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EFFECT OF PROXIMAL SELECTIVE VAGOTOMY ON GASTRIC PROSTAGLANDIN CONTENT IN THE SHAY-RAT ULCER MODEL

S. Dubecz jr., D. Szombath,* I. Rózsa,** H. Lippert,***
T. Gáti,*. M. Ihász

Third Department of Surgery, *Institute of Pathophysiology, Semmelweis University Medical School*, Second Department of Surgery Postgraduate Medical University, Budapest, Hungary, **Department of Surgery, "Charité", ***Humboldt University, Berlin, GDR

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During Shay-ulcer formation damages to the barrier of the gastric mucosa develop even before the appearance of macroscopic ulceration. Proximal selective vagotomy prevents these damages. Following pyloric ligation the prostaglandin content of the mucosa changes in parallel with the injuries of the mucosal barrier: TXB_2 content of the forestomach increases, while $PGF_{2\,alpha}$ content of both the forestomach and the antrum decreases. Following PSV operation the 6-keto-$PGF_{1\,alpha}$ content of the mucosa decreases, whereas $PGF_{2\,alpha}$ and TXB_2 contents exhibit no alteration. As a combined effect of proximal selective vagotomy pretreatment and pyloric ligation the 6-keto-$PGF_{1\,alpha}$ and $PGF_{2\,alpha}$ contents of the mucosa remain low and the TXB_2 increase, otherwise detectable after pyloric ligation, does not take place.

Keywords: proximal selective vagotomy (PSV), potential difference (PD), TXB_2, 6-keto-$PGF_{1\,alpha}$, $PGF_{2\,alpha}$

It has benn demonstrated in our previous experiments that mucosa barrier suffers damages during Shay-ulcer formation [20]. Injuries could be detected as early as the 6th hour following pyloric ligation. Injuries of the mucosa barrier were also detected following PSV operation: these injuries however, proved to be transiet [6].

In the present experiments the changes of the prostaglandin content of the mucosa were measured 10 hours after pyloric ligation. It is well-known from the works of Mozsik that in this period, and in even earlier periods as well, biochemical alterations take place in the cells of the mucosa: ATPase activity of the membrane and glycoprotein content of the stomach increase, tissue ATP content decreases and in the period preceding ulcer formation also the metabolism of nucleic acids exhibits alterations [12].

In the Shay-ulcer modell, ulcer formation takes place 17 to 24 hours after pyloric ligation. In the present experiment the changes of the prostaglandin content of the mucosa were determined in such a moment when bio-

Correspondence should be addressed to
Sándor Dubecz
Third Department of Surgery, Semmelweis School
1085. Budapest. Nagyvárad tér 1. Hungary

chemical alterations had already taken place but the macroscopic ulceration had not yet developed.

The present experiment was aimed at answering the following question:

Does or does not PSV operation alter in the Shay-ulcer modell the injuries of the mucosa barrier and the changes of prostaglandin content?

Materials and methods

The experiments were carried out on CFY rats (LATI) weighing 200 g. The animals were kept starving for 24 hours before the experiment, but water was given ad libitum. Median laparotomy was performed under ether anaesthesia, the pylorus was lifted and — by securing the integrity of the blood vessels — ligated according to the method of Shay [18, 19]. The PSV operation was carried out as previously described [3, 6]. Measurements of PD values were carried out according to the method of Gáti et al. [8]. Prostaglandin content of tissues samples obtained from the forestomach and the antrum was determined by RIA method [4, 5, 7]. The following kits manufactured by the Isotope Institute of the Hungarian Academy of sciences were used: ^{125}I-6-keto-PGF$_{2\,alpha}$, ^{125}I-TXB$_2$ and ^{3}H-PGF$_{2\,alpha}$.

Prostaglandin contents are given in pg/mg wet tissue weight units [2]. It should be noted that other authors suggest that prostaglandin content should be referred to 1 mg protein [14].

The animals used were divided into to following groups:

PD measurements

1. untreated, control group (18 animals)
2. measurements 10 hours after pyloric ligation (10 animals)
3. measurements 10 hours after PSV operation (12 animals)
4. measurements 10 hours after pyloric ligation and PSV operation (10 animals).

