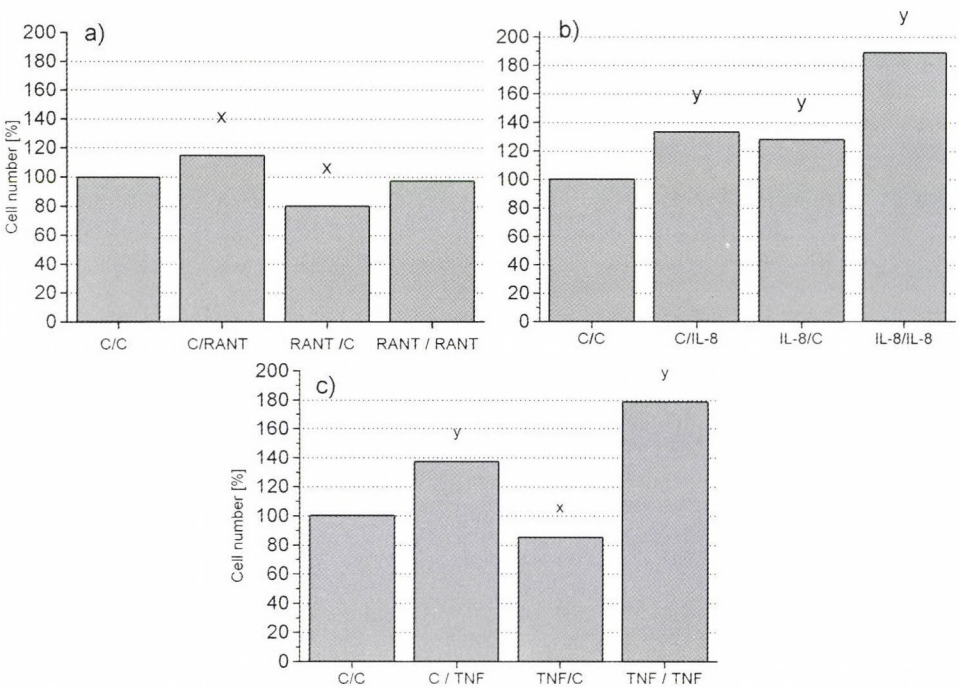

Fig. 14. Chemotactic responsiveness of subpopulations selected via chemotactic selection to 75 ng/ml RANTES (a), 1 ng/ml IL-8 (b) and 1 ng/ml TNF-α (c)

In this relation a the CC chemokine IL-8, the CXC chemokine RANTES and TNF-α were tested. The two chemokines are *per se* chemotactic in different target cells of mammals [23, 56], but the third cytokine TNF-α is also chemoattractant to special cells [60]. Concentration course studies documented that all the three cytokines can induce chemotaxis of *Tetrahymena,* too [34], and the sensitivity to IL-8 was higher than in the mammal reference, while RANTES worked in the same range, which shows that cytokines have also deep phylogenetical backgrounds in their receptor mediated functions.

On the basis of previous results chemotactic selections were done with the optimal concentrations of the cytokines and following consecutive transfers the chemotactic responses were analysed again towards the selector cytokine in the 70th generation. The differences of chemotactic responsiveness (Fig. 14) show that the two structurally divergent chemokines (IL-8 and RANTES) have various potencies in chemotactic selection: the CC chemokine IL-8 and TNF-α are good selectors as subpopulations of the chemotactically selected with them have significantly enhanced response, while the CXC chemokine RANTES cannot select via chemotaxis, its chemotactic character proved to be uncertain in long-lasting experiments.

Other still unpublished data also suggest that the mechanism mentioned above – to select high-responder subpopulations via chemotaxis – is a ligand specific process and chemotactic selections with amino acids, oligopeptides or peptide hormones are linked to specific signaling mechanisms.

In the future, the major purpose of our work will be to clarify the afferent or efferent mechanisms determining the succesful selection via chemotaxis, therefore our investigations are essentially focused to different points of the interactions between receptor and ligand.

CONCLUSION

In our works reviewed above the chemotaxis was used as a functional test of mostly surface membrane receptor mediated processes. The aim of our work was/is to characterize the ligands required for chemotactic responses and to get information about dynamics of signaling mechanisms making possible these reactions. Using *Tetrahymena* the survey proved that our model is as sensitive object for these experiments as the other cells applied previously in higher ranked organisms. Concerning the types of ligands we conclude that amino acids and oligopeptides are distinguished upon their physicochemical characteristics (e.g. in proline containing dipeptides – lipophylicity, residual volumes, statistical distribution of sidechain distances). The sensitivity of the model is not strictly dependent upon the size of the interacting molecule as small or large vertebrate hormones could work as attractant (e.g. histamine, STH) or repellent (e.g. serotonin, TSH) signals as well, but it perceives slight changes in the interacting signal sequences of the molecule (e.g. insulin variants). Studies of hormonal imprinting suggest that chemotactic responses following the pretreatments are also molecule dependent and the phylogenetically selected signal

molecules have a unique potency to modify chemotactic responsiveness even in the offspring (e.g. ACTH). Finally our chemotactic selection studies open a new field in investigations of ligand–signaling relations as it makes possible to evaluate the durability of chemotactic characters of subpopulations selected with chemotactic, however, different molecules (e.g. chemokines).

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RECEPTOR TYROSINE KINASE, AN AUTAPOMORPHIC CHARACTER OF METAZOA: IDENTIFICATION IN MARINE SPONGES*

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In the present review we summarize sequence data obtained from cloning of sponge receptor tyrosine kinases [RTK]. The cDNA sequences were mainly obtained from the marine sponge *Geodia cydonium*. RTKs *(i)* with immunoglobulin [Ig]-like domains in the extracellular region, *(ii)* of the type of insulin-like receptors, as well as *(iii)* RTKs with one extracellular speract domain, have been identified. The analyses revealed that the RTK genes are constructed in blocks [domains], suggesting a blockwise evolution. The phylogenetic relationships of the sequences obtained revealed that all sponge sequences fall into one branch of the evolutionary tree, while related sequences from higher Metazoa, human, mouse and rat, including also invertebrate sequences, together form a second branch. It is concluded that the RTK molecules have evolved in sponges prior to the "Cambrian Explosion" and have contributed to the rapid appearance of the higher metazoan phyla and that sponges are, as a taxon, also monophyletic. Due to the fact that protein tyrosine kinases in general and RTKs in particular have only been identified in Metazoa, they are, as a group qualified, to be considered as an autapomorphic character of all metazoan phyla.

Keywords: Receptor tyrosine kinase – autapomorphic character – Metazoa – sponges – monophyly – evolution – *Geodia cydonium*

1. PORIFERA [SPONGES] AS LIVING FOSSILS

Metazoa are multicellular, heterotrophic and diploid organisms that develop anisogamously. The diploid zygote, the product of the sperm and the egg, develops by mitotic cell divisions to a ball-like larva, blastula [30, 59]. All metazoan phyla meet these criteria. However, the questions *(i)* about the relationships between the different metazoan phyla in general and between the lowest metazoan phylum, the Porifera (sponges), and those of higher invertebrates in particular, as well as *(ii)* for the ances-

* Dedicated to Professor György Csaba for his 70th birthday.

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The sequence reported here is deposited in the EMBL/GenBank data base: *Geodia cydonium* scavenger RTK, *GCSCRTK,* under accession number 18562.

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tor(s) of Metazoa among the protists remained still under debate. Some authors favoured the idea that sponges had unicellular ancestors different from those of other Metazoa *(polyphyly)* [30], while other scientists, e.g. Morris [32], believed that multicellular animals evolved only once *(monophyly)*. It had been assumed that sponges evolved from a member of the phylum Zoomastigina (perhaps from choanoflagellates (Craspedomonadida) with *Protospongia haeckeli,* a problematic extant Metazoa [16], as a possible link to the sponges [25].

A solution of the problem of "monophyly versus polyphyly" of the metazoan kingdom, gave rise to many theories [61]; it demands *(i)* answers on evolutionary intermediates between the primitive phyla, *(ii)* analyses of fossil records and *(iii)* a refutation of the possibility of polyphyly. In the past embryological, histological and ultrastructural data had been discussed and their results were taken as arguments for a monophyletic origin of animals [32, 52]; others considered this evidence not convincing [61]. Palaeontological data are at present critically assessed [5].

One powerful approach which is particularly helpful in answering questions on the presence or absence of corresponding structures in sister groups is to collect molecular data from the respective taxa. However, a clear distinction is necessary. Until now many nucleotide [nt] sequence data have been gathered from genes of members of different phyla, encoding small and large ribosomal RNA. They have been used to build phylogenetic trees to resolve deep branches. The outcome in most reports was the statement that bootstrap statistics to support monophyly is very low [4] or even not at all significant [51].

A second, separate concept used nt sequences and their deduced amino acid [aa] sequences from protein-coding genes to obtain reliable insights into the branching order of the metazoan phyla from a potential common ancestor [48]. Our group has introduced this approach to establish the phylogenetic position of Porifera, within the metazoan kingdom. The data obtained are compatible with the view that all Metazoa incuding sponges are of monophyletic origin [33, 39]. In the last three years we have extended the knowledge on the composition of genes from sponges with main emphasis on *(i)* identification of further genes, crucial for the establishment of a metazoan "Bauplan" and their use to position the sponges within metazoan systematics and *(ii)* the identification of "transition" modules in deduced aa sequences from sponges, which may help to understand the construction of the complex molecules seen in higher animal phyla.

1.1. The phylum Porifera comprises two subphyla: Hexactinellida and Cellularia

It has been proposed that the Porifera, the most ancient metazoan phylum, should be divided into two subphyla, the Cellularia comprising the classes Demospongiae and Calcarea, and the Symplasma with only the class Hexactinellida [49]. This classification reflects the fact that species belonging to the Cellularia are composed of uninuclear cells, while those in the Hexactinellida have syncytial tissues [30]. This fun-

damental structural difference raises the question whether the ancestors of the Metazoa in general, and the Porifera in particular, were colonial flagellates [20) or syncytial ciliates [14].

1.2. Monophyletic origin of animals

We postulated that animals – like sponges – which are positioned at the border between Protozoa and Metazoa – are especially rich in molecules having already the functions of those in higher Metazoa and also in ancestral molecules comprising modules for structural and functional proteins found in higher phyla. This approach was successful. We found that the structures of the characteristic metazoan genes and proteins required for *(i)* tissue formation (galectin, collagen, integrin), *(ii)* signal transduction (receptor tyrosine kinase), *(iii)* transcription (homeodomain- and MADS-box-containing proteins [34, 35]), *(iv)* immune reaction (heat shock proteins, proteasome, proteins featuring scavenger receptor cysteine-rich domains [34, 35]) and *(v)* sensory tissue (crystallin) are present in the marine sponge *Geodia cydonium* (Fig. 1); they all display high homology to sequences from members of higher metazoan phyla.

Based on the presented sequence data it is reasonable to adopt the view that Porifera should be placed into the kingdom Animalia together with the (Eu)Metazoa [32–35]. This conclusion implies that all animals evolved from one ancestor, meaning that animals are of monophyletic origin. In addition, as taken from the first sponge genes, especially the one coding for the receptor tyrosine kinase [RTK] (see below), it is now established that modular proteins, composed by exon-shuffling, are common to all metazoan phyla; a detailed description is given elsewhere [37]. This mechanism of exon-shuffling is apparently absent in plants and protists [45]. If this view can be accepted then the "burst of evolutionary creativity" [45] during the period of the "Cambrian Explosion" which resulted in the big bang of metazoan radiation [27] was driven by the process of modularization. During this process the already existing domains were transformed into mobile modules allowing the composition of mosaic proteins (Fig. 1).

2. RECEPTOR TYROSINE KINASES: GENERAL

Protein tyrosine kinases [PTKs] represent a large group of enzymes that specifically phosphorylate tyrosine residues [15]. They play important roles in the response of cells to different extracellular stimuli and are essential proteins most notable for control of growth and differentiation. Many PTKs serve as receptors and signal transducers for circulating peptide hormones and growth factors. PTKs together with Ser/Thr kinases represent the largest known protein superfamily.

All PTKs possess a highly conserved tyrosine kinase [TK]-domain which is specific for the phosphorylation of the aa tyrosine only [19]. Members of the PTKs are

further classified on the basis of their structural (functional) similarities, and divided into more than twenty subfamilies [19]. PTKs are divided into two major groups, the receptor tyrosine kinases [RTKs], which are membrane spanning molecules with similar overall structural topologies, and the non-receptor TKs, also composed of structurally similar molecules.

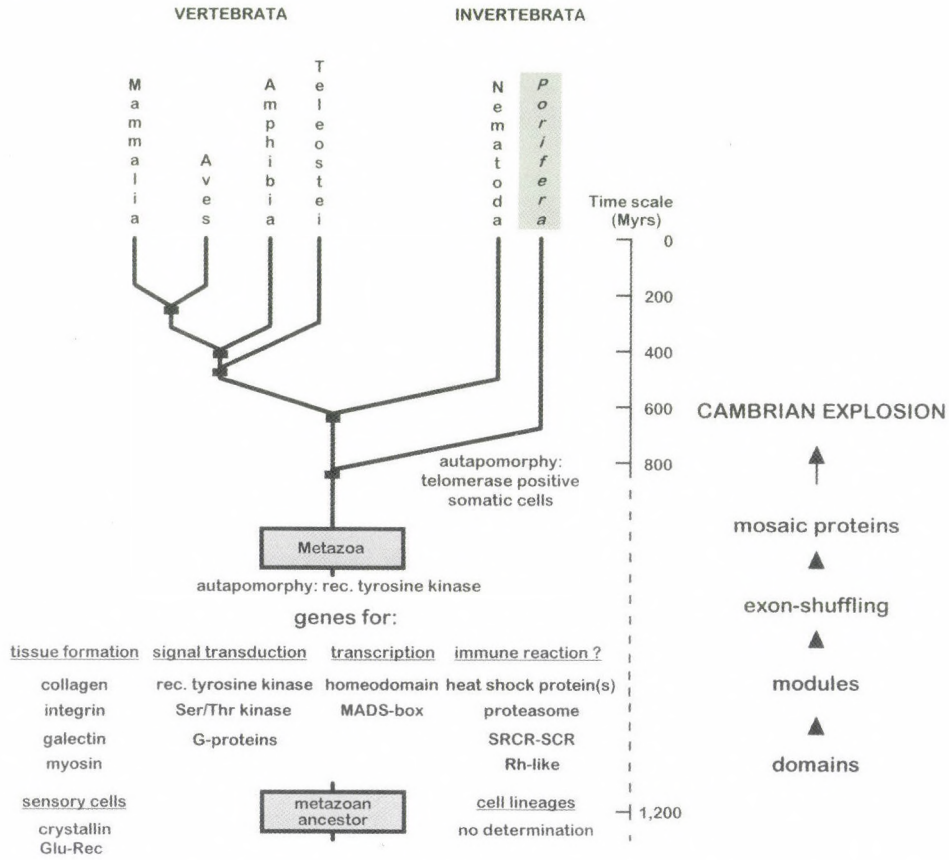

Fig. 1. Phylogenetic relationship of Porifera within the animal groups based on molecular biological data obtained from sequences of "metazoan" proteins required for tissue formation, signal transduction, transcription, immune [potential] as well as sensory reactions. The cell lineages in sponges are less strictly determined than in higher Metazoa, almost all cells from sponges are telomerase-positive [22]. It is proposed that the "Cambrian Explosion" of metazoan radiation became possible after the creation of the evolutionary important mechanism of modularization of distinct protein domains, thus allowing the formation of mosaic proteins by exon-shuffling; this process happened approximately 1,000 MYA

3. RECEPTOR TYROSINE KINASES: SPONGES

Until recently RTKs have been described only in higher invertebrate and vertebrate species [reviewed in 11]. Recently the phylogenetically oldest RTK was cloned from the marine sponge *G. cydonium* [reviewed in 38].

3.1. RTK with Ig-like domains

The *G. cydonium* RTK was identified and found to contain in the putative aa structure *(i)* the extracellular part with a Pro/Ser/Thr-rich region, and two complete immunoglobulin [Ig]-like domains, *(ii)* the transmembrane domain, *(iii)* the juxtamembrane region and *(iv)* the catalytic tyrosine [TK]-domain [41, 53–56]; Fig. 2A.

A similarity search with the *G. cydonium* TK-domain aa sequence deduced from the gene as well as from the cDNA sequence was performed. All 50 sequences most similar to sponge RTK contained the TK-domains of PTKs. The dendrogram of the alignment of TK-domains of 15 PTKs is shown in Fig. 2B. All RTKs fall in one branch of the tree while the non-receptor TKs are grouped in a second one; the sponge RTK forms a separate branch, which splits off first from the common tree of metazoan PTKs [10]. An estimation of the time of divergence of the sponge RTK from RTKs of other metazoans revealed 650-665 MYA was calculated [56].

3.2. Introns late

The RTK from *G. cydonium* has also been cloned at genomic level [10]; Fig. 2A. Surprisingly, only two introns have been found in the RTK gene, the first one located between the two Ig-like domains and the second intron between the second Ig-like domain and the transmembrane region in the extracellular part. Both introns are of medium size [≈180 bp and 508 bp, respectively]. The rest of the gene, that is the transmembrane domain, the juxtamembrane region and the catalytic TK-domain is encoded by one single exon only. All RTK genes studied – with the exception of the *G. cydonium* RTK – contain introns in their TK-domain. Some of these genes are more than 100 kb long with over 20 introns, as in those of the insulin receptor subfamily of RTKs [57].