Determination of prostaglandin content of samples obtained from the forestomach and the antrum

5. untreated, control group (16 animals)
6. tissue sampling 10 hours after pyloric ligation (10 animals)
7. tissue sampling 10 hours after PSV operation (10 animals)
8. tissue sampling 10 hours after pyloric ligation and PSV operation (10 animals)

The results obtained are presented in the form of column diagrams. Student's unpaired t-test was used for the statistical analysis.

Results

Changes of PD values are shown on Fig. 1. Following pyloric ligation PD value decreased (2nd column). The decrease could be prevented by PSV treatment (4th column).

Figure 2 shows the changes of gastric PGF$_{2\,alpha}$ content. The column-pairs show the changes of the prostaglandin content of tissue samples obtained from the forestomach and the antrum. PSV pretreatment exerted no effect on the pyloric ligation-induced decrease of PGF$_{2\,alpha}$ content (4th column-pair). PSV had no effect on its own on the PGF$_{2\,alpha}$ content of the mucosa.

Changes of 6-keto-PGF$_{1\,alpha}$ content are shown on Fig. 3. This PG content did not change following pyloric ligation (2nd column-pair). The

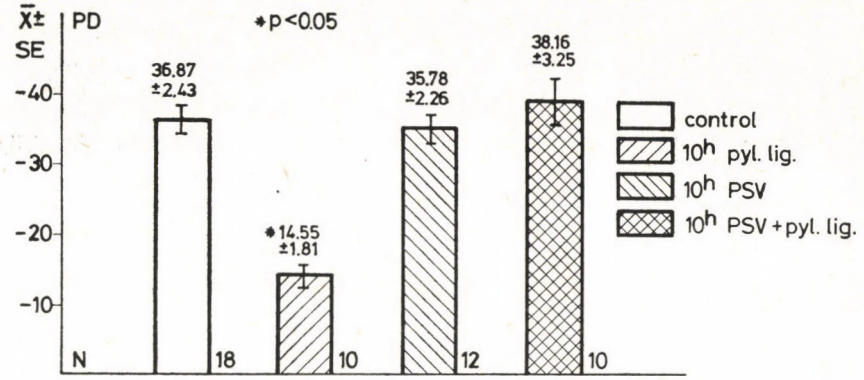

Fig. 1. Effects of 10-hour-long pyloric ligation and proximal selective vagotomy on transmucosal PD values of the rat stomach

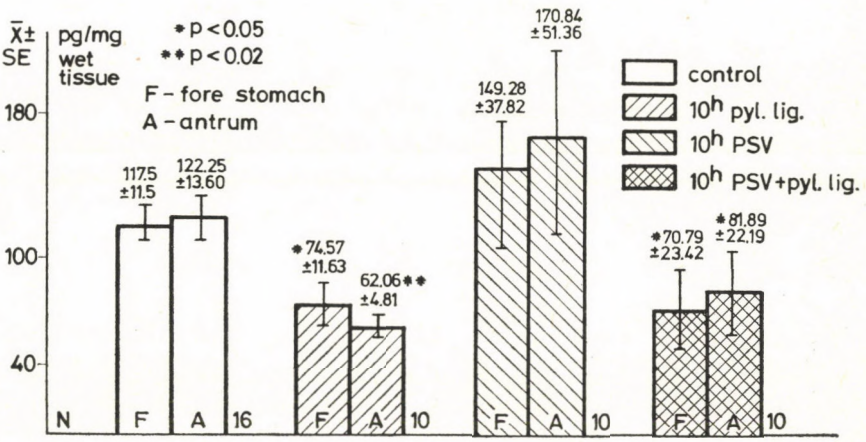

Fig. 2. Effect of pyloric ligation and proximal selective vagotomy on $PGF_{2\,alpha}$ content of the rat stomach

6-keto-$PGF_{1\,alpha}$ content of the mucosa decreased significantly upon the effect of both pyloric ligation (3rd column-pair) and pyloric ligation + PSV operation (4th column-pair).

Figure 4 demonstrates the changes of TXB_2 content. The results obtained indicate that PSV pretreatment (4th column-pair) prevented the TXB_2 content increase observes in the forestomach following pyloric ligation (2nd column-pair).