According to the exon theory of genes, also called the "introns early" view [7, 8, 12], this common ancestral gene was present already in pieces composed of exons and introns. On the basis of the exon theory of genes [12] it was proposed by Seino and coworkers [57] that the putative ancestral TK-domain may have been assembled from 13 exons; consequently, introns must have been lost in a more or less random fashion from individual genes. One can only speculate about the structure of the common ancestral gene for all TK-domains of RTKs, including the *G. cydonium* RTK. If this assumption were correct, then (at least) in the sponge *G. cydonium* all

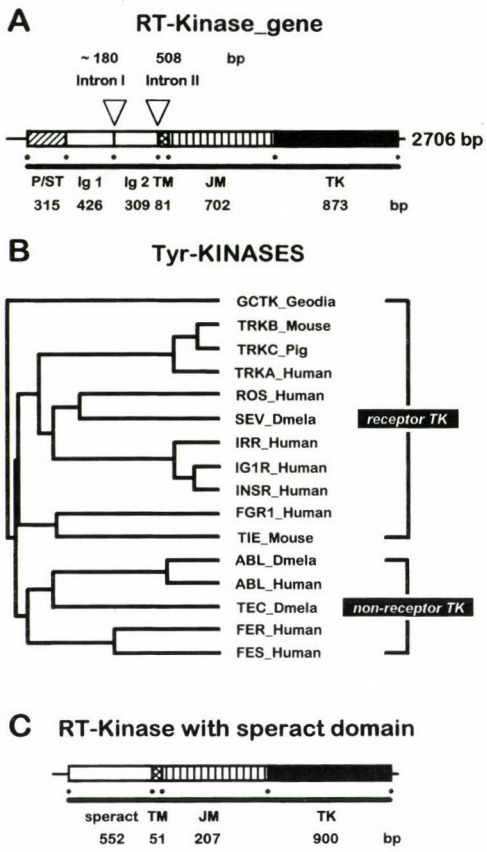

Fig. 2. Receptor tyrosine kinase from *G. cydonium.* **A.** Structural topology of *G. cydonium* RTK, comprising the Pro-Ser-Thr rich domain [P/S/T], the Ig-like 1 and -2 domains [Ig-1 and Ig-2], the transmembrane domain [TM], the juxtamembrane region [JM], and the TK-domain [TK]. The lengths of the segments are given in bp. The coding region of the receptor posseses only two introns which are located between Ig-1 and Ig-2, and Ig-2 and the transmembrane domain. **B.** Dendrogram deduced from multiple alignment of the TK-domain of RTK gene of *G. cydonium* [*GCTK*_Geodia] with those of ten receptor TKs: Neurotrophin-4 receptor [*TRKB*_Mouse]; NT-3 growth factor precursor [*TRKC*_Pig]; nerve growth factor receptor [*TRKA*_Human]; ROS proto-oncogene tyosine kinase [*ROS*_Human]; *D. melanogaster* sevenless receptor PTK [*SEV*_Dmela]; insulin receptor-related receptor [*IRR*_Human]; insulin-like growth factor 1 receptor precursor [*IG1R*_Human]; insulin receptor precursor [*INSR*_Human]; basic fibroblast growth factor receptor 1 precursor [*FGR1*_Human] and TIE protein-Tyr-kinase [*TIE*_Mouse] as well as five non-receptor TKs: *DASH/ABL* proto-oncogene Tyr-kinase from *D. melanogaster* [*ABL*_Dmela]; ABL proto-oncogene Tyr-kinase [*ABL*_Human]; *D. melanogaster* SRC protein Tyr-kinase [*TEC*_Dmela]; FES/FPS-related PTK [*FER*_Human]; and FES/FPS protein-Tyr-kinase [*FES*_Human]. **C.** Structure of the deduced aa sequence of the cDNA coding for the RTK with speract domain from *G. cydonium.* The extracellular domain, including the speract domain, the transmembrane domain [TM], the juxtamembrane region [JM] and the TK-domain are delimited

introns must have been eliminated from the TK-domain during the long period of the sponge's separate evolution.

However, it is also – perhaps more – likely that introns, found in the TK-domain of existing protein tyrosine kinase genes were introduced after splitting off the sponge line from the common, primitive ancestral metazoan organisms. The most logical way to explain the results obtained from the analysis of the ancient *G. cydonium* RTK gene is to accept the "introns late" hypothesis [3, 37, 40].

3.3. Increased expression of RTK during autograft fusion in the sponge G. cydonium

Regeneration in reponse to tissue transplantation is a suitable model to study histo(in)compatibility reactions not only at the cellular level, but also at biochemical (immunocytochemical) and molecular levels. In spite of the fact that transplantation experiments with sponges are among the pioneering approaches to understand self/self- and self/non-self recognition since the works of Wilson [63], insights into the molecular events proceeding after tissue transplantation in these animals are presented first in our studies. We have used the sponge *G. cydonium* because most potential adhesion molecules which might be involved in tissue recognition have been identified from this species [reviewed in 34–37]. Antibodies were raised against the recombinant protein comprising the intracellular part of the RTK from *G. cydonium,* including the juxtamembrane region and the TK domain. Western blotting studies revealed that the anti-RTK antibodies recognizes in membrane extracts specifically a polypeptide of a size of 135 kDa, closely corresponding to the size of the deduced 3.3 kb long cDNA [10].

To determine a potential differential expression of the genes selected, RNA was isolated from the zone of fusion after autografting. Samples were taken after 5, 7 or 10 days. From earlier studies it is known that within the first two days the grafts, auto-, allo- as well as xenografts show an initial fusion reaction [47]; Fig. 3A and B. After 5 days grafts that are not autologous are slowly rejected; this process of rejection with the synthesis of cytotoxic factors resulting in a destruction of the transplant is completed latest at day 10 after grafting. Experiments of this kind have been performed with the marine sponges *Callyspongia diffusa* [17] or *G. cydonium* [41, 47]. The experiments revealed that the expressions of the genes encoding *G. cydonium* α-integrin and β-integrin are strongly upregulated 5 days after grafting of allogeneic tissue. Almost the same kinetics is found for the change in expression of the RTK (Fig. 3C and D). In contrast, no upregulation is seen for the expression of the galectin and of HSP70; in fact the level of transcript for galectin was close to zero in the fusion zone. A similar change as seen for the steady-state level of the transcripts could be demonstrated also immunohistochemically. The cells accumulating at the border between the two autografts are brightly stained with antibodies against integrin and RTK, but not with those raised against galectin. The staining pattern for HSP70 was almost as strong as in the control region [62].

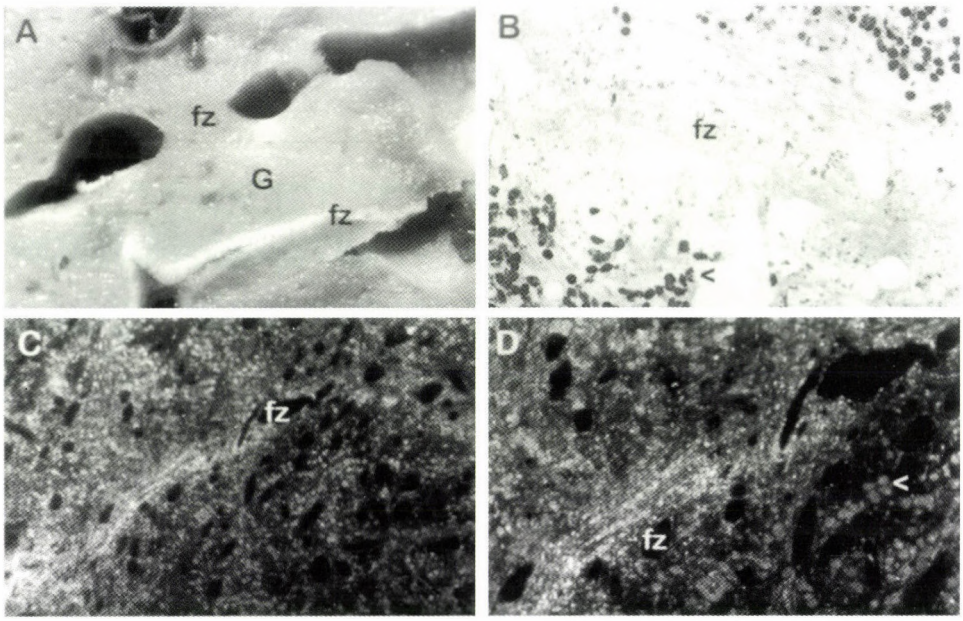

Fig. 3. Histological analysis of autograft fusion in *G. cydonium.* **A.** An autograft [G] was inserted into the "host". Several days later the specimens were cut. The graft completely fused with the "host". The fusion zone is marked [fz]. Magnification: ×2. **B.** Staining of a section through the fusion zone with Ziehl's fuchsin/aniline blue; ×100. **C–D.** Immunofluorescence images of sections through the area comprising the fusion zone [fz]. Sections were incubated with the anti-RTK antibodies, McAb-RTK-IVG8, and subsequently with FITC-conjugated anti-mouse IgG. Clusters of spherulous cells are marked [<]. Magnifications: E: ×50; F: ×100

The function of the RTK region comprising the Ig-like domains in sponges is not known. Until now, we were not able to identify a ligand for this receptor. Hence it remains unsolved if the RTK interacts in a homo- or heterophilic manner. The RTKs in mammals are activated by growth factors that stimulate metabolic responses [reviewed in 11]. At present, we are only at the beginning of the understanding which factors are involved in outside-in signalling in sponges. Several putative morphogens, e.g. an activin-related molecule, have been cloned from *G. cydonium* whose function will be soon investigated. We assume that a diffusible ligand, perhaps mediating a long-range signaling, causes upregulation of the sponge RTK which, in turn, causes cell growth.

3.4. RTKs: insulin-like receptors

Recently, we could demonstrate for the first time that in all three classes of the phylum Porifera one distinct subfamily of the RTKs is present which contains the TK class II signature with the consensus pattern D-[LIV]-Y-x_3-Y-Y-R [45 [Prosite]). By

choosing appropriate primers for polymerase chain reaction, sequences were obtained from sponges which have to be grouped to the insulin receptors [InsRs] from vertebrates [59], the insulin-like growth factor I receptors [IGF-I-Rs] from vertebrates [60], the InsR-related receptors from vertebrates [58], and the InsR homologue from *Drosophila melanogaster* [9]. These are all members of the class II RTKs which display the following consensus for InsRs, GF-I-Rs and InsR-homologues R-D-[IV]-Y-E-[TS]-D-Y [15] within the subdomain VII.

It was found that TK domains of InsR-(like) molecules are present in the hexactinellid sponge *Aphrocallistes vastus,* the demosponge *Suberites domuncula* as well as the Calcarea *Sycon raphanus.* From *S. raphanus* three full-length clones of InsR-like molecules have been isolated (to be published). All sequences were used for phylogenetic analyses which revealed that the sponge InsR-like molecules are statistically significantly separated from the related molecules of higher Metazoa and allowed an assessment in which evolutionary order the three classes of Porifera appeared.

A phylogenetic tree was constructed and rooted with the sequence of the catalytic domain of the RTK from *G. cydonium* (Fig. 4A). It shows that all sponge sequences fall into one branch, while the selected sequences of InsRs, IGF-I-Rs or InsR-related sequences from invertebrates and vertebrates are grouped together into a second one. This relationship is statistically very robust as analysed by bootstrapping. Hence, support for monophyly of Porifera can be deduced. In consequence, the presented findings, based on the data obtained with the catalytic domains of the InsR-like molecules from sponges, shed new light on the assumed uncertain position of sponges [51]. In addition, the data given do not support earlier notions which suggested that the phylum Porifera might be paraphyletic [4].

3.4.1. Rate of evolution of the catalytic domains of sponge InsR-like molecules

Using our data collected on the percentage of aa identity among the polypeptide sequences from the different sponge species on one hand and the sponge sequences in comparison to those from higher metazoa, on the other allow a comparative approach to determine the time of divergence of the sponge classes from a common ancestor. This estimation, which bases on the number of point mutations per 100 aa within given polypeptides, might reflect the time of divergence of two taxa. The evolutionary rates – expressed as k_{aa}-values – vary between different proteins. As shown by Kimura [21], k_{aa}-values vary from 8.3×10^{-9} for fibrinopeptides (reflecting a value of 8.3 aa substitutions per site per 10^9 years) to 0.01×10^{-9} for histon H4, with an overall average k_{aa}-value of 1×10^{-9} [meaning 1 aa substitution per site in 10^9 years]. In a previous study it was calculated that the galectin protein from the sponge *G. cydonium* [48] has a k_{aa}-value of 1.7×10^{-9} [18], while a k_{aa}-value of 1.24×10^{-9} was calculated for the RTK from *G. cydonium* [56] from the same animal.

Using the data from the catalytic domains of the InsR-like molecules, a k_{aa}-value of 0.91×10^{-9} (corresponding to a divergence time of 455 million years ago [MYA])

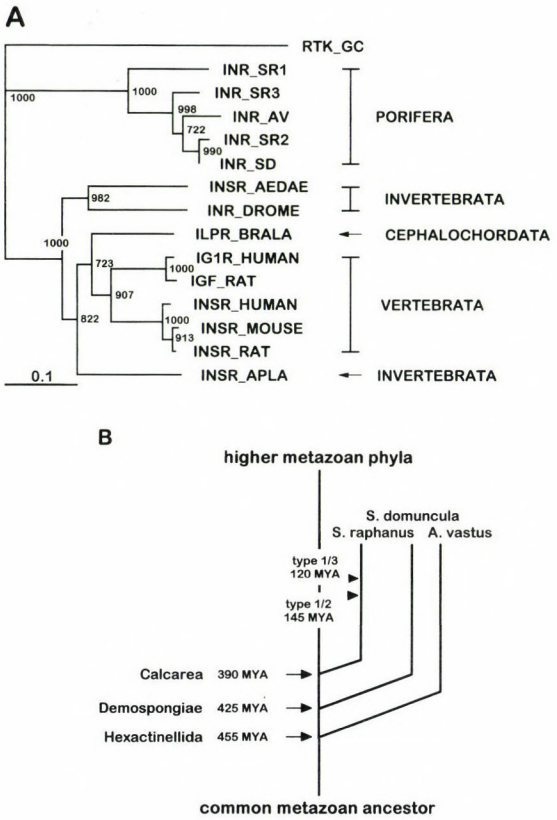

Fig. 4. **A.** Rooted phylogenetic tree of catalytic domains of the insulin-like receptors as well as of the related sequences within the class II RTKs. The deduced aa sequences of InsR homologues from the polypeptides of the three classes of the phylum *Porifera (sponges), (i)* the Demospongiae *S. domuncula* (INR_SD), (*ii*) the Calcarea *S. raphanus* type 1 (INR_SR1), *S. raphanus* type 2 (INR_SR2), *S. raphanus* type 3 (INR_SR3), and *(iii)* the Hexactinellida *A. vastus* (INR_AV) have been aligned with the related sequences for *invertebrates,* the insulin-like receptor precusor from the mosquito *Aedes aegypti* (INSR_AEDAE) and the InsR homologue from the fruit fly *Drosophila melanogaster* (INR_DROME) as well as the mollusc *Aplysia californica* InsR (INSR_APLA), one *cephalochordate,* the insulin-like peptide receptor precursor from amphioxus *Branchiostoma lanceolatum* (ILPR_BRALA) and from selected *vertebrates,* the human insulin-like growth factor 1 receptor precursor (IG1R_HUMAN), the human InsR precusor (INSR_HUMAN), the InsR precursor from the house mouse *Mus musculus* (INSR_MOUSE), the IGF-I-R I receptor precursor from the rat *Rattus norvegicus* (IGF_RAT) and the InsR precusor from *R. norvegicus* (INSR_RAT). In addition, the RTK domain from the sponge *G. cydonium* was used for the comparison. The numbers at the nodes refer to the level of confidence as determined by bootstrap analysis. Scale bar indicates an evolutionary distance of 0.1 aa substitution per position in the sequence. The catalytic domain of the receptor TK domain from *G. cydonium* (RTK_GC) was used as the outgroup sequence. **B.** Proposed branching order of the three classes of the phylum Porifera, Hexactinellida, Demospongiae and Calcarea, from a common metazoan ancestor. In addition, the separation of the three types of the *S. raphanus* InsR-like molecules, type 1 from type 3, and type 1 from type 2 is also indicated. The dates of the approximate time of divergence are indicated

between the human IGF-I-R and the hexactinellid sponge *A. vastus* is calculated. A corresponding k_{aa}-value of 0.85×10^{-9} (425 MYA) is calculated between the human receptor and the molecule from the demosponge *S. domuncula* and a k_{aa}-value of 0.78×10^{-9} (390 MYA) between the human and the calcareous sponge *S. raphanus*. Applying the same approach the type 1 and type 2 InsR-like molecules from *S. raphanus* diverged 145 MYA and type 1 and type 3 120 MYA from the common ancestor (Fig. 4B).

The data given here, indicate that the Hexactinellida branched off first from a common metazoan ancestor (455 MYA), while the Demospongiae diverged later (425 MYA), while finally the Calcarea appeared (390 MYA). This order of evolution of the three classes of Porifera, which is outlined in Fig. 4B, strongly supports recent data obtained with sequences from protein kinase C [24] and heat-shock proteins [23].

3.4.2. Phylogenetic relationship of sponge taxa

In all three classes of the phylum Porifera molecules of the group of InsR-related molecules exist. This finding implies that already animals of the lowest metazoan phylum contain growth factor receptors which allow to react *(i)* to nutrient cues and also *(ii)* to neighbouring, individual cells, with a complex intracellular signalling reaction. The InsR-homologues, which are putative transmembrane receptors, presumably allow the transduction of signals through the cellular membrane.

From the evolutionary point of view the present contribution sets three points. First, it adds the fact that molecules similar to the InsR-homologues have evolved prior to the "Cambrian Explosion". Due to recent findings that the Porifera existed already earlier than this event [26], it can be assumed that this class of key molecules involved in the complex network of intracellular signalling, was at least one major driving force which allowed the other metazoan phyla to arise. Second, the phylogenetic analyses indicate that based on the autapomorphic character for Metazoa, the RTKs, sponges as a taxon are monophyletic; the Hexactinellida have been calculated to be the oldest class, followed by the Demospongiae and finally the Calcarea. Third, already in sponges epidermal growth factor [EGF]-like domains are present which allow their insertion into potential cell surface receptors and also into matrix molecules (to be published).

3.5. RTK with speract domain: a novel molecule

Proteins featuring scavenger receptor cysteine-rich (SRCR) domains comprise a superfamily, which includes one invertebrate and several vertebrate protein members [reviewed in 50]. The SRCR domain consists of a 110 aa-residue motif with conserved spacing of six to eight cysteines, which apparently participate in intradomain disulfide bonds. Proteins of this superfamily feature one to eleven SRCR domain repeats [50]. The first molecule identified as a member of this superfamily was the

sea urchin *(Strongylocentrotus purpuratus)* speract [sperm egg-peptide receptor] that featured four SRCR repeats [6]. Other members of the superfamily have so far been found only in vertebrate sequences, such as macrophage type I scavenger receptor, macrophage M130 antigen, complement factor I [CFI], CD5), CD6, T-cell antigen WC1, cyclophilin-C-binding protein and its homologue MAC2-binding protein, a recently cloned sea lamprey *(Pteromyzon marinus)* protein, a macrophage bacteria-binding receptor [MARCO] and CRP-ductin-α [for references 43]. All cloned members of the SRCR superfamily are either cell-surface or secreted proteins and most of them, with the exception of speract, appear to be involved in host defense functions either expressed on T-cells, B-cells, and macrophages, or as soluble proteins [50].

The first putative SRCR protein from the marine sponge *G. cydonium* was recently identified [43]. Our data indicate that the recombinant polypeptide of the SRCR molecule promotes aggregation of cells *in vitro* [2].

3.5.1. Cloning of the cDNA encoding the G. cydonium *scavenger RTK*

The complete cDNA was obtained by library screening. The *G. cydonium* cDNA library in λZAP Express™ was used [48]. *E. coli* XL 1-Blue MRF', served as host for library plating. Plaque lifting and hybridization were performed according to standard procedures [1]. The temperature for the overnight hybridization with the insulin receptor-related cDNA from the sponge *S. raphanus* as a probe ([nt 728–1372 of the cDNA, *SRINR1* [type 1], corresponding to the subdomain I to IX of catalytic domain: aa_{222} to aa_{436}]; accession number Y17877, [to be published]) was 42 °C, with 50% formamide. Positive signals were detected using alkaline phosphatase conjugated anti-digoxygenin antibody. *In vivo* excision of the recombinant phagemids pBK-CMV from λZAP Express™ phages was performed using the Rapid Excision Life Kit (Stratagene). *E. coli* XLOLR served as host for the infection/amplification of *in vivo* excised pBK-CMV. The clones obtained were termed *GCSCRTK.*

The novel *G. cydonium* nt sequence, *GCSCRTK* (accession number Y18562) is 1915 nt long and has a potential open reading frame [ORF] of 1710 nt encoding a 570 aa long-deduced protein sequence, termed scavenger RTK, SCRTK_GC.

3.5.2. Deduced aa sequence of the scavenger RTK

The deduced aa sequence of the cDNA encoding the scavenger-RTK, SCRTK_GC, of 570 aa in size, has a calculated peptide mass of 62,874 Da and an isoelectric point of 4.58 (45; Physchem program). The putative sponge scavenger-RTK comprises the extracellular domain, the transmembrane domain, the juxtamembrane region and the TK-domain (Fig. 2C).

In the extracellular domain, the characteristic speract receptor domain signature is found (Fig. 5). The borders of the catalytic subdomains I to VII [according to 15], could be defined also unequivocally for this sponge sequence (Fig. 5). In subdomain

I: the ATP-binding site [consensus: GxGxxGxV], in subdomain II: the residue Lys in the consensus VAxK which is required for kinase activity, in subdomain VIb: the aa D [Asp] and N [Asn] as well as in subdomain VII: the DFG tripeptide are present. The DFG segment has been implicated in ATP binding [13] and represents the most conserved portion within the catalytic domain. The residue Y [Tyr] in subdomain VII undergoes phosphorylation (tyrosine kinase phosphorylation site). Signatures within subdomains III, IV, V, VIa, VIII, IX, X and XI are in general less well conserved. The TK specific active-site signature, D-L-A-T/A-R-N, characteristic for both vertebrate and invertebrate TKs [13] is found in subdomain VIb. Within the subdomain VII the signature for the TK class II receptors with the consensus pattern is found, D-[LIV]-Y-x_3-Y-Y-R [45 [Prosite]).

```
MNVECLGNESNISECSHSDINHVGVCLHADDAGVICEAPPGSCRDGDIRVEDGTVGTVDS  60
                                                        |- SPE
VSGNIEVCYTGLWGTVCATVWDETNGLVACRQLGYAELEVTEKRPLMGDEARLQSLLSGV 120
                                SPE -|
NCSGSEERLADCDHDGVDMGDTTGCARAFVRCLKQIPTGVTVVDSSTSMGDTTASIIGGS 180

VGATVLLLIVVIVVVIATIIVLKEQAVRKKMNYSLENARQTPSVPDYIEMYTQGQASNYL 240
    [~~~~~~TM~~~~~~~]|- JM
EPVSSRAQVDMYSKWRGPLKGLLLAEDQLILTESLGEGAFGRVYKGSMKTGDDDDRTVEV 300
                            JM -|- TK I          I • II
AIKTLKDISQQENIDNFVQESILMLGFNHPNVLELLGVCFDTTDRHPLIVLPFMANGDLR 360
        II • III      III • IV            IV • V
SYLMSKRDPSVTTKFTHFPQGLDEERALVMCLEVARGMEYLSNSSFVHRDLAARNCMVSG 420
                          V • VIa        VIa • VIb
ELTVRVADFGLSRDVYSKDYYRMGTKTMLPVKWMAPESLADNIFTVKSDVWSFGVVCWEV 480
VIb• VII            VII • VIII       VIII • IX
FSLVAPYPGIGNHEMSDYIGGGRRLKIPRLCPREIYELMEQCWNEESNKRPNFSELVSQL 540
   IX •
ERVIDTRLNPHYMNMNNGTTQQNGHAVQPQ                                570
```

Fig. 5. G. cydonium scavenger-RTK. Deduced aa sequence, SCRTK_GC, showing the following building blocks: The extracellular speract domain [|- SPE -| (the characteristic aa are in superscript); the transmembrane domain [~~TM~~], the juxtamembrane region [- JM -] and the TK-domain [- TK -]. Below the sequences the borders of the subdomains are shown [I to IX]; the nomenclature follows that of Hardie and Hanks (15). In addition, the ATP-binding signature (in subdomain I), the tyrosine kinase specific active-site signature (in subdomain VIb), the TK class II signature (in subdomain VII) as well as the TK phosphorylation site (Y in superscript; subdomain VII) are marked (double underlined)

CONCLUSION

In conclusion, the demosponge *G. cydonium* is a surviving species of a phylogenetic lineage that according to paleontological data evolved more than 550 MYA [31, 36]. The spicules, one morphological characteristic of the taxon Geodiidae, changed very little since that time. According to the determinations of the rates of aa substi-

tutions of the deduced sequences from the *G. cydonium* galectin(s) and the RTK, this taxon diverged 800 to 665 MYA from other metazoans.

Until 1993, when the first cDNA, coding for a sponge adhesion molecule, was isolated and characterized [48], Porifera were considered as merely colonies of unspecialized cells – "individual flagellates" – which require only cells that secrete adhesive glycoprotein and bind to it [28]. Now that several cDNAs coding for "metazoan" adhesion molecules and receptors have been cloned, it has become clear, that these proteins show high homology to the corresponding molecules from higher metazoan phyla.

One major step in the evolution of the Metazoa from Protozoa was the production of molecules forming the extracellular matrix. These molecules provide the scaffold and the support for tissues and organs. Until now, only constituents of the ECM have been detected in sponges [36]. However, these elements alone are not sufficient to allow the development of tissues and organs; receptors are required for a tuned and controlled interaction between cells and between cells and extracellular molecules. In sponges, two main signalling receptors have been detected, *(i)* a receptor tyrosine kinase and *(ii)* elements of seven-spanning G protein-linked receptors [34–36]. Hence, the animals, belonging to the lowest metazoan phylum, the Porifera, are provided with an elaborated intercellular communication network allowing a coordination of growth, differentiation, tissue formation and allorecognition.

Until now RTKs have been identified only in Metazoa, including the Porifera as the lowest phylum. Therefore, this molecule is qualified to be used as an autapomorphic character for Metazoa. Recently, further autapomorphic characters have been identified in sponges, e.g. integrin receptors [42, 62] or the metabotropic glutamate receptor [46].

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STRESS-RESPONSIVE GENE EXPRESSION IN *TETRAHYMENA*

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Cells properly respond to extracellular stimuli and circumstantial environment. The unicellular eukaryotic protozoan *Tetrahymena* is a potentially useful animal cell model system for studying the molecular mechanism of adaptation to environment. *Tetrahymena* is exposed to fluctuations in temperature, pH, amounts of nutrients and concentration of dissolved gases in natural habitat. For example, the cells adapt to cold environment by increase in unsaturated fatty acids in membrane phospholipids to maintain proper membrane fluidity. To accomplish this modification, the activity of fatty acid desaturase is increased upon a down-shift in temperature. We have cloned Δ9 fatty acid desaturase which is involved in this process and shown evidence that its mRNA level increased in response to cold environment. Moreover, in order to examine other genes responsive to clod stress, we have adopted mRNA differential display technique to temperature shift-down of *T. thermophila.* We have cloned two kinase genes, NIMA (never-in-mitosis in *Aspergillus nidulans*)-related protein kinase (*Tp*Nrk) and MAP kinase-related kinase (MRK). Interestingly, these genes were also shown to be expressed by the osmotic stress.

Keywords: Tetrahymena – stress response – signal transduction – desaturase – MAP kinase

INTRODUCTION

Microorganisms should adapt the extreme environments by changing the membrane components such as proteins and lipids. Also gene expression can be modulated during the adaptation process. Only microorganisms capable of adapting can survive. A unicellular eukaryote, *Tetrahymena* has a potent adaptability to the environmental stresses, e.g. temperature, osmolarity, chemicals. Therefore, this cell has proved to be a potentially useful model system for studying the molecular mechanisms of the stress adaptation. In fact, we have previously demonstrated the marked adaptive modification of phospholipid acyl chain composition in various membranes of *Tetrahymena* cells in response to the cold stress [18, 19, 23]. The principal effects of

* Dedicated to Professor György Csaba for his 70th birthday.

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decreasing temperature are an increase in linoleic and linolenic acids and a decrease in palmitic acid, producing a generally higher degree of unsaturation. Such pronounced alterations in membrane phospholipids play an important role in adapting the membrane fluidity to the cold stress. Our and Thompson's groups proposed the induction theory and the fluidity-mediated control theory, respectively, for regulation of fatty acyl desaturase activity [17, 23]. To examine the possibility of desaturase induction, we have cloned cDNA of Δ9 desaturase enzyme and determine its expression during the adaptive response to cold temperature. In addition to the Δ9 desaturase gene, we could observe expressions of other genes, NIMA (never-in-mitosis in *Aspergillus nidulans*)-related protein kinase (*Tp*Nrk) and MAP kinase-related kinase (MRK), by using the differential mRNA display. These two kinase genes were also found to be expressed by the osmotic stress.

In this article, we will describe these genes expressed by the cold and osmotic stresses.

FATTY ACID Δ9 DESATURASE

In response to cold environment, *Tetrahymena* cells modify their membrane phospholipid composition and also their fatty acyl chain composition (Fig. 1) to maintain adequate membrane fluidity [18, 19, 23]. The latter adaptation is achieved by increase in the activity of fatty acid desaturases. It appears that introducing the first double bond into fatty acids in membrane phospholipids has the greatest effect on membrane fluidity and successive unsaturation reactions have smaller effects [11]. The enzyme which introduces the first double bond into palmitic or stearic acid is Δ9 acyl-CoA desaturase in animal cells. When *Tetrahymena* cells were cooled from normal growth temperature to 15 °C at a rate of 0.8 °C/min, there was a quick increase in palmitoleic acid at the expense of palmitic acid in membrane phospholipids, followed by a progressive increase in polyunsaturated acids, e.g. linoleic and γ-linolenic acid. During this cold adaptation, the activity of microsomal palmitoyl- and stearoyl-CoA desaturases showed transient increases with a peak at 2 h following the shift-down [25]. Two theories have been proposed for the increases in desaturase activity; self-regulation by membrane fluidity [23] and enzyme induction [17]. To gain further insight into the regulation of desaturase activity, especially the latter theory, we have attempted to clone cDNA encoding Δ9 acyl-CoA desaturase from the protozoan. In order to clone Δ9 acyl-CoA desaturase from *Tetrahymena,* degenerate primers based on conserved regions of Δ9 desaturases from *Saccharomyces cerevisiae,* rat and mouse were constructed and used in RT-PCR (reverse-transcriptase-polymerase chain reaction). The amino acid sequences HRLWSHR (residues 161–167 in yeast, 119–125 in rat and 116–122 in mouse and) and HN(F/Y)HH(A/E) (residues 325–330 in yeast, 297–302 in rat and 294–299 in mouse) were chosen for the 5' primer and the 3' primer, respectively. A 547 bp fragment was specifically amplified by PCR using these primers and *T. thermophila* cDNA as a template. *T. thermophila* λgt10 cDNA library was screened with plaque hybridization technique, using ^{32}P-labeled

this DNA fragment as a probe. The isolated cDNA contained an open reading frame of 292 amino acids with estimated molecular weight of 34 kDa [14]. Desaturases are classified into three types [13]. In plants and cyanobacteria, acyl-lipid desaturases introduce double bonds into fatty acids which are bound to glycerolipids. Higher plants also contain acyl-ACP desaturases which catalyze desaturation reaction while fatty acids are bound to ACP (acyl carrier protein). In animals and yeasts, fatty acids are desaturated in a CoA-bound form and the involving enzymes are known as acyl-CoA desaturases. The deduced amino acid sequence of the open reading frame of the cloned cDNA was highly homologous to those of Δ9 acyl-CoA desaturases of other organisms (Fig. 2) and also to those of Δ9 acyl-lipid desaturases of cyanobacteria. However, it was very different from Δ9 acyl-ACP desaturase of plants. The amino acid sequences in the central portions of the Δ9 acyl-CoA desaturases (corresponding to the region between Gly^{63} and Trp^{263} in *Tetrahymena* enzyme), shown in black boxes in Fig. 2, are highly conserved. Among amino acids, histidine (11 out of 16), proline (6 out of 8) and arginine (7 out of 9) residues are found in the aligned conserved positions. *Tetrahymena* desaturase possesses three histidine-cluster motifs, one HXXXXH (residues 69–74) and two HXXHH (residues 106–110 and 246–250), which are characteristics of Δ9 acyl-CoA desaturases [13]. Histidine clusters are assumed to bind iron atoms and to play an important role in introduction of double bond into fatty acids. By hydropathy analysis, two long hydrophobic regions preserved in desaturases are present in *T. thermophila* desaturase at the aligned positions. These regions could potentially form two membrane-spanning domains. This assumption is consistent with the finding that Δ9 acyl-CoA desaturase is tightly bound to microsomal membrane. Moreover, four smaller hydrophobic regions are found at the identical positions among *Tetrahymena, Amblyomma,* rat, mouse and yeast desaturases.