Discussion

The pathogenic role of hydrogen ions in the development of gastroduodenal ulcers is well-documented. The nearly 80-year-old statement of Schwarz holds true even nowadays: there is no ulcer without acid [17].

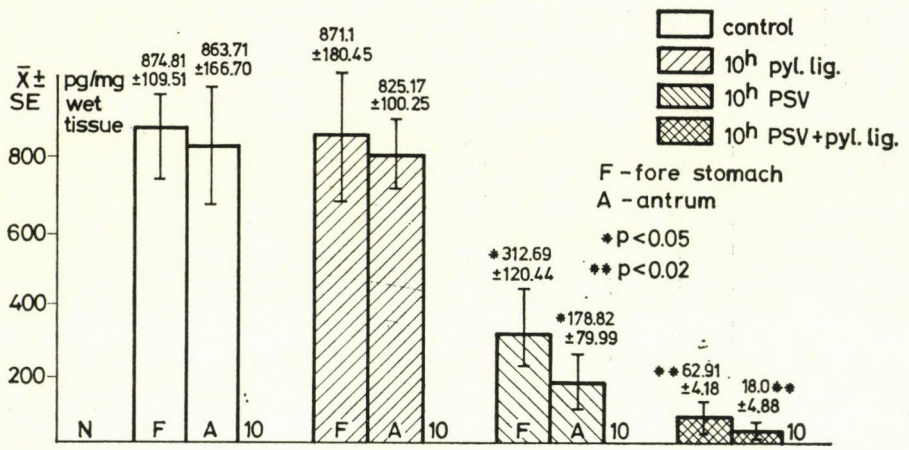

Fig. 3. Effect of pyloric ligation and proximal selective vagotomy on the 6- keto-$PGF_{1\,alpha}$ content of the rat stomach

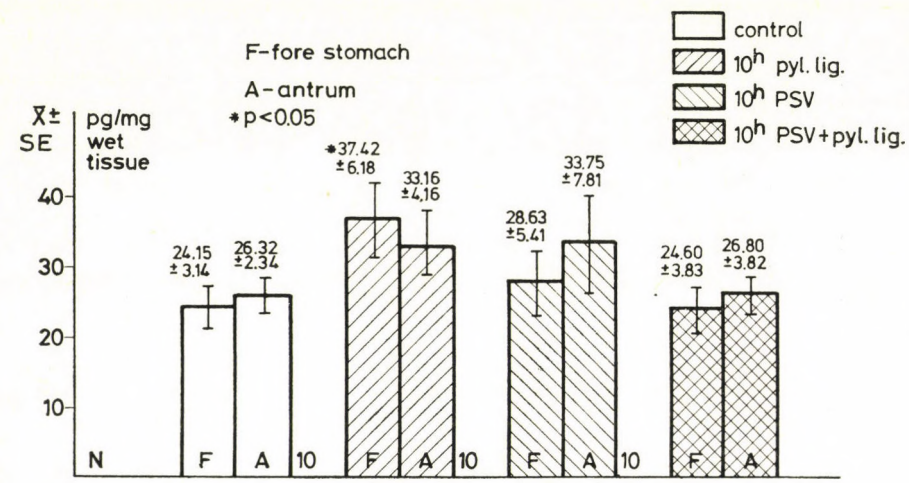

Fig. 4. Effect of pyloric ligation and proximal vagotomy on the thromboxane B_2(TYB_2) content of the rat stomach

According to the investigations of Brodie the pyloric ligation-induced increase of gastric secretion is caused by vago-vagal reflex which can be inhibited by vagotomy [1]. Truncal vagotomy inhibits the hydrogen ion hypersecretion developing after pyloric ligation and protects against Shay-ulcer formation [9, 10, 19].

It has been known for a long time that PSV operation decreases gastric secretion and contributes to the healing of duodenal ulcers. According to the data of Schwamberger [16] the protective effect of truncal vagotomy against Shay-ulcer is more pronounced than that of selective vagotomy. This obser-

vation has been explained in connection with the devascularisation of lesser curvature, which intervention is carried out during selective vagotomy [16].