Our previous studies showed that microsomal palmitoyl- and stearoyl-CoA desaturase activity increased during cold adaptation and reached a peak at 2 h after tem-

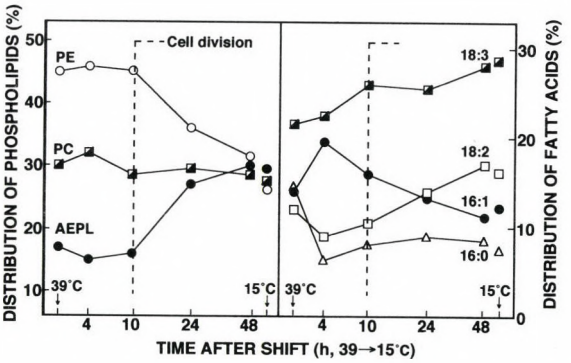

Fig. 1. Proportional changes in polar head group and fatty acid composition during acclimation in *Tetrahymena* NT-1 cells after the growth temperature shift from 39 to 15 °C. PE, phosphatidylethanolamine; PC, phosphatidylcholine; AEPL, 2-aminoethylphosphonolipid

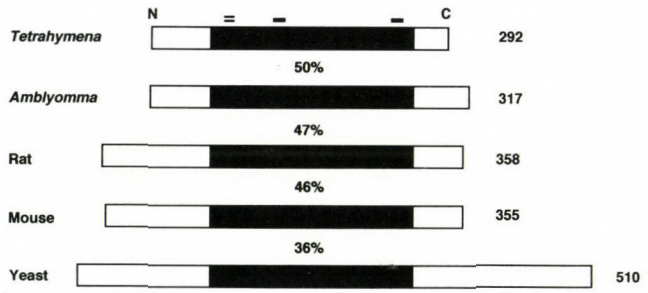

Fig. 2. Comparison of amino acid sequences of *T. thermophila, Amblyomma,* rat, mouse and yeast Δ9 desaturases. The deduced amino acid sequence was compared with those of Δ9 acyl-CoA desaturases from *Amblyomma,* rat, mouse and yeast. Analysis was performed by DNA analysis program DNAsis. The homologous central regions are shown in black boxes (identity to *Tetrahymena* enzyme is shown by %). The positions of conserved HXXXXH and HXXHH motifs are shown by double lines and bold lines, respectively

perature shift-down [25]. In order to examine whether transcriptional control is involved in the regulation of desaturase activity, expression of desaturase mRNA was examined by Northern blot analysis. A single band of 1.4 kb was recognized by the ^{32}P-labeled desaturase cDNA fragment as a probe. When *T. thermophila* cells were cultured in normal temperature (35 °C), the mRNA level was low. However, in response to cold stress, the level of the desaturase mRNA was upregulated (Fig. 3). Its increase reached at the maximal level immediately after temperature was cooled down from 35 °C to 15 °C at a rate of 0.8 °C/min (Fig. 3). The level of mRNA gradually decreased thereafter with time but remained above the basal control level for at least 5 h. Furthermore, we examined the temperature at which expression of the desaturase mRNA was induced. During the course of the cooling process at a rate of 0.8 °C/min, samples were taken at every 2 °C intervals and their extracted RNAs were analyzed with Northern blotting. An increase in the level of desaturase mRNA was detectable at 27 °C and became more evident at lower temperatures, indicating that the expression is highly sensitive to the lowered temperature from the previously grown temperature. Similar findings have been reported in cyanobacteria cell, which induce an increase in mRNA level of Δ12 acyl-lipid desaturase in cold environment [13]. Cold-induced expression of Δ9 acyl-CoA desaturase was also described in carp [24]. Therefore, the increase in the levels of desaturase mRNA was considered to be due to enhanced expression, but the possibility of its increased stability cannot be ruled out.

In order to investigate whether the temperature shift-down activates the transcription of desaturase gene, the nuclear run-on analysis was performed. Cells were harvested at 0 min (35 °C), 15 min (23 °C) and 25 min (15 °C) during the course of the cooling process (0.8 °C/min). To quantify the transcription rate, the RNAs transcribed in nuclei were labeled with ^{32}P-UTP and were isolated from these cells. ^{32}P-labeled RNAs were then hybridized to desaturase cDNA which was immobilized on

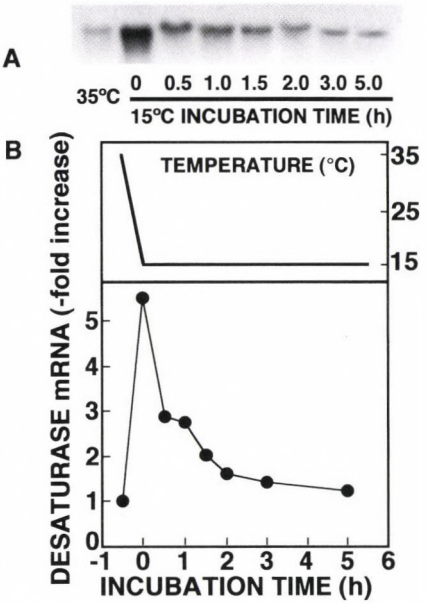

Fig. 3. Northern blot analysis of Δ9 desaturase mRNA following temperature shift-dow to 15 °C. *T. thermophila* cells were grown at 35 °C to the logarithmic phase and was cooled from 35 °C to 15 °C over 25 min (0.8 °C/min). Total RNA was extracted at the indicated time points with the guanidine thiocyanate method. Δ9 desaturase mRNA was detected by Northern blotting with ^{32}P-labeled Δ9 desaturase cDNA fragment as a probe. (A) Typical autoradiogram from three experiments. (B) The density of the bands, shown as -fold increases from the control value (means±S.D.) were monitored with a densitometer (Atto Densitograph, Tokyo, Japan)

the nylon membranes. During temperature shift-down, the level of ^{32}P-labeled actin RNA (reference control) was not significantly changed. However, transcription of the desaturase gene was elevated 2.0 ± 0.5-fold at 23 °C and 2.6 ± 0.8 at 15 °C (mean ± S.E. of three experiments). These results indicate that increases in desaturase gene expression by chilling is, at least in part, is due to transcriptional activation.

To gain further insight into the mechanism for regulation of the desaturase mRNA, its stability was examined. An RNA synthesis inhibitor actinomycin D (10 μg/ml) was added to the cells growing at 35 °C or at 15 °C immediately after temperature shift-down. Total RNA was isolated every 30 min and the amount of desaturase mRNA was measured by Northern blot analysis. The half-life of desaturase mRNA in cells growing at 35 °C was less than 45 min. However, at 15 °C it was prolonged to more than 60 min (73 ± 9 min, mean ± S.E. from 3 experiments), indicating that elevation of the desaturase mRNA by temperature shift-down is due to its increased stability. This and the above run-on analyses have provided evidence that the increases in both transcription and mRNA stability are responsible for the elevated mRNA level of Δ9 desaturase.

NIMA-RELATED PROTEIN KINASE

Differential display is a potentially useful method to detect altered gene expression in complex RNA populations [8, 9]. In this method, differential gene expression can be detected relatively easily as differences in the intensities of cDNA fragments following PCR amplification and electrophoretic separation on a gel. To investigate further the signal transduction pathway that mediates the cold stress response in *Tetrahymena,* we have attempted to isolate some differentially expressed genes which may be involved in this complex process by differential display analysis. RNA

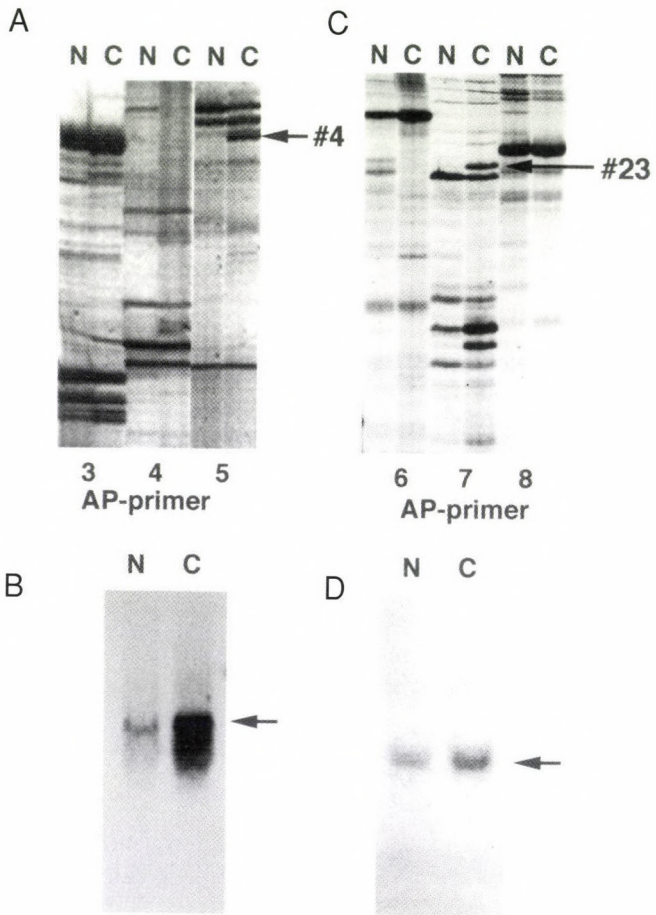

Fig. 4. Differential display fingerprinting and Northern blot of differentially expressed cDNA fragments. (A, C) Autoradiogram of typical differential display. Differentially expressed cDNA fragments (termed #4 and # 23) are indicated by arrows. (B, D) Northern blots were performed with these cDNA fragments as probes. 30 μg total RNA was subjected to Northern blot analysis. N, samples from cells cultured at 35 °C (normal temperature); C, samples from cells shifted-down to 15 °C (cold temperature)

was extracted from *T. thermophila* cells grown at 35 °C and cells 1 h after shifted to 15 °C with the rate of 0.8 °C/min. These RNA samples were subjected to differential display analysis using 10 arbitrary primers (AP-1~AP-10) and two anchored primers ($GT_{15}MG$ and $GT_{15}MA$). Approximately 1,500 bands ranging from 100 to 400 bp size were successfully amplified. Most of the bands did not display significant changes in the cells cultured at 35 °C and 15 °C, but several bands showed different intensities (Fig. 4). Differential expression was confirmed by Northern blot analysis using these cDNA fragments as probes. A cDNA fragment (termed #4, about 500 bp) amplified with $GT_{15}MG$ and AP-5 recognized about 2.3 kb mRNA which was markedly expressed in cells shifted to 15 °C as assessed by Northern blotting (Fig. 4). Using this cDNA fragment as a probe, the cDNA was obtained from *T. pyriformis* λgt10 cDNA library which contained 2408 nucleotides encoding a protein with 561 amino acids [26]. It contains all 11 conserved subdomains in the protein kinase family [7]. Homology analysis showed that it has a high structural homology with NIMA (never-in-mitosis in *Aspergillus nidulans*)-related kinases, so-called Neks and Nrks, which have regulatory roles in the cell cycle in various types of cells [4, 10, 21, 22, 28]. We designated this clone as *Tp*Nrk (*T. pyriformis* NIMA-*r*elated protein *k*inase), based on its amino acid identity with the catalytic domain of NIMA-related kinases. In addition to the primary sequence similarity at the catalytic domain, *Tp*Nrk and the NIMA-related kinases are also similar in their overall structural arrangement, with their kinase domains at the extreme N-terminus, followed by a long basic C-terminal extension. This can be regarded as one of the structural characteristics of the NIMA-related protein kinases. The phylogenic tree analysis also shows that *Tp*Nrk is a member of NIMA-related kinases (Fig. 5). As confirmed by Northern blotting, the

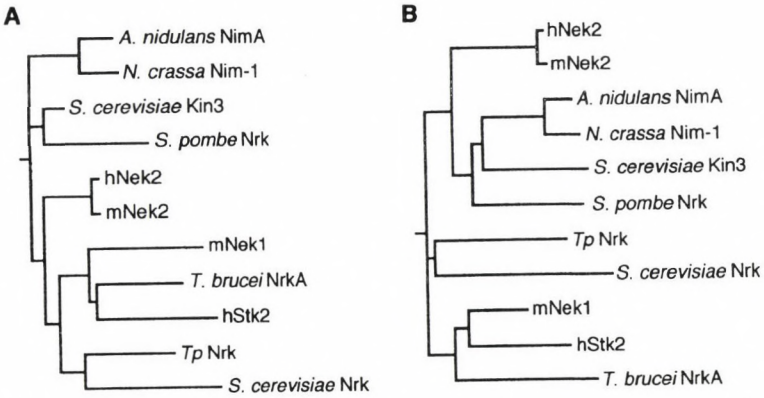

Fig. 5. Phylogenic tree analysis of *Tp*Nrk. The construction of the phylogenic tree was based on the aligned amino acid sequences of the whole protein sequences (A) or the catalytic domain of the kinases (B). The accession numbers of these kinases are listed below. Mouse Nek1 (P51954), human Nek2 (P51955), mouse Nek2 (U95610), human Stk2 (P51957), *A. nidulans* NimA (P11837), *N. crassa* Nim-1 (P48479), *S. cerevisiae* Nrk (P38692), *S. cerevisiae* Kin3 (S11185), *S. pombe* Nrk (Z98975), *T. brucei* NrkA (L03778)

level of *Tp*Nrk mRNA increased in response to cold stress. Interestingly, its expression was also induced by osmotic stress, such as high concentrations of NaCl and sorbitol. These results suggest that may be involved in the adaptation processes to stresses.

Since the NIMA-related kinases are involved in the regulation of cell cycle [4, 21, 22, 28], the changes of *Tp*Nrk mRNA during synchronous cell division were examined. *Tetrahymena* cells were synchronized by cyclic heat treatment. Following the heat treatment, 80% of the cells exhibited synchronous cell division at 75 min after the end of heat treatment (EHT). The amount of *Tp*Nrk mRNA promptly increased just after EHT, with a peak at 30 min after EHT, and then decreased sharply before the synchronous cell division at 75 min after EHT. The level of the *Tp*Nrk mRNA began to increase thereafter with relatively lower level compared with the first synchronous cell division. These results indicate that *Tp*Nrk may also play some roles in the cell cycle regulation in *Tetrahymena,* as other NIMA-related kinases in other types of cells and at least that its mRNA expression is under tight cell-cycle control.

MITOGEN-ACTIVATED KINASE-RELATED KINASE

The second cDNA fragment (termed #23, 348 bp) obtained by differential display analysis with $GT_{15}MA$ and AP-7 primers recognized about 1.7 kb mRNA which was markedly expressed in cells shifted to 15 °C (Fig. 6). Using this cDNA fragment as a probe, we isolated cDNAs from *T. thermophila* and *T. pyriformis* λgt10 cDNA libraries [15]. Their deduced amino acid sequences (430 amino acids) showed 90% identify and possess eleven conserved amino acid domains (I–XI) characteristic of protein kinases [7]. Comparison of amino acid sequences with data bases by NIH BLAST search revealed that they were similar to mitogen-activated protein kinases (MAPKs) [3, 16]. Members of the MAPK superfamily are thought to be important signal transducing molecules that transmit signals from the cell surface to the nucleus [3, 16]. In addition to classical MAPKs (also called to as ERKs, extracellular-signal-regulated kinases), recent studies have identified two other members of the MAPK superfamily: p38 and JNK (c-Jun N-terminal kinase, also known as stressactivated protein kinase, SAPK) [1, 5, 6]. p38 and SAPK are activated by inflammatory cytokines and cellular stresses such as ultraviolet light and high osmolarity. The phosphorylation cascades made up of MAPKK kinase, MAPK kinase, and MAP kinase are conserved among various signal transduction pathways from yeast to vertebrates. However, very little information has been obtained regarding these kinases in protozoa. This is the first demonstration of the cDNA encoding a protein which possibly belongs to MAPK family in protozoa.

The deduced amino acid sequence of the cloned *T. thermophila* cDNA showed 40% sequence identity with human ERK1, 39% with *Arabidopsis thaliana* ATMPK3, 39% with *Nicotinia tabacum* NTF3, 38% with yeast HOG1 and 38% with human p38. *Tetrahymena* kinase is more closely related to the stress-activated MAPKs such as p38 and JNK than ERKs. Members in a MAPK family possess the

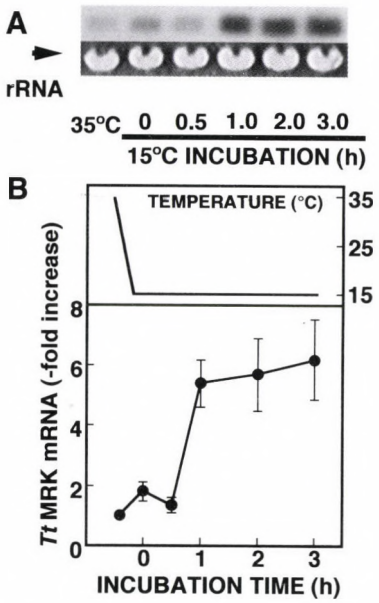

Fig. 6. Northern blot analysis of MRK mRNA following temperature shiftdown to 15 °C. *T. thermophila* cells were grown at 35 °C to the logarithmic phase and was cooled from 35 °C to 15 °C over 25 min (0.8 °C/min). MPK mRNA was detected by Northern blotting with ^{32}P-labeled MRK cDNA *Bgl*II fragment (544-871) as a probe. (A) Typical autoradiogram from three experiments. (B) The density of the bands, shown as -fold increases from the control value (means±S.D.) were monitored with a densitometer (Atto Densitograph, Tokyo, Japan)

conserved residues Thr-X-Tyr in subdomain VIII and phosphorylation of both Thr and Tyr residues is required for enzymatic activity [3, 16]. To be noted, in *Tetrahymena* Thr is conserved but Tyr is replaced by His. Recently, Nlk, a murine homologue of *Drosophila* Nemo MAPK-related protein is reported [2]. The amino acid sequence of this kinase is homologous to the MAPKs but Nlk contains the sequence Thr-Gln-Glu in place of Thr-X-Tyr. In data bases, the partial sequence of "*Plasmodium falciparum* MAPK-like protein" is deposited (GenBank accession number X98689), which also has His at the corresponding position (Thr-Ser-His). Therefore, we will call this *Tetrahymena* kinase as *M*APK-*r*elated *k*inase (MRK). In prokaryotes there is a histidine kinase autophosphorylation system, known as the two-component system (His-Asp phospho-transfer mechanism), which is widely distributed [27]. Recently, the HOG1 pathway, one of the MAPK cascades in yeast that mediates the response to osmotic stress, appears to be initiated by a similar two-component osmosensing system, namely the SLN1-YPD1-SSK1 pathway [20, 27]. A similar two-component signal transduction pathway that mediates the ethylene response is also found in higher plants. Therefore, it is tempting to speculate that His^{228} may be involved in the regulation of *Tetrahymena* MRK, although further work is necessary to confirm this.