It has been demonstrated in our previous experiments that truncal vagotomy prevents those damages to the mucosa barrier which would otherwise develop during Shay-ulcer formation [7]. Lesion of the mucosa barrier can be detected after PSV operation; however, they can not already be demonstrated in the 6th hour following surgery [6].

PSV treatment prevents the pyloric ligation-induced damages to the mucosa barrier [6].

It is known from the works of Robert that prostaglandins — even in doses which are but small fractions of those inhibiting gastric secretion — protect against the development of experimental mucosa lesions [13]. According to the investigation cited endogenous prostaglandin mobilization can be evoked by pretreatment with substances which are just mildly irritative to the mucosa. This prostaglandin mobilization provides protection against necrosis-inducing agents. The former mechanism is called direct, while the latter adaptive cytoprotection [14]. Different prostaglandins may exert opposite physiological effects [15]. PGA_1, PGE_1 and PGE_2 are vasobronchodilators, while PGF_{2alpha} is a vaso-bronchoconstrictor. PGA_1, PGE_1 and PGI_1 inhibit the aggregation of thrombocytes, whilst the vasoconstrictor TXA_2 stimulates thrombocyte aggregation and exerts ulcerogenic effect in the stomach [22]. PGF_{2alpha} has no antisecretory effect, but protects against indomethacin-induced ulcer [21]. According to the investigations of Sreagne in rats the PGE_2 content is significantly higher in the forestomach than in the antrum. As far as the PGE_2 content of the mucosa is concerned no difference can be found between truncal and proximal selective vagotomies [2].

Miller and Teppermann reported on the observation that lesions of the mucosa barrier which were evoked by the administration of 5 mM acetylsalicylic acid could be prevented by PGE_2 treatment. According to their view, PGI_2 may protect the integrity of the mucosa barrier [11].

When discussing the results obtained in the present experiments it should be noted that these experiments were acute ones in which PSV prevented the development of an acute gastric ulcer caused by hypersecretion. It should also be mentioned that the changes of prostaglandin content may presumably take place earlier than the 10th hour chosen here for the determination. In the future we wish to investigate also on the time course of the changes of prostaglandin content following PSV.

Since TXB_2 increases the aggregation of thrombocytes and exerts ulcerogenic effect, so in the preulcerous state the increase of TXB_2 content seems to be of importance. The PSV secured protection of the mucosa may partly be explained be the beneficial effect of the intervention on mucosal PG content.

A conclusion can be drawn, accordingly during the formation of Shay-ulcer PSV influences the lesions of the mucosa barrier and alters mucosal prostaglandin content. After pyloric ligation PSV inhibits both the decrease of PD and the increase of TXB_2 content of the forestomach.

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THE INFLUENCE OF ELECTROCOAGULATION OF THE SEPTUM AND SECTION OF THE ENTORHINAL CORTEX ON GENERAL BEHAVIOUR AND MEMORY IN CATS

T. N. Oniani, N. G. Nachkebia, A. Y. Nachkebia,
E. V. Chkhartishvili, L. T. Oniani

I. S. Beritashvili Institute of Physiology, Georgian Academy of Sciences,
Tbilisi, USSR

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In chronic experiments on cats it was shown that the lesion of the medial part of the septum does not result in the "septal syndrome"; the ratio of the different sleep-wakefulness cycle phases remains within the baseline values; the acquisition, retrieval and extinction of instrumental alimentary conditioned reflexes proceed normally; the delayed conditioned reflexes are impaired. Massive septal lesion, including its lateral part, leads to the development of the "septal syndrome"; there are changes in the structure and percentage of the different sleep-wakefulness cycle phases; the acquisition and extinction of instrumental alimentary reflexes with the sound discrimination are markedly retarded; the performance of delayed conditioned reflexes is completely destroyed. The section of the entorhinal cortex produces an increase in the number of repeated errors and perseverative movements during performance of instrumental alimentary reflexes, the deceleration of the acquisition and extinction of instrumental alimentary reflexes, complete disturbance of the delayed conditioned reflexes and does not affect the sleep-wakefulness cycle. The problems of the role of the hippocampus and its main inputs in the regulation of the short-term operative memory in the "pure form" as well as the significance of the descending influence of the hippocampus on the regulation of general animal behavior were also discussed.