To gain further insight into the role of MRK in response to cold stress, its mRNA expression upon temperature shift-down was examined by Northern blot analysis. A single band of 1.7 kb was recognized by the ^{32}P-labeled MRK cDNA as a probe. Although its mRNA level was low in cells grown at 35 °C, the cold stress increased mRNA expression of the kinase; the increased expression was detectable immediately after shifted-down to 15 °C from 35 °C (Time 0 in Fig. 6). The maximal level was obtained 1 h after the temperature shift and was maintained for at least 2 h.

JNK and p38 type-MAPKs are known to be activated in response to environmental stresses [1, 5, 6, 20], such as temperature change and osmotic stress. In plant *Arabidopsis thaliana,* the mRNA level of ATMPK3 (*A. thaliana* MAPK) was increased in response to cold stress as well as high-salinity stress [12]. Therefore, expression of *T. pyriformis* MRK by osmotic stress was examined. As expected, its mRNA increased when cells were exposed either to NaCl (25–100 mM) or sorbitol (100–200 mM). Increases in its mRNA expression were detectable, when cells were incubated with 25 mM NaCl or 100 mM sorbitol for 1 h. At 100 mM NaCl the increase in its mRNA was seen at 30 min. This and above results indicate that expression of MRK mRNA is induced by cold and osmotic stresses.

In order to examine whether the kinase activity of MRK was changed in response to stresses, its activity was measured by the immune complex kinase assay. A polyclonal rabbit antibody against the C-terminal 14 amino acids was obtained. The antibody, but not preimmune serum, recognized a protein of about 50 kDa in *T. pyriformis* lyaste by Western blotting. The antibody was also found to immunoprecipitate the kinase. The kinase activity of the immunoprecipitate was measured with myelin basic protein (MBP) as a substrate. The activation of MRK by osmotic stress was detectable within 15 min and lasted for at least 60 min. At 30 min MBP phosphorylation activity was increased by 2.5-fold and 1.8-fold, when cells were exposed to 50 mM NaCl and 100 mM sorbitol, respectively (Fig. 7). However, significant increases of MRK protein were not observed by Western blotting within 60 min after osmotic stress (Fig. 7). The activity of MRK increased before the increase of mRNA and

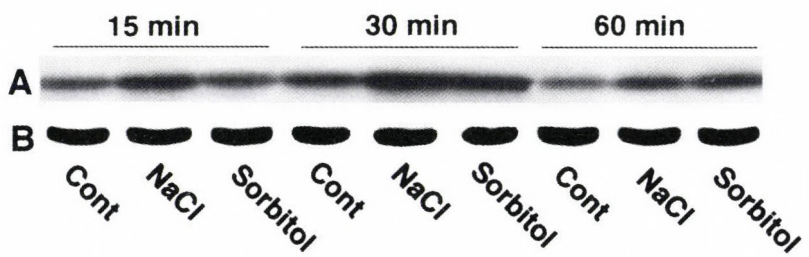

Fig. 7. Activation of MRK in response to osmotic stress. *T. pyriformis* cells were incubated in control medium or medium containing 50 mM NaCl or 100 mM sorbitol for the indicated periods of time. (A) The kinase activity in immunoprecipitate with antibody against MRK was determined with MBP as a substrate. Phosphorylated MBP was resolved by SDS-PAGE and detected by autoradiography. (B) The protein levels were analyzed by Western blotting. The results shown are the typical data from three separate experiments

protein became evident, indicating that the kinase was activated by osmotic stress. From these findings, we would like to propose that MRK is a member of the phosphorylation cascade involved in stress signaling to the nucleus in *Tetrahymena.*

EPILOGUE

We have successfully cloned the gene encoding Δ9 fatty acid desaturase, which is essential for membrane homeoviscous adaptation and showed the evidence that its mRNA is induced by temperature shift-down in *Tetrahymena.* In addition, two kinases possibly involved in the stress signaling could be identified. However, the molecular mechanism(s) for the signal transduction via cold stress leading to desaturase gene expression remains to be disclosed. It is of great interest to define the thermosensitive factor in the signal transduction. In *S. cerevisiae* osmosensing system, SLN1 is identified as an osmosensor [27]. Therefore, it is reasonable to speculate that a sensor for lowered temperature may initiate the signaling cascade to nucleus. The membrane fluidity could act as a sensor for the lowered temperature (cryosensor), but further experiments are required to prove this hypothesis.

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IMPRINTING: PERINATAL EXPOSURES CAUSE THE DEVELOPMENT OF DISEASES DURING THE ADULT AGE*

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Since the early reports linking the development of clear cell cervicovaginal adenocarcinoma in young women with diethylstilbestrol treatment of their mothers during pregnancy, it became clear that perinatal exposure to several substances may induce irreversible alterations, that can be detected later in life. Current evidence suggests that these substances induce, by the mechanism of imprinting, alterations of the differentiation of several cell-types, resulting in the development of disease during the adult age.

The most known delayed effects to prenatal exposure to agents displaying hormone action, pollutants, food additives and natural food components, substances of abuse and stress by the mechanism of imprinting are described. Among them, estrogens, androgens, progestins, lead, benzopyrenes, ozone, dioxins, DDT, DDE, methoxychlor, chlordecone, parathion, malathion, polychlorobiphenyls, pyrethroids, paraquat, food additives, normal food constituents, tetrahydrocannabinol, cocaine and opiates.

It is concluded that perinatal exposure to several agents causes irreversible changes that determine health conditions during adulthood. Several diseases developing during adulthood probably were determined during early stages of life, under the effect of exposure or preferential mother's diet during pregnancy. Regulations to avoid these early exposures may contribute to an important improvement of health conditions of humankind.

Keywords: Perinatal exposure delayed effects – imprinting – cell reprogramming – pollutants – environmental substances

INTRODUCTION

It is widely known that prenatal exposure to various substances causes delayed adverse effects such as mutagenicity, teratogenicity and carcinogenicity. Since the first reports linking the development of clear cell cervicovaginal adenocarcinoma in young women with diethylstilbestrol treatment of their mothers during pregnancy [37, 38, 114], confirmed in studies with experimental animals [79, 115], it became

* Dedicated to Professor György Csaba for his 70th birthday.

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clear that prenatal or neonatal exposure to this synthetic estrogen induces permanent changes in some cell types. These changes become evident as late as after puberty onset as an enhanced risk for malignant cell transformation, probably under the effect of postpubertal increased estrogen levels in the blood.

Based on the above information, György Csaba and his coworkers started an experimental study that allowed the demonstration that exposure of fetuses to hormones or xenobiotics displaying hormonal action during critical periods of their development induces permanent changes in the action of related hormones [15, 16]. These changes can be detected later in adulthood as a modification in the activity of receptors and in the intensity of responses mediated by them [19, 22]. This effect of hormones during fetal or neonatal life permanently modifying the ability of the cells to react to hormone stimulation during adulthood, was named by Csaba "imprinting" [15, 19].

This process, however, does not only involve a change in quantity and quality of hormone receptors in affected cells once they reach maturity; it involves several biochemical, morphological and functional changes as well. Taking into consideration the hypothesis that the process discovered by Csaba really involves a modification of the routes of normal differentiation of these cells, we proposed to rename it as "imprinting of paths of heterodifferentiation" [109]. Imprinting may be also considered as a cell reprogramming process.

Subsequent studies lead to the finding that not only hormones, but additionally several pharmaceutical agents, pollutants, stress, food additives, some natural components of food, and several substances present in plants, display the ability to induce imprinting or cell reprogramming following interaction with the different cell-types at precise stages of their fetal or neonatal development [109].

It was proposed that the changes in cell differentiation induced by this mechanism may favor, later in adulthood, the development of various diseases, such as neoplasias, endocrine abnormalities, infertility, immune diseases, psychic alterations, or changes in personality and behavior [109]. The enormous importance of this process in the determination of health conditions later on in life takes place in the fact that it is not only generated by agents that it is easy to avoid. It is also induced by early exposure to a myriad of agents and conditions that it is very difficult to detect and avoid, such as stress, very low concentrations of pollutants and natural substances contained in food.

The present report reviews the best known imprinting inducing agents relevant to human pathology, specially those affecting human health during adulthood. These are: substances displaying sex hormone action, various pollutants, food additives, natural food components, drugs of abuse and stress.

AGENTS DISPLAYING SEX HORMONE ACTION

Effect of perinatal exposure to diethylstilbestrol and other estrogens on the female genital tract

The earliest information on the effect of prenatal exposure to diethylstilbestrol in humans and in experimental animals was the gynecologic malignancies that develop after puberty or during adult age in daughters of mothers treated with this compound during pregnancy [37, 38, 79, 114, 115].

In addition to increased carcinogenic activity, other alterations were described in the genital tract of female offspring after maternal exposure to diethylstilbestrol and other estrogens during pregnancy. In experimental animals, perinatal exposure to diethylstilbestrol, allylestrenol, estradiol-17β or estradiol benzoate induce permanent changes in steroid hormone activity [1, 2, 11, 19, 30], histological alterations in the female genital tract [2, 24], including gross abnormalities [24], development of paraovarian cysts [36] and infertility [71]. In the human species, women prenatally exposed to diethylstilbestrol present histological alterations in the genital tract [37], including gross abnormalities [7, 74], development of paraovarian cysts [36], endometriosis [99], an increased frequency of abortions [114] and infertility [7, 74, 99]. We previously suggested that changes in steroid receptors, explaining the modifications of the responses to hormone stimulation and most of the above changes, probably reflect the imprinting of routes of heterodifferentiation of genital tissues following perinatal exposure to diethylstilbestrol or other estrogens [109]. In the mouse, the precocious appearance of estrogen receptors in uterovaginal epithelium [2] may explain the postpubertal increase in adenocarcinomas derived from this tissue. In the human, the abnormal localization of uterine epithelium in the cervix and vagina was considered as one of the factors increasing risk of malignancy development [85]. Further, the decrease in estrogen receptors in the rodent uterus following neonatal treatment with diethylstilbestrol, allylestrenol, estradiol-17β or estradiol benzoate [1, 11, 12, 19, 30, 72] may explain the persistent underdevelopment of rat uterine glands [72] and suggest an explanation for uterine hypoplasia in humans [72], in addition to a decrease in the ability of the uterus to respond to estrogen stimulation [1, 11, 12, 30].

The existence of separate groups of estrogen receptors that mediate separate groups of estrogenic responses through independent mechanisms of hormone action [102, 106, 107] and the finding that perinatal exposure to other sex steroids induce selective interference with some responses to estrogens but not others [3, 73] suggests that estrogenic compounds, including phytoestrogens, may selectively interfere with the development of some parameters of estrogen action but not others. Preliminary observations from our Laboratory support this possibility.

Effect of perinatal exposure to androgens on the female genital tract

Perinatal exposure of experimental animals to high levels of androgens causes changes in the normal development of fetal genitalia [4, 67], failure in ovulation and corpus luteum formation [4, 46, 93], polycystic ovary development [4, 46, 52], presence of a constantly cornified vaginal epithelium [39, 46, 47, 49], changes in uterine physiology (including abnormal hormone-induced uterine growth [4, 30, 39, 65, 67, 93]), a permanent alteration in the hypothalamic cyclic center [5], and sterility [4, 66, 92]. Some of the above changes were not always supported in the literature by other investigators. While some investigators did not detect changes in estrogen receptor levels [11, 12, 30] or in estrogen action [30] in the uterus of neonatally androgenized rats, others reported a decrease in receptor levels [65] and an impairment in hormone action [11, 12, 65, 67, 93]. Biochemical techniques not discriminating between changes in the different uterine cell-types were used in these studies. Considering the possibility of dissociation of responses to estrogen under different experimental conditions [28, 35, 101, 103–107], studies were performed in our Laboratories on estrogen action in the uterus of prenatally androgenized rats, using morphometrical techniques that discriminate between responses in the different uterine cell-types. We found that prenatal androgenization inhibits estrogen induced luminal and glandular epithelium hypertrophy, and potentiates endometrial edema, eosinophil migration to the uterus [3] and the mitotic response [108], but does not modify myometrial hypertrophy in the prepubertal rat uterus [3]. This dissociation of responses to estrogen can be explained by the independence between the different mechanisms of estrogen action in the uterus [28, 35, 102–107] and the independent regulation of hormone action in every uterine cell-type [101, 106].

The mechanisms involved in the changes of uterine physiology following prenatal exposure to androgens are not well understood (see review in [109]). The most conspicuous effect, the selective inhibition of estrogen action in luminal and glandular epithelium cells in androgenized rats, may explain the decrease in fertility observed in these animals [4, 5]. If this effect is confirmed in humans, it may explain changes in fertility in daughters of patients treated with androgens or other steroids during pregnancy and alert population to the possible risk of the ingestion of meet from animals grown with synthetic androgens.

Neurobehavioral effects of perinatal exposure to synthetic androgens, estrogens or progestins

The development of adult sex behavior and other sex-dependent personality characteristics is dependent on the presence of sex hormones during precise stages of intrauterine development in some regions of the brain. These hormones determine paths of neuronal differentiation that are normal for each gender. Following prenatal exposure to abnormal levels of sex steroids or substances displaying agonist action, the most conspicuous alterations appear in the central nervous system, including bio-

chemical changes and neurobehavioral alterations. Prenatal exposure to low level of synthetic estrogens determine an outer-directed personality in the adult, one that is more group-oriented and group-dependent, less individualistic and more consciously identified with its group or social environment. Exposure to synthetic progestins causes an inner or self-directed personality in the adult, one that is more independent, self-assured and self-sufficient, more individualistic and less concerned with social environment [84]. Perinatal exposure to higher levels of sex steroids or non-steroidal synthetic agonists such as diethylstilbestrol determines, in humans (see [109] for a review), lifelong alterations in sex-dimorphic behavior (gender role), temperamental sex differences and sexual orientations in adults, as well as sex-dimorphic play behavior in children and a decrease in orientation towards parenting in adult women.

Other effects of prenatal or early postnatal exposure to synthetic estrogens or androgens

Besides the effects described above, neonatal androgenization abolishes clock-timed gonadotrophin release in prepubertal and adult female rodents, induces changes in tuberoinfundibular dopamine nerve activity and several biochemical alterations in the forebrain, hypothalamus and cerebellum, including alterations in opioid control of noradrenaline release in specific brain areas and changes in gonadotropin, oxytocin and prolactin secretion (see review in [109]). Prenatal exposure to estrogens affects subsequent transport of α-aminobutyric acid into rat brain [64]. This reflects important changes in the differentiation and development of the central nervous system and suggests an explanation for the behavioral changes in exposed experimental animals or humans.

Androgenization induces permanent alterations in testosterone metabolism in the hypothalamus-pituitary-gonadal axis in male rats. Neonatal exposure to estrogens determines developmental, structural and functional alterations in the testis, prostate and seminal vesicles (see review in [109]).

The immune system is also affected by exposure to sex hormones. Estrogens cause changes in the development of the rat thymus gland, including premature involution of its cortex [63]. Diethylstilbestrol persistently alters NK cell activity in the mouse [53] and humans [27]. Alterations in immune responsiveness [118] and increased occurrence of autoimmune disease [81], in addition to the increased frequency of diseases suggesting impaired immune function, such as respiratory tract infections, asthma, arthritis, and lupus [121], were reported in women exposed *in utero* to diethylstilbestrol.

POLLUTANTS

Pollutants best known to induce imprinting mechanisms following perinatal exposure are lead, benzopyrenes, ozone, dioxins and several pesticides.

Lead

The most conspicuous delayed effects caused by prenatal, early postnatal or infant age exposure to lead by the mechanism of imprinting occur in the central nervous and the female reproductive systems. These effects persist through life. They include infertility (possibly through alterations in uterine estrogen receptors and ovary LH receptors), learning impairment, lower IQ scores and neurobehavioral changes.

It is well known that exposure to lead, at childhood or during adult age, depresses fertility and causes reproductive disfunctions [76, 80, 86]. Although the infertility developing during adult age may recover following blood lead levels decrease, the one developing after perinatal or childhood exposure usually persists through life. This alteration in frequent in humans under the effect of environmental or occupational exposures, and may widely affect human population, as it was hypothesized for the Roman Empire times. In fact, Gilfillan suggested that the declining birth rate and apparently increased incidence of psychoses in Rome's ruling class, which may have been at the root of the Empire's dissolution, were a result of exposure to lead in food and wine [31].

In the experimental animal model, it was found that prenatal exposure of female rats cause a persistent alteration of ovary gonadotrophin receptors and of steroidogenesis that can be detected later in life [120]. In prenatally exposed animals, the number and characteristics of uterine estrogen receptors differ from that found in non-exposed animals [119]. An accute [110] or chronic [111] exposure of female rats to lead, at concentrations found in human blood under occupational conditions, selectively affects several but not all responses to estrogen in the uterus. Preliminary results from our Laboratory suggest that prenatal exposure to lead also cause selective changes in some responses to estrogens but not others (Tchernitchin & Tchernitchin, unpublished observations). These alterations explain, at least in part, the known fertility inhibition in lead exposed experimental animals [44] and humans [76, 80, 86].

In experimental animals, prenatal exposure to lead causes a permanent increase in the affinity of δ- [70] and μ-opioid [58] receptors, but not κ-opioid receptors [58] in the rat brain. This change parallels the impairment of opioid but not non-opioid stress-induced antinociception in developing rats [49]. It has not been investigated whether these changes also occur in humans; if they do, they may explain behavior changes that occur in exposed population [6, 77, 87], and perhaps may explain increased frequency of addiction to opioid or other abuse drugs in high lead contaminated environments [109]. The finding that the dopamine and 5-hydroxyindoleacetic acid response to amphetamine is enhanced in lead-exposed animals [62], suggests that the response to other stimulant abuse substances may be enhanced as well [109]. Our hypothesis on the role of early exposure to lead on abuse drug addiction [109] was confirmed by the finding of opiate deprivation syndrome that occurs in prenatally exposed rats but not in those were not exposed [59].

In experimental animals, exposure to lead impairs learning [68]. In humans, it causes deficits in central nervous system functioning that persists into adulthood,

including learning impairment, deficit in psychometric intelligence scores, lower IQ scores, poorer school performance, increased school failure, reading disabilities and poorer eye-hand coordination [6, 77, 87]. According to the U.S. Department of Health & Human Services, a deficit in the IQ scores can already be detected in children with lead blood levels as low as 9 μg/dL [88]. Further, exposure to lead causes the development of an aggressive behavior, and it was recently shown that there exist a correlation between bone lead levels and history of delinquent behavior in an American population [78]. Imprinting of paths of heterodifferentiation in central nervous system cells may explain at last in part the above alterations.

Taking into consideration the proposed role of lead in the Roman Empire dissolution, through the declining birth rate of the ruling class and increased incidence of psychoses, it is necessary to pay attention to the widespread lead contamination that currently affects many human communities throughout the world. Besides its effects on reproductive functions, it is relevant its damage to central nervous system, impairing mental abilities, inducing aggressive behavior, favoring delinquent behavior and a tendency to addition to drugs of abuse. This way, lead may also cause an enormous damage to our societies. We previously proposed [109] that during the second part of this century, an important epidemic of addiction to drugs of abuse developed, first, in large cities from the U.S.A., then, in large cities from Western Europe, and currently in most large cities from the developing Third World. It does not affect rural or small town population localized far away from large cities. This addiction to drugs of abuse paralleled a simultaneous increase in criminality. According to our hypothesis, these changes can be explained, at least in part, by the increase in lead pollution in large cities, but not in small towns, that followed the massive use of leaded gasoline in these places. Perinatal exposure to lead would have originated changes in brain opiate, estrogen and other receptors and subsequent neurobehavioral alterations.

Benzopyrenes

These are present in air particulate material, and are mainly originated from the combustion of diesel gasoline. It has been shown that perinatal exposure to benzopyrenes induce, by the mechanism of imprinting, a persistent decrease in glucocorticoid receptors in thymus [18]. Recently, it has been shown that exposure to benzopyrenes during the adult age also cause a persistent decrease in glucocorticoid receptors in thymus, which is the first documentation of imprinting mechanisms induced during de adult age [20]. These findings mean an alteration in glucocorticoid regulation of the immune function in lymphoid organs. They explain the relative decrease in the immune defense mechanisms against bacterial and viral infections and their increased severity in population exposed to high particulate pollution in cities like Santiago, Chile. Further, this mechanism may explain the increase in premature mortality and the correlation between mortality and PM10 particulate air concentration in the three subsequent days following increased particulate pollution in Santiago [82].

Ozone

This air pollution component, present in many large cities, is originated by a photochemical process from nitrogen oxides, under catalysis by ultraviolet light and volatile hydrocarbon compounds. It has been found in experimental animals that prenatal or early postnatal exposure to increased ozone concentrations causes persistent delayed selective neurobehavioral effects [21]. Although these effects were not confirmed in the human species, the findings warn on a possible damage of the central nervous system in perinatally exposed population, that can be detected later in life and could be responsible of behavioral alterations or damage to mental abilities.

Dioxins

Prenatal exposure to 2,3,7,8-tetrachlordibenzo-p-dioxin (TCDD) induces thymus gland atrophy and immune suppression, due to an alteration of the differentiation of the lymphocyte stem cells [26]. It also causes a reduction in ejaculated and epididymal sperm numbers and sex accessory gland weights in offspring with normal testosterone blood and androgen receptor levels [34]. It also determines a morphologic and behavioral demasculinization and feminization, and a decrease in fertility [34].

Pesticides

Perinatal exposure delayed effects caused by imprinting were described for organochlorine pesticides (polychlorobiphenyls, DDT, DDE, methoxychlor, chlordecone), organophosphate pesticides (parathion, malathion), pyrethroid insecticides (cyhalothrine, cypermethrine) and the herbicide paraquat.

Perinatal exposure to polychlorobiphenils causes persistent behavioral changes in rats [83], a decrease in fertility in *Peromyscus polionotus* [69] and, in humans, a delay in cognitive development [60] and alterations in nail development [45]. There is scarce information on the effects of perinatal exposure to DDT. It induces changes in muscarinic acetylcholine brain receptors [51] and altered behavior during adulhood following neonatal exposure to low doses [25]. Based in reports that it displays estrogenic action [10,29], it is possible to expect similar effects to that displayed by estrogens. The metabolite of DDT, p,p'-DDE displays high affinity for androgen receptors and potent anti-androgenic action [56]. The decrease in sperm cells count in Denmark and a smaller penile size in Apopka Lake (Florida, U.S.A.) alligators were attributed to this agent [95]. Perinatal exposure to methoxychlor, a pesticide with estrogenic action, increases the number of atretic follicles and induces early vaginal opening [100]. Perinatal exposure to chlordecone causes masculinization [96] and persistent changes in blood corticosterone levels [14].

Prenatal exposure to the organophosphate pesticide parathion causes a persistent inhibition of Mg-dependent renal ATPase, which is possible to detect later in life

[50]. Malathion, in turn, causes changes in cholinesterase activity and facilitates addiction to ethanol during adulthood [48]. Perinatal exposure to the pyrethroid cypermethrine causes a decrease in thymus CD4 and CD8 sybtypes, decreases the thymocyte proliferating ability and affects thymocyte differentiation [91]. It also increases blood NK cells and blood cytotoxic activity dependent of antibody [90]. The pyrethroid cyhalothrine causes a delay in testis descent [33]. Prenatal exposure to the herbicide paraquat causes a delayed increase in hepatic peroxidation [32], and counteracts, during adulthood, paraquat-induced increase in liver glutathione reductase [94].

FOOD ADDITIVES AND NATURAL FOOD COMPONENTS

A number of food components may induce imprinting if individuals are exposed perinatally to them at increased concentrations. High perinatal lipid feeding alters hepatic drug metabolism during adult life [54], induces cholesterol homeostatic memory [9] and alters blood estrogen levels during adulthood, which, in turn, favor the development of breast cancer [43]. Early exposure of experimental animals to glucosamine and mannose may alter insulin production by pancreatic beta cells during adulthood [17]. A perinatal diet high in sodium chloride determines increases in salt intake and sodium excretion in the adult [13]. Further, the activity of various enzymes during adulthood are determined by the alimentary conditions during pregnancy of their mothers. Levels of liver and lung gamma glutamylcystein synthase and liver glutation peroxidase are decreased in the offspring of diet protein deficiency exposed mothers [61]. Based on the above findings, it was suggested that dietary factors in early life modify the extent of adaptive responses in later years [9], and determine the prevalence of various diseases during adulthood within the different ethnic groups displaying specific food preferences [109].

Some foods or beverages contain active agents (see [109] for a review). For instance, coffee, besides caffeine, also contains estrogenic agents that may induce imprinting mechanisms. Prenatal caffeine determine in the adult rat increased activity and decreased emotionality; higher doses of caffeine may have the opposite effects. Exposure to pregnant rats to caffeine inhibits differentiation of testis interstitial tissue and Leydig cells and reduces testosterone synthesis by fetal testis, which may in turn imprint paths of heterodifferentiation in other tissues.

Many processed foods and beverages contain exogenous compounds that may imprint paths of heterodifferentiation (see [109] for a review). For instance, caffeine is added to some soft drinks in several countries and pregnant women are usually not warned about it. Nitrates or nitrites, added to foods, may cause discrimination learning deficit and impaired retention behavior following prenatal exposure. Many food color additives and other substances used to improve organoleptic properties of food have not been yet evaluated for health risks following perinatal exposure.

SUBSTANCES OF ABUSE

Tetrahydrocannabinol

Prenatal exposure to delta 9-tetrahydrocannabinol causes biochemical and functional modifications in the central nervous system, that lasts through life. Among them, alterations in brain tyrosine hydroxylase expression [8], in the pituitary-adrenocortical axis [89] and neurobehavioral changes [75, 89].

Cocaine

Prenatal exposure to this substance determines a persistent reduction in dopamine release in the mesocortical region [116], causes neurobehavioral changes [40] and selectively decreases the sensitivity to cocaine for some but not all responses to the drug during the adult age [41]. It also causes a persistent alteration of norepinephrine release from cardiac adrenergic nerve endings [97].

Opiates

Prenatal exposure to morphine determines changes that persist through life. In the rat, affects noradrenaline and dopamine turnover rate in various central nervous system regions [112, 113]; in humans imprints sequellae in the area of attention [42].

STRESS

Prenatal exposure to maternal stress alters the developing immune system and causes a depression of cellular and humoral immune responses, which persist through life [55, 98]. Prenatal exposure to maternal stress alters morphine- and stress-induced analgesia in male and female rats [57]. This may be explained by persistent decrease in μ-opioid receptors in the striatum but not in other brain regions in prenatally exposed adult rats [47]. Prenatal stress also affects adult sex behavior both in experimental animals and in humans. In rats, it feminizes and demasculinizes male behavior [117]. In humans, it increases homosexuality in males [23]. Stress may interact via hypersecretion of various maternal hormones, which in turn cause the above described alterations via the imprinting mechanism.

CONCLUDING REMARKS

Taking into consideration the above information, it is clear that prenatal or early postnatal exposure to several chemicals, including natural food components and various polluting agents, causes irreversible changes that determine the future health conditions during adulthood. It can be assumed that an important number of adult age dis-

eases were determined during early stages of life, under the effect of these chemicals or agents present in the preferential mother's diet during pregnancy. It is possible to foresee that the knowledge of prenatal exposure delayed adverse effects will allow to avoid them during sensitive stages of human development, i.e., late fetal and early neonatal ages. Further, the finding of mechanisms for antagonizing the effects of imprinting opens the possibility to interfere with prenatal exposure delayed adverse effects, in situations where it is not possible to avoid the contact with the imprinting-inducing agent. This new field of environmental pathology and medicine may therefore contribute to an important improvement of health conditions of humankind.

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ORIGINS OF SIGNALLING AND MEMORY: MATTERS OF LIFE *VERSUS* DEATH*

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This review focuses on the principles in cell-cell communication and cellular ability to respond to external chemical changes which have been so crucial for the development of life on planet Earth. We now know that the capacity of free-living organisms which evolved more than a billion years ago to respond to intercellular signal molecules, originating either from themselves or from other sources in their vicinity, is so similar – possibly even more sophisticated – to that of the cells in our own body, and these findings have had a major impact on our struggle to understand how life has evolved and how it can be maintained. Attention is drawn to the very important topic of mechanisms in cell death, being seen as an aggressive and very powerful instrument in the continuance of life and ability of life to proliferate into a plethora of new species, and use insulin-related material as our paradigm. Such signal molecules (hormones) may have played a major role in cellular maintenance throughout evolution.

Keywords: Evolution – eukaryotes – signalling – hormones – ligands – receptors – memory – hormonal imprinting – life – death

INTRODUCTION

Common requirements for signalling of cell life and death throughout eukaryotes

An enormous range of constitutive signal (messenger) molecules that can be elaborated by eukaryotes is currently being revealed. Each of the different types or species of messenger will necessarily have at least one relatively specific recognition or "binding" site, commonly referred to as "receptors". The interaction of the signal molecule as the ligand with its appropriate receptor sets off one or more relatively

* Dedicated to Professor György Csaba for his 70th birthday.

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well-defined intracellular pathways which result in some form of action being taken by the cell. Defining a "signal" molecule is not that simple, especially where a molecule can be a powerful signal to some particular action (or inaction), and yet be consumed as a necessary molecule for metabolic processes (some sugar molecules might fit this bill). However, one other feature of a signal molecule is that it is usually required at extraordinarily low concentration to elicit a biological response effect, e.g. pheromones. What all this means is that the cell, literally every cell, is probably capable of "tuning in" to a phenomenal number of chemical signals, as well as a range of physical signals in its environment. It will not be just listening in, but will be constantly responding to the symphony or cacophony of the whole universe brought to its surface and its matrix via the immediate neighbourhood to an extent dependent upon its ability to "perceive" the signals (i.e. its receptor complement). And presumably, should this noise level become relatively constant, it will continue the same activity while this state persists, that is until a spike of a particular signal suddenly occurs. If it has no means to answer to the signal, we assume it does not receive any information. While this introduction to signalling may sound a little banal to the expert in the field, it is included because it leeds to the first enunciation in this article, in which we discuss our views in relation to the seminal work of Professor Csaba in the world of cell signalling. Whether this is profound or not will remain to be seen, but we can state that in biology there may be a law which is semi-analogous to Newton´s first law of motion; to paraphrase, a cell in a relatively constant environment will continue what it is doing *until signaled otherwise.* This suggests that unless a physical force or a chemical prod is given, its behaviour/metabolic pattern remains relatively set.

Positive versus negative defaults

Remain at the fundamental philosophical level, the next issue concerns the basic signalling mechanisms which are necessary to makes cells do "other things". Trying to scale this down to the minimum, there will be pressures which make cells speed up or slow down, some affecting the whole of metabolism, many affecting some specific activity of the cell (a differentiated function, such as secretion of a particular protein). There are signals which make cells move towards or away from them. But the rock bottom of all cell responses, and the options which are puzzled over the most, relate to cell proliferation. The simplest summing up comes from Hinselwood [34], and concerns the bacterial cell. But it is probably a universal truism, which is that a cell either grows or it dies. This suggest that cells are essentially survival "machines" which are in positive drive otherwise they will inevitably through inaction "rust" and fall apart. The current debate, which is by no means resolved despite the strong case made by Raff [78, 79] and many followers, is whether the same cell should need to be constantly signalled to proliferate, in the absence of which (or default) death occurs. But we also know that cells can be actively signalled to die, and therefore the default is not necessarily passive, but must also have an active mode. A diametrical-

ly opposite, as by Sonneschein and Soto [85], is that cells, in general, are highly gregarious, and will be communicating with each other on probably many different wavelengths at any moment in time. They will be able to distinguish some signals which have become characteristics to themselves, i.e. of their kith and kin, and also to more distant relations. On some wavelenghts they will be able to tune in and respond to what is going on in all *other* types of cells around. In protecting themselves in their own environmental niche, they may develop signals for avoidance of other cells, and even signals which spell death to another species, but we must not forget that the vast majority of basic signalling molecules, which are similar throughout all eukaryotes, will be designed to instruct cells to respond to, and thereby avoid, many of the perils of existence for living forms, i.e. they are geared for survival.

Why do cells tend to cluster together and not diffuse/disperse in the locality and further afield, if indeed this is true? Supposing it is, the most basic requirement probably is that cells which reproduce by simple fission, despite being very well nourished, loose their vigour and their proliferative potential after a time. Exchange of pieces of DNA between cells of the same species will allow them to circumvent in many cases this loss of vigour, although exactly what is going on in "hybrid vigour" remains very much a mystery to most cell biologists. Cells cannot conjugate to exchange DNA without having a means which draws them together, and cells which find places where they can proliferate also find that the food substances contain molecules which attract them to that area. When cells proliferate, they release signals which encourage others to survive and grow.