Keywords: septo-hippocampal system, entorhinal input, short-term memory, general behaviour

The hippocampus and closely related structures are thought to be of paramount importance in the organization of learning and memory [18, 21, 22, 23, 35] as well as in the regulation of the orientation reflex [7] and motivated emotional processes [25, 30]. It is naturally assumed that the hippocampus as well as the other cortical structures may fulfil these functions by way of their inputs and outputs through which information arrives, on the one hand, about the events of the external and internal world of the organism and, on the other, the hippocampus can interact and effectively affect other structures of the brain. Over the last two decades the researchers' attention has been directed at the elucidation of the functional organization of the septal inputs

Correspondence should be addressed to
T. N. Oniani
I. S. Beritashvili Institute of Physiology, Georgian Academy of Sciences
Tbilisi, Sovietunion

and outputs. This interest seems to be inspired by the fact that the septum occupies a strategic place between the mesodiencephalon and the archipaleocortical structures and its destruction leads to drastic changes in the background electrical activity of the hippocampus [29, 30], to the development of the widely known septal syndrome [3, 4, 12] and to the impairment of different learning tasks [8]. In the analysis of the neurophysiological mechanisms responsible for the organization of learning and memory great attention is attached to the dynamics of the hippocampal theta rhythm, that is the integrative electrophysiological phenomenon reflecting different levels of functioning of the septohippocampal system. It is considered that the development of the hippocampal theta rhythm is associated with the recording, storage and retrieval of information in the brain [1, 14, 17, 29, 35, 36]. However, works have appeared recently [5, 24, 25, 26] in which the special significance of the development of the hippocampal theta rhythm in the organization of learning and memory is questioned. Furthermore, the view seems to have been revived that the development of the hippocampal theta rhythm is related to the non-specific activation of the brain, which in turn is obligatory for the realization of all the steps of organization of learning and memory [9, 10, 34].

For the involvement of the hippocampus in the integrative activity of the brain its cortical input from the entorhinal cortex is of no less importance. In Bennett's view [5], the influence of the entorhinal input, resulted in the desynchronization of the electrohippocampogram, must be of greater importance for the memory organization than the influence of the septal input leading to the hypersynchronization of the electrohippocampogram in the range of the theta rhythm.

It follows from the foregoing that further investigation employing a variety of approaches and techniques are necessary in order to solve the problem of (a) the participation of the hippocampus and its inputs in the memory organization, and (b) the significance of the dynamics of the hippocampal theta rhythm for the learning and consolidation of memory traces. Bearing this in mind, we have undertaken a study of the influence of electrocoagulation of the septum and of section of the entorhinal cortex on: 1) the general behaviour of animals, 2) the structure of the sleep-wakefulness cycle, 3) the acquisition of the instrumental alimentary reflexes, 4) the extinction of instrumental alimentary reflexes, and 5) delayed conditioned responses.

Methods

Animals

The experiments were carried out on 30 mature cats of both sexes. Before the commencement of the learning sessions the animals were adapted to the experimental chamber and observation was made of their general behaviour. The amount of daily food intake was also

measured. Later on, the cats that had easily adapted to the experimental set, had a good appetite, and were not distinguished for aggressiveness or apprehensiveness, i.e. were steady, were selected for the experiments.

Implantation of electrodes

Metal electrodes were stereotaxically implanted with the object of recording the electrical activity from the neocortex and the hippocampus and for electrocoagulation of different nuclei of the septum. Surgery was made under Nembutal anesthesia (35 mg/kg). The coordinates of the cat's brain atlas of Jasper and Ajmone-Marsan were used [11].

Procedure of acquisition and extinction of instrumental alimentary reflexes

Instrumental alimentary reflexes were trained in the experimental chamber consisting of two compartments. The rear part of the chamber with the area of 0.3 m^2 served as the starting place where the animals were kept in the intertrial period. In the front compartment with the area of 1 m^2, outside, at the front-side walls, feeders were placed from which the animal received food by raising the suspended door. A 500 Hz tone served as a conditioned signal for one feeder, and clicks for the other. In response to the former the animal was expected to go to the right, in response to the letter, to the left. The source of sound was placed on the top of the front wall at the very ceiling of the experimental chamber. Five seconds after the onset of one or the other signal the door of the starting section was opened and the cat was given the possibility of food-procuring.