Much of our biology is based on premises which have been generated from above not below, that is they have been deduced from what we have spent most of our time studying most intensively, which is the biology and behaviour of man. This is very much due to the drive for healthy existence and the fact that research which is human orientated has always been given far greater resources than other endeavours in the life sciences. In the early days of cell biology, investigators looked at all sorts of interesting organisms and their cells, but most culturing has been based on human (or more generally mammalian) cells. We learned about signalling from these, despite – or perhaps because of – the fact that they have often been obstinately stubborn to begin proliferating *in vitro*. From the start, some "factor" (our euphemism for an "unknown substance") had to be provided. And this has led now to signalling being seen as important. But the media which were used to grow cells allowed little chemical dissection of the nature of these substances because the additives were so ill-defined which did get cells started. Only in the last 10–20 years it has started to move from alchemy.

ANTIQUITY OF SIGNAL MOLECULES

We have already dealt with a whole range of signalling phenomena and molecules that occur in protozoa [9], demonstrating their extraordinary similarity to those of most other groups of organisms, and noting that these are truly transphylal in nature.

But this is nothing new; it has just taken a considerable length of time for the "news" to filter down through the biological fraternity to the point where many can now begin to accept it – or, at least, to acknowledge it. In a sense, it is a latter-day case, rather like that of Darwin, whose ideas were scorned because they suggested there was a strong case for man "descending" from apes, with whom we now know we share about a 99% homology in our DNA. One of the difficulties was the possibility that some of the messengers,* which human cells, protozoa, and even bacteria have in common, included insulin, ACTH, and somatostatin [57], to name three, might have been acquired by single-celled organisms. As more conjunction came to light, the theory that parasitic unicellular organisms might have acquired mammalian genes rather than possessed them constitutively, i.e. as an integral part of the genome since the origin, became increasingly implausable. Furthermore, and as will be discussed below, unicellular eukaryotes had appropriate binding sites or receptors to all these ligands, and the internal response pathways which were also closely homologous with those of "higher" eukaryotes. While implication, inference and similarity (discerned from cooperative approaches) provide relatively solid evidence, experimental work on the physiological and behavioural necessities of signals and signalling systems need to go deeper, and the genetic basis understood.

Early names which are important in this work include Professor Jesse Roth and his collegues working at NIH, whose findings with regret never have been considered either serious or important in the States. Others, notably Professor György Csaba and his colleagues in Budapest, who have pursued such matters as the remarkable signalling processes in protozoa over his entire career, and throughout the eukaryotic world. Fortunately, their findings are now increasingly seeing the light of day, and many of us are aware that we have been oblivious to the obvious for too long. Recognition has fortunately again come within his life-time and we dedicate this article to the outstanding efforts of Professor Csaba in a long and distinguished career.

Signal molecules cannot exist alone; pathways that handle with them

Like all major revelations, we are only just beginning to appreciate the impact and the enormous vista of new work which these findings opens up. Hormones, pheromones, chemoattractants, neurotransmitters, growth and survival factors, in other words any chemical signal, which will be found throughout the eukaryotic world. In the prokaryotes, fungi, and plants, as also in some subdivisions of the liv-

* It is incumbent upon us to define our terms; a signal molecule or messenger is hard to define. Hardie [31] bewailed this same situation: "I also believe it to be unfortunate that there is not a collective term to describe neurotransmitters, hormones, local mediators and growth factors, other than clumsy phrases such as 'extracellular signal molecules'. The lack of a short and distinctive word for these entitles genuinely hampers people from thinking about them in an integrated way." But now we have seen the problem, perhaps it has been solved for us. Having identified the major *personnae dramatis,* we may have to add other smaller groups to this list; these are also signal molecules, but their sphere of operation must be seen as both extracellular and intracellular.

ing kingdom yet to be appreciated and more fully understood (e.g. archaebacteria), signalling is a must for survival, but there have been evolutionary divergences and dichotomies, and similarities have been lost. The whole of the molecular evolution concerning the basic signals of life has yet to be uncovered, and promises to be a trail which will tell us probably as much about the evolution of the eukaryotes themselves as any other approach. For the cataloguers, there is a lot of necessary work – much as himself Darwin undertook at his level of inspection – in taking all cell types of all extant creatures, then listing and comparing on massive databases the repertoires and homologies of their ligands, receptors and response pathways. And for the physiologists and ecobiologists, there is the problem of seeing how all these signals and signalling systems interact not just within and about a single cell, but within a population of cells, within an organism, within a population of organisms, and then within a whole ecosystem. And for the experimentalists, there will be one over-riding phenomenon in all of this business which begs an early answer, and we will chose this one, not only because it has already been a focus of interest, but because the answer to a particular problem in this will probably give us an important insight into the whole issue.

Commonalties in signalling and its pathways

Should we have recognised the (relatively) universal eukaryotic language of communication earlier? Biologists spent too long revealing in the diversity of nature rather than concentrating on the similarities at the core. Apart from DNA, they often start from a position of sheer disbelief in the first instance, that mechanisms in yeast, plants and animal cells have an extraordinary consistency, as examplified finally by the genetics of cell cycle regulatory proteins; it was a major breakthrough to show that a gene coding for one particular cell cycle engine component, i.e. cdc34 from yeast could be cloned and transfected into cell cycle aberrant malignant human cells and restore normal regulation of proliferation [71]. The universality of the cell cycle regulatory mechanism is now beyond question. Are we going to take each major system of a eukaryotic cell and start from the stand-point that they are different *until proven alike?* Endless questions arise from these findings of signalling systems, too many to recount here, but one final one which usually comes to the fore in all such discoveries and revelations is: "is there anything in it to benefit man?". If *Tetrahymena* insulin can quickly tell us a lot more about human insulin, the answer will be a vigorous affirmative. Whatever we learn about insulin in "lower" cells (that have a generation time of 2 hours and could have gone through about 10 doubling of their offspring in the time our cells would have divided just once, and are therefore evolutionary light years ahead of us in many respects) is inevitably going to help mankind, although the extent has yet to be gauged. The fact that we can do very clean experiments in very highly controlled environments in completely chemically defined nutrient media using organisms which grow exceedingly quickly, have the same essential amino acid requirements as human cells, and which have excellent

genetics strongly supports their exploitation in speeding up our understanding of signal processes generally, as well as providing potential biotechnological advances which will also benefit mankind [93]. Of course, *Tetrahymena* is not *Homo sapiens,* and the critics will rightly clamper as usual for caution in translating effects from one system to another. But let us not continue to see everything as only worthwhile if it has been proven in man.

For the sake of a focus in this paper, which hopes to draw comparisons and will assist in letting the reader appreciate how questions asked of protozoa might, like *Schizosaccharomyces pombe* in human cancer, equally benefit mankind, we will continue to concentrate on insulin and insulin-like molecules. Since we started talking about the issue of life and death, we are dealing with signals which are crucial to the maintenance of the living state. In man, insulin-related compounds regulate a vast array of cellular processes, including cell proliferation, chemosensory behaviour, and neurotransmission, and insulin is generally seen as crucially important in the homeostatic mechanism regulating blood sugar level, which can be a matter of life and death. Let us ask the same question of *Tetrahymena* with respect to insulin: are we dealing with glucose regulation in this protozoa and/or are there some more fundamental processes which we have been missing ever since its discovery in man? If the answer to this question is yes, then we are going to witness some dramatic revelations about how cells integrate their physiological processes and in turn affect one another in the company that they keep, whether it is in a swarm of protozoa around a hay infusion in a pool, or in a group of bronchial epithelial cells lining the passages of a human lung. If this example fails to strike home, then consider the finding of opioid receptors in *Tetrahymena* [72, 97] and ask the same sort of question: what would these be required for in a free-living protozoan? And there are many other queries, which seriously need to be elucidated, and we have come up with ten questions, some of these being discussed in the following:

1. Given that we have no "fossil record" of *Tetrahymena,* what were the early ciliates like some hundreds of millions of years ago when they evolved from an early eukaryotic predecessor?
2. Did these early forms have receptors to opioids, cannabinoids, insulin, somatostatin, ACTH, etc?
3. Given that an organism such as *Tetrahymena* has a short generation time under adequate nutrient conditions, and must regularly indulge in conjugation to maintain hybrid vigour, this species must have far greater evolutionary scope as a free-living organism than our own cells and bodies, so is it not probably that the receptors themselves and the pathways they elicit are part of the "normal" machinery of cells, characteristically as living matter being highly responsive to its environment, and much more so than in "higher" organisms which had developed an internal homoestasis?
4. Given that receptors evolved which allow cells to alter their behaviour in responses to the environment, will not their functions in early species teach us much more about the underlying mechanisms by which metabolism is redi-

rected according to the "signals" perceived in the environment? The basic signals are to attract (e.g. for conjugation or symbiotic purposes) or to repel, which aims at warding off threat of other organisms taking advantage of a vulnerable cell? Are all other signals not a specific form of one or the other of these?

5. Is the basis for improvement in the response of a cell on re-exposure to a specific signal due to some alterations in receptor topography, dimerisation, cooperativity, especially during development, or is it related to some other form of "memory" within the cell?
6. Is "hormonal imprinting" a memory trace which can persist through many vertical generations by virtue of the fact that it is one of the above, or is it a memory trace in DNA, protein, or some other internal (molecular) network?
7. What is this in common with memory as commonly understood in higher organisms, and is it not the same problem fundamentally, or is it more like immunological memory?
8. How specific are the receptors of *Tetrahymena* relative to those of "higher" organisms, and what are the lowest concentration at which ligands affect an appropriate response?
9. How are the responses to any particular signal co-ordinated in such a way that a coherent response results?
10. What especially can we learn about the effects of insulin in *Tetrahymena* (or yeast, or any other species which has been studied) with regard to (9), since the metabolic implications of receptor activation lead to such plethora of metabolic changes that it becomes difficult to dissect which are the primary (direct) and which are secondary (indirect, knock-on effects)? Inbried, what is the initial effect of insulin upon, e.g. glucose metabolism?

DISCOVERY ON INSULIN SIGNALLING IN "LOWER" EUKARYOTES

Insulin-related material is an ancient signal substance

It is now evident that unicellular organisms and cells from metazoan sources share great homology as to basic processes in the regulation of programmed cell death, survival or proliferation, chemosensory behaviour, and differentiation [9], and insulin or insulin-related compounds may well have been important as multifunctional signal molecules regulating these processes early in evolution, being the prerequisites to the development and maintance of functions in the metazoan organisms. Müller et al. [66–68] recently showed that human insulin in the yeast *Saccharomyces cerevisiae* elicits physiological and molecular properties similar to those in vertebrate cells as to the regulation of glucose metabolism. Furthermore, it was found that human insulin at picomolar levels is a growth factor in cells of *S. cerevisiae* when cultured in a 120-fold dilution of Wickerhams medium [94] in which glucose was replaced

with glycerol as the only carbon source [74]. In these experiments the lag phase was reduced from weeks to a few hours. *Neurospora crassa* produces and secretes insulin-related material [55, 58], and insulin induces numerous metabolic responses such as growth enhancement, increased rates of glycolysis, glucose oxidation, alanine synthesis, glycogen synthesis, and modulated intracellular Na^+ levels [25, 28, 29, 61].

Human insulin stimulates cell survival [4, 11] and the adaptory element of chemosensory behaviour [44, 47] in cells of the protozoan ciliate, *Tetrahymena thermophila* and *T. pyriformis,* and insulin is active from low picomolar to nanomolar levels. *T. thermophila* releases autocrine signal molecules, which prevent its death in a nutritionally complete, chemically defined medium (CDM, [87]) [5, 82]. At low initial cell densities in CDM these factors become too diluted and cells die by a process which we have related to programmed cell death (PCD) because of common particular features to this type of cell demise [6, 12], but this in turn can be circumvented by low picomolar levels of human insulin [8]. Adaptation reflects the ability of the cells to "remember" (short-term memory) a certain concentration of insulin thereby preventing them from swimming towards lower concentrations [53]. It has also been shown that insulin-like material can be found both in *Tetrahymena* cell isolates and in their growth media [55, 83], and that insulin – among many other responses – regulates glucose uptake in these cells [22], just as it stimulates glucose metabolism in yeast [66]. This suggests that transduction mechanisms associated with insulin signalling in *Tetrahymena* share characteristics similar to those present in a wide range of organisms including vertebrates.

Insulin receptors

Signalling requires a distinct set of receptors, and since the effects of insulin in unicellular organisms are so very similar to those seen in mammalian organisms, we might assume that receptors for insulin share similar characteristics throughout evolution. The mammalian insulin receptor is a tetrameric tyrosine kinase that consists of disulfide-linked heterodimers, each being composed of an extracellular 130 kDa alpha-subunit (IRα) that is covalently linked to a transmembrane beta-subunit (IRβ). Binding of insulin to the alpha-subunits induces activation of the beta-subunit kinase domain and autophosphorylation. Müller et al. [68] reported a putative plasma membrane insulin receptor in yeast of a monomeric structure with an apparent molecular mass of 53 kDa, being phosphorylated *in vitro* on serine residues upon stimulation with human insulin. In addition, they found evidence that the binding of insulin to this receptor can activate a tyrosine kinase which is a constituent of a 300 kDa non-covalently subunit of a hetero-oligomeric receptor kinase complex, involving the tyrosine phosphorylation of a 70 kDa membrane protein [67]. *N. crassa* has a 66 kDa plasma membrane protein which could act as an insulin receptor, although it contains no detectable protein kinase activity [46]. However, insulin promotes the *in vivo* and *in vitro* phosphorylation of at least 14 distinct proteins, of which at least six are phos-

phorylated on tyrosine residues, and one of these (a 38 kDa protein) cross-reacts with an antibody against the human insulin receptor autophosphorylation site [45].

Gomes et al. [27] found that human insulin-like growth factor I stimulates the *in vivo* tyrosine phosphorylation of several proteins in cultures of promastigotes and amastigotes of the parasitic flagellate, *Leishmania (Leishmania) mexicana.* These includes a 185 kDa protein in cells of promastigotes and two proteins of 40 and 60 kDa in amastigotes, suggesting that tyrosine phosphorylations are regulated during the life cycle of these flagelates by stage-specific kinases [27]. Little is known about the nature of insulin receptors in *Tetrahymena.* Previous reports have shown that *Tetrahymena pyriformis* binds insulin, and that treatment with insulin leads to an alteration of metabolic functions as well as an increase in the binding of this molecule to the cell surface and nuclear membrane [19]. Christopher and Sundermann [14] found proteins in cilia, plasma membrane and organelles, being immunologically similar to the α-subunit of the mammalian insulin receptor. Further evidence for the presence of insulin-receptor like molecules in *Tetrahymena* comes from studies in *T. thermophila,* in which a 53 kDa protein was shown to cross-react with an antibody raised against the human insulin receptor β-subunit (IRβ) [11].

Insulin-mediated signal transduction

Insulin-mediated signal transduction in *Tetrahymena* was suggested to involve the activity of phospholipase D [49, 51], turnover of inositol phospholipids (PI, PIP, PIP_2) and glycosyl-phosphatidylinositols (GPIs) [48], and nitric oxide and cyclic GMP [10, 43, 50]. Straarup et al. [86] also suggested a PKC-like activity to be associated with autocrine regulation of cell survival and proliferation in a manner similar to that of insulin-mediated inhibition of cell death. The potent PKC inhibitor, staurosporine, inhibits phosphorylation of the PKC-specific substrate, myelin basic protein peptide fragment 4–14 [86], and induces programmed cell death with characteristics of both apoptosis and autophagic degeneration [10]. Cell demise could be circumvented by addition of membrane permeable analogues of cGMP, while insulin had no effect [86]. Kovács et al. [49] showed that the tyrosine phosphatase inhibitor, orthovanadate, and the tyrosine kinase inhibitor, genistein, enhanced and inhibited PLD activity, respectively, suggesting that PLD activity is regulated via a PTK activity. Indeed, recent findings from studies in *T. thermophila* strongly support the conclusion that as in mammalian cell systems, PTK activity plays a major role in insulin-mediated signal transduction in cell survival, proliferation and chemosensory behaviour. Thus, genistein and other PTK-inhibitors block insulin-induced chemoattraction [53] and inhibit cell survival in low-density cultures [11]. Furthermore, Christensen et al. [11] have now identified at least three proteins (p53, p57 and p59) being tyrosine phosphorylated *in vivo* upon addition of insulin to cultures of *T. thermophila* in chemically defined nutrient medium, the former phosphoprotein, co-migrating with the cellular protein which is immunological similar to the β-subunit of the human insulin receptor.

Cilia – a sensory site for intercellular signal molecules?

The surface of cells is the first site to be affected by the environment, and in many organisms organelles such as villi and cilia greatly extend the area of this contact. For several different reasons, cilia have properties which lend themselves not just to the role of moving cells or their environment past them, but as sites which contain higher than average receptor densities for many different purposes. They can be responsive to physical and chemical pertubations. While the ciliary membrane of ciliates, as will be discussed below, are notably sites of, e.g. insulin bindings, it is likely that many others will also be similar located. But cilia processes, which are enriched in receptor density, have been found elsewhere, notably in primary or centrosomal cilia, which are now known to be quite common throughout eukaryotic cells. The most speculative paper on this subject comes from Poole et al. [77], but more recent work on nodal cilia, primary cilia in *C. elegans* and other species are quickly turning this prediction into a reality (for a review see [92]). Before providing further circumstantial evidence for a sensory role for primary cilia, as if those in the eye and the kidney were not in themselves an adequate demonstration, it is worthwhile discussing what cilia in "lower" organisms, in particular in ciliates such as *Tetrahymena* (Fig. 1), are doing in terms of the role in cell survival and proliferation.