The procedure of extinction of instrumental alimentary reflexes to two feeders was started after the elaboration of a 100% discrimination of conditioned responses. Over one session every conditioned signal was presented without food reinforcement as many times as it was required for the obtaining of the acute extinction of the reflexes. Chronic extinction was achieved only in the run of the multisession experiments.

Measurements of delayed responses was started when a 100% discrimination of conditioned signals had been reached. One or the other conditioned signal was delivered for 10 s and the animals were allowed to leave the starting section only at a certain time after its cessation. The time after which the cats approached the signalled feeder correctly and the errors did not exceed 5—10% was considered a maximum of delay.

Control animals

In 10 control cats before any surgery the rate of acquisition and extinction of instrumental alimentary reflexes as well as the duration of delayed conditioned reflexes were measured. The mean data obtained in these cats served as a standard for comparison with those obtained in the experimental ones.

Septal lesion

The septum was lesioned by electrocoagulation. For this purpose, bipolar electrodes with 2 mm difference in length were implanted on each side of the brain, in the septum. Direct current with the intensity of 8—15 mA was passed for 30—40 s. The extent of septal lesions varied depending on the intensity of direct current and duration of its passage.

Section of the entorhinal cortex

The entorhinal cortex was sectioned with a special knife designed in this laboratory [19]. It consisted of two parts. The guiding part was made of a syringe needle 2 mm in diameter and placed stereotaxically according to the coordinates A-5.5, L-10 into the necessary points of the skull. The mobile part of the knife was a pilot, smaller in diameter, to the end of which a steel wire 3—4 mm long, bent at a right angle, was attached. When put into the guiding part the wire became straight but upon leaving it the angle recovered and the total ablation of the entorhinal cortex was performed by means of a circular motion of the knife.

Polygraphic recording

Recording of electrical activity in the neocortex and hippocampus was effected on an ink-writing electroencephalograph of the firm "San'ei". In order to identify different phases of the sleep-wakefulness cycle, apart from the electrical activity of the studied structures, recording was made also of the cervical muscles and eye movements.

Histology

To specify the localization of deep electrodes and the extent of septal lesion and section of the entorhinal cortex after the termination of the experiments direct current (20 mA for 20 s) was passed through them. Fixation of the brain was made by perfusion of neutral formalin through the carotid artery exposed under Nembutal anesthesia (35 mg/kg). For further fixation the brain was removed from the skull and was again placed for some days in neutral formalin. Then the exact location of the electrodes and the extent of the septal lesion were determined in frontal serial sections.

Statistics

The data were statistically treated. The mean values were calculated, their standard deviations and the significance of differences in the mean values were checked by Student's t-test.

Results

Effects of septal lesion

1. Effect of septal lesion on the motivational-emotional reactions and the sleep-wakefulness cycle. On the basis of the character of the effects obtained by septal lesion and their comparison with the data of morphological control

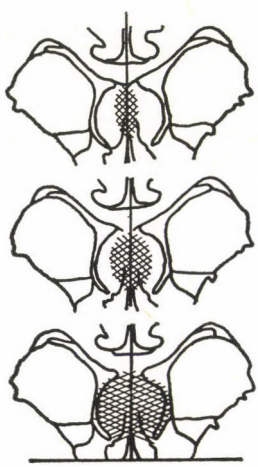

Fig. 1. Schematic drawing reflecting the extent of lesion in the septum and section of the entorhinal cortex. A — a lesion of only the medial septum, B — a massive lesion of the whole septum, C — section of the entorhinal cortex

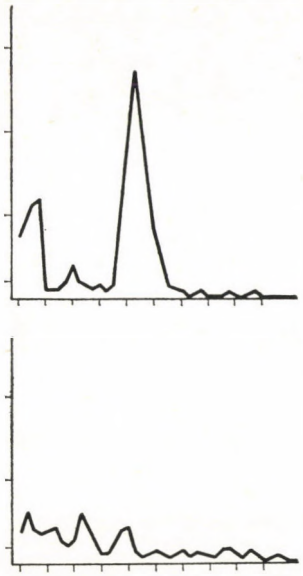

Fig. 2. Effect of medial septum lesion and section of the entorhinal cortex on the spectral density and power of the electrohippocampogram. Ordinate — relative amplitude. Abscissa — frequency in Hz. A — prior to and C — following the medial septum lesion, B — following section of the entorhinal cortex