Fülöp and Csaba [26] reported that insulin binds to the cilia of *T. pyriformis,* and these also contain proteins which are immunologically similar to the α-subunit of the mammalian insulin receptor Christopher and Sundermann [14]. Furthermore, the ciliary membrane contains a ~67 kDa insulin-like protein, which was suggested to function as both precursor to a soluble form and membrane-bound binding site/receptor [13]. These observations strongly support the idea, that the cilia may have adapted a sensory role in line with the findings that the level of phosphotyrosine compared to those of phosphoserine and phosphothreonine, dramatically increases as we move from the whole cells to the cilia and the ciliary membrane [3]. In mammalian cells, only about 0.1% of phosphoproteins in eukaryotic cells are phosphorylated on tyrosine residues, and PTK activity is highly associated with signalling processes. Indeed, the membrane structure of ciliates resembles that of chemosensory neurons and the olfactory epithelium in mammalian cells [36, 62], and it is therefore possible that mechanisms of adaptation in *Tetrahymena* share similarities to, e.g., neuronal activities.

Other intracellular signal events may take place in the cilia other than those associated with PTK-activity, and they may act as secondary messenger systems in insulin-mediated signal transduction in *Tetrahymena.* Such systems include cGMP-dependent processes. Thus, insulin induces a rapid production of cGMP in the cilia of *T. pyriformis* [43], in line with the findings that cilia have binding sites for insulin [26] and that guanylate cyclase activity [39, 81, 90] and cGMP-dependent protein kinases [69, 70] are present in the cilia.

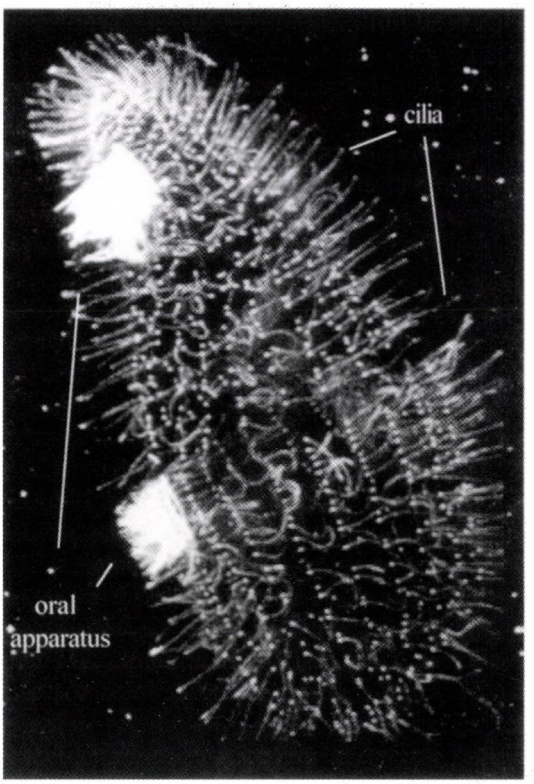

Fig. 1. Cilia of *Tetrahymena thermophila* labelled with anti-tubulin. Note: the cell is undergoing division, showing two oral apparatus. The cilia are clearly demonstrated in both oral apparatus as well as on the surface of the cell (IDS staining)

Receptor imprinting in Tetrahymena *and evolution of signal molecules*

In contrast to the controlled environment in which cells from metazoan organisms exist, free-living unicellular organism exhibit all the means necessary at their disposal to maintain their own survival in a rapidly changing environment, sensing a practically infinite variety of dissolved materials, not to mention physical insult. Such means may thus be seen as of fundamental character, allowing cells to respond as quickly and as efficiently as possible, to react to new conditions in their vicinity. This also implie that single celled-organisms must possess a much broader spectrum of surface receptors and signalling pathways than those in individual mammalian cells, being highly specialized to deal with a limited number of specific actions within the body. One way of doing this is to memorise the first encounter with a ligand, originating either from cells of its own kind or from other types of organisms in their vicinity. This will ensure that the cell can respond in a much more efficient way upon

a second encounter with the molecule. Csaba has been the main pioneer in this field, using *Tetrahymena* as a model organism for evolutionary studies on the development of hormones/growth factors and their receptors. He and co-workers found that *Tetrahymena* grown in a rich nutrient medium upon pretreatment with a hormone of mammalian origin, e.g. insulin, increases their sensitivity to the molecule both by altering their binding capacity of the molecule and by demonstrating a decreased threshold value to response upon a second encounter of the signal molecule [19–21]. This phenomenon has been termed "hormonal imprinting", arising from the encounter of a vast number of molecules, which also evoke cellular responses in mammalian cells, suggesting that the binding sites/receptors have characteristics similar to their mammalian counterparts.

Imprinting may have far-reaching implications of how signal molecules and their receptors arose in evolution, in which an increased binding capacity may be seen as the first meeting of the presumptive hormones and their presumptive receptors [21]. Csaba argued that signal receptors arose *"because the external environment of the individual cell is transformed at the multicellular level to an internal environment, in which the random variety of environmental molecules is replaced by a predetermined set of ligands (signal molecules)"*. Lenhoff [54] suggested that amino acid type hormone receptors originated from amino acid (food) receptors, and both oligopeptides and single amino acids provoke imprinting [21], supporting the hypothesis of Lenhoff, and we might as well suspect all sorts of cell-released compounds including waste- and by-products as candidates for presumptive signal molecules. Indeed, there are several examples from studies in unicellular organisms – prokaryotes as well as eukaryotes – showing that compounds generally regarded as such, can be as important in intercellular signalling events as seen with complex peptide-signal molecules. One example comes from studies on the myxomycete, *Dictyostelium discoideum,* in which slime mold formation, besides being regulated via intercellular extruded compounds such as cyclic AMP and specific polypeptides, is dependent on autocrine and paracrine action of NH_4^+ [84]. And when we take a look at neurotransmitters, being single (modified) amino acids or similar small molecules, we realize that signalling elements in unicellular organisms and nerve cells can be very similar. In fact, cells of the unicellular flagellate *Trypanosoma cruzi* have a nitric oxide-dependent guanylate cyclase system, which is activated via NMDA receptors [76]. In the case of insulin, it also seems plausible that compounds similar to this signal molecule already existed very early in evolution. Thus, as for *Tetrahymena* and *Neurospora crassa,* material closely resembling vertebrate insulin was found in prokaryotes of *E. coli* and *Halobacteria,* and this material possesses bioactivity in adipocytes, incorporating glucose into lipid [55, 56, 58, 75, 83].

MEMORY; THE PROBLEM OF ITS NATURE IN EARLY CELL SIGNALLING

Molecular mechanisms in and evolutionary aspects of imprinting

As mentioned in the above, imprinting is the "memory trace" which cells retain of an encounter with a ligand, such that it will respond more quickly (or more efficiently) the next time it meets it, and what is more intriguing is that it is retained through innumerable (vegative) divisions (i.e. by the progeny) of the cell [21]. After studying this phenomenon in protozoa, the immediate thought is, does this occur in all cell types? Do our own cells respond to insulin or other signal compounds at their first encounter and "know" that molecule no matter how many subsequent divisions are undertaken in growth and development? Quite possibly: the developing vertebrate olfactory system alter the sensory surface according to environmental conditions so as to enhance sensitivity to molecules of particular relevance (e.g. [35], and biological mechanisms may exist to "memorise" the metabolic effects of early nutritional environments in mammals, i.e. "metabolic imprinting" [91]. With an impressive accomplishment, Csaba and colleague professors have taken the data on hormonal imprinting from *Tetrahymena* and applied it in principle to higher systems, even to medicine in man, and has obviously shown that this approach can be a fast route to new knowledge and new applications. We are confident that these achievements will be recognised and reviewed by others in this celebration issue, honoring the work of Professor Csaba.

We seem to be moving inexorably in this discussion to the suggestion that this is the cell or the organism having much the same thing as an immunological memory, a recognition of and ability to respond to previously encountered signals, although this is now to hormones and factors, not antigens. The latest ideas on such memory traces in cells is that of Bray [2] based upon groups on interacting proteins operating as an intracellular "neural" network, although as yet there is no firm evidence that proteins are indeed involved, the ideas being largely speculative.

Csaba [21] suggested that imprinting could arise from either (*i*) gene arrangement similar to that produced during the formation of antibody coding genes, (*ii*) rearrangement of methylation pattern of the appropriate genes, influencing the expression of receptors, or (*iii*) changes in the membrane subpatterns forming the receptors. Christopher and Sundermann [14] found that insulin imprinting is associated with an altered composition of proteins in the ciliary membrane, supporting the conclusion that cilia, not only possess the necessary components in insulin signalling, but are implicated in complex processes associated with molecular changes in hormonal imprinting. However, we still have many questions to be answered, before we can draw a clear picture of the molecular mechanisms and the role of imprinting as to the evolutionary aspect of signalling events in memory tracing.

DEATH SIGNALLING

Introductory remarks

As we move toward the end of this review, we realise that there are so many aspects of cell signalling which need to be taken into account to fully understand the many complex events, that eventually have been so crucial for the development of life. But that constant partner of life which cannot be fully defined without viz. death, is as much a biological process as any other, barring pure accident. We also have to set it apart from most other biological happenings for the very obvious reason that it is final. In our Introduction, we discussed some substantial aspects of cell survival versus death, but we feel that this subject is of decisive importance for us to understand a very significant part of evolution, and we therefore continue our considerations on the role of death. It should come as no surprise, even to biologists, that it is highly controlled event in cell populations. Cell biologists have for so long been overly concerned with life, especially proliferative mechanisms, to the neglect – until quite recently – of cell death, other than in simple descriptive terms.

Mechanisms in programmed cell death

The death of specific cells at specific moments, intervals and sites play a crucial role in morphogenesis, in shaping tissues, organs and the entire body of metazoan organisms. This is seen in embryos of plants and animals developing into adult organisms [37, 78, 79], in tadpoles metamorphosing into frogs [89], and during the life cycle of invertebrates [33, 63]. Myxomycetes of *Dictyostelium discoideum* assemble into a multicellular aggregate, differentiating into a foot-plate, a stalk and a fruiting body with spores during starvation [60] and the programmed cell death of specific cells serves to form the mature body [16]. Possibly, any cell at any time can be "forfeited" to die to ensure the developmental processes in a predictable manner from its interactions with other cells via intercellular signal molecules. A celebrated case is that of the nematode, *C. elegans,* in which exactly 131 of its 1090 somatic cells die during the development of the adult hermaphrodite [24].

Developmental cell death is executed by a multiplicity of destruction mechanisms. Once the cell has been committed to die, it eliminates itself by a distinct set of morphological and enzymatic changes. In his review, Clarke [15] distinguishes four modes of death in metazoans: apoptosis, autophagic degeneration, non-lysosomal vesiculate disintegration, and a cytoplasmic type associated with cell lysis, although we might expect many more modes of cell demise, especially when we include the programmed cell death of unicellular organisms, since such cells encounter environmental changes, which vary much more than those of cells in multicellular organisms.

Apoptotic cell death is characterized by membrane blebbing, cell shrinkage, chromatin condensation, and nuclear and cellular fragmentation, with the products of these last processes eventually being engulfed by neighbouring cells [41, 42, 96].

While it has typically been used to describe mammalian cell death, it has been described in a vast range of different organisms, including unicellular eukaryotes (for reviews see [1, 9]). Apoptosis involves the action of a discrete class of cysteine proteases (caspases), of which the prototypes are interleukin-1β-converting enzyme (ICE) in human cells, and the product of the ced-3 gene in *C. elegans* [38]. Other key regulators are the human Bcl-2 protein family, comprising both suppressors (e.g. Bcl-2, Bcl-X_L and Mcl-1) and promoters (e.g. Bax and Bak) [3], and the nematode suppressor ced-9 (a Bcl homologue), and the ced-4 promoter [33, 63]. Studies with yeast cells suggest that these regulatory units are conserved throughout the eukaryotes [30, 32, 59, 80, 88]. This balance between promoters and suppressors must all be linked to specific, and carefully controlled, signalling systems to ensure the right outcome in the right place at the right time.

Tetrahymena thermophila also has similar "death pathways" to those originally described in metazoan cells. During mating and the conjugation process the old macronucleus is removed by a process similar to that seen in the nucleus of mammalian cells undergoing apoptosis [23, 65, 73], and vegetative cells exposed to the PKC-inhibitor, staurosporine, die by programmed cell death associated with characteristics of both apoptosis and autophagic degeneration [10]. In the absence of appropriate levels of autocrine survival factors, i.e. at low initial cell densities in chemically defined nutrient medium, *T. thermophila* also die in a programmed manner, but cell demise differs from other known morphological modes of active death, being set off by a quick disintegration, lasting less than 50 msec [12]. However, the latter type of cell death has widened our understanding as to the role of signalling for cell survival as a function of nutrient availability and *de novo* transcriptional and translation processes: the level of autocrine signal molecules required to support survival and proliferation, dramatically decreases for cells kept under circumstances in which the rate of metabolic processes and macromolecular synthesis is reduced, as seen in medium without an essential amino acid or in starvation-buffer, or in full medium with low oxygen availability. Thus, cells resuspended in a medium without signals, but which otherwise would support maximal growth and short generation times, could be very more fatal than cells going through a poorer medium, and this living on the "knife-edge" between life and death may well be regarded as an example in nature of a selective process in which only the fittest and most competitive cells are able to survive for the "benefit" of the species itself.

Final remarks

How cell death occurs has become very important, as evidenced by the exponential growth in reports devoted to the subject and the advent of new journals specifically catering for the thousand of investigators absorbed in the topic, and how it occurs is being rapidly uncovered in molecular terms. It also seems self-evident that cell death has a function, or serves a "purpose", and teleological arguments are frequently presented in the literature; many reasons for its occurrence appear very persuasive, but

these speculations are not necessarily correct. There is no question that cell death processes evolved and were selected for by many of the forces which shape nature; but it is equally evident that they must be carefully controlled since the consequence of something going wrong is extinction. There could be some basic underlying mechanism, which has arisen once or several times during the period that life has existed on this planet. It is difficult to be persuaded by the available evidence that the same mechanisms, for example, has evolved in both prokaryotes and eukaryotes, and unfortunately data on prokaryotic cell death death lags seriously behind that gleaned from eukaryotic systems [40, 96].

Some years ago it would be difficult to believe that eukaryotic systems as diverse as yeast, protozoa, blowfly and mammalian cells would necessarily have a lot in common with respect to cell death; for one thing, it was not generally appreciated that "free-living" cells had the ability to control their own life-span, death being met purely by accident. But if death is not accidental (or perhaps incidental), then it must have some sort of control. It seems logical that it has been there since the advent of the eukaryotes, and probably involves some particular pathway(s), which has since become much more diversified and sophisticated in terms of its checks and controls, but retains its basic character. The alternative hypothesis would be that different pathways have arisen throughout eukaryotic cell evolution, and therefore many significant differences will be found in the metabolic fates of different cell types. In this overview of the situation as far as current knowledge can take us without entering into wild speculation, it is increasingly clear that the former is going to be upheld.

Control over cell death, where the "sentence" is delivered by the subject itself, had led to the frequent use of the word "suicide", a word that has unfortunate overtones of conscious action; in modern parlance this leads us to surmise that the cell has a "program" which can be switched on under the appropriate circumstances. The "circumstances" are now being most keenly studied, especially the nature of the signal which throws the cellular switch. We can safely assume that the one which sets a cell off on its final journey is going to be a very specific one, and that there will be escape or rescue points before the point of no-return is reached. The problem is one of finding the master signal among all the checks and balances which is associated with directing the cell activites down the death pathway, and delineating in molecular physiological terms what is indeed the point of no return.

Although many instances can be cited of death occurring in a highly selective manner among populations of cells, we must also assume that it is because those cells are in some particular spatial and temporal arrangement, which leads to their demise, and that if other cells had been moved to these same positions at appropriate times, they would also have undergone the same fate, which is simply a long way of saying that all cells have the capacity to bring about their own death in a controlled way, but that circumstances can also be a deciding factor. However, our proposition suggests a particular interesting and simple experiment to perform. The expression "programmed cell death" (PCD) is used for this situation, one form being apoptosis. We need to bear in mind that some damaging event has occurred which is beyond cell repair. Every cell has the equipment to clear itself away, e.g. a lysosomal system; this

is also common to all eukaryotic cells. A distinction here is that the cell is being "killed", and the plethora of reactions which it might undergo have been the subject of great deal of "cellular pathology" since before the time of Virchow. If we can reasonably accept that cells have evolved more than a single system by which they can dispatch themselves, and that all cells carry at least both these two main types, then we should have a far better understanding and insight into how cells are selected to die and actually carry out the process than we currently seem to possess.

Raff [78] has stated the first law of cell biology: all cells need to be signaled to survive. This implies that all cells have a default, i.e. they die if they are not signaled, and we just agreed that they have the wherewithal to do this. Wheatley and Christensen would proffer as the second law of cell biology the following: cells need signals, nutrients, factors and co-factors in order to grow, differentiate and carry out all the functions which they undertake, and *under a constant environmental conditions they will continue what they are doing until they are signalled to do otherwise.*

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