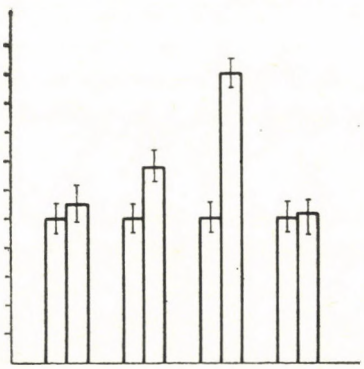

Fig. 3. Changes in the food motivation due to septal lesion and section of the entorhinal cortex. Ordinate — the amount of food intake in gramms. The first bars represent data obtained prior to and the second ones — following the surgery. A — the lesion of the medial septum, B — massive septal lesion, C — section of the entorhinal cortex.

of the lesion extent (Fig. 1), the experimental animals were subdivided into two groups. The first group comprised animals in which the lesion was restricted to the medial nucleus. Cats with a massive lesion of the whole septum fell into the second group.

In both groups the electrical activity of the hippocampus was altered equally, as evidenced by a total abolishment of the hippocampal theta rhythm

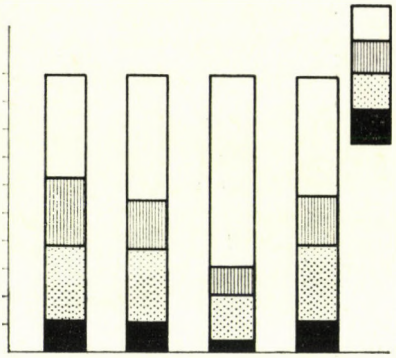

Fig. 4. Effect of septal lesion on the slepp-wakefulness cycle. A, B — before the surgery, C. D — after the lesion of the medial septum, E, F — after a massive lesion of the septum. W — wakefulness, LSWS —light slow wave sleep, DSWS — deep slow wave sleep, PS — paradoxical sleep.

during the performance of the motivational-emotional reactions against the background of wakefulness, as well as during the development of paradoxical sleep (Fig. 2). Behavioural reactions and the structure of the sleep-wakefulness cycle altered in a different way. In animals with the lesion placed in the medial septum, food motivation remained almost unaltered (Fig. 3A), while it increased significantly in animals with lesion of the entire septum (Fig. 3B). Sometimes the amount of food intake increased two times. The components of the so-called "septal syndrome", described in rats, i.e. rise of sensitivity to stimuli to which the animal had earlier reacted slightly or paid no attention, development of the perseverative movements etc., developed in those cats only in which the lateral nucleus was lesioned in addition to the medial septum.

Depending on the volume of lesion the structure of the sleep-wakefulness cycle altered variably. Figure 4 illustrates a 24-h structure of the sleep-wakefulness cycle before lesion (A), after lesion of only the medial septum (B), and after the massive lesion of the septum (C). As can be seen, the structure of the sleep-wakefulness cycle and percentage of its different phases considerably alter in the cats with a massive septal lesion, this being expressed in the increase of amount of wakefulness and decrease of the paradoxical phase. Destruction of only the medial nucleus does not result in any appreciable changes.

2. The effect of septal lesion on previously elaborated instrumental alimentary reflexes. After the acquisition of stable instrumental alimentary reflexes the control animals without septal lesion usually developed a pronounced hippocampal theta rhythm in response to a conditioned signal and during approach to the feeders. It might be assumed that this theta rhythm is in some way casually associated with the retrieval of conditioned reflexes,

i.e. readout of memory traces. However, in cats with lesions only in the medial nucleus of the septum, in which the hippocampal theta rhythm disappeared altogether, the previously elaborated conditioned reflexes found to be unaltered, i.e. a 100% discrimination of conditioned sounds was maintained (Fig. 5, dotted line).

A different picture is observed if, in addition to the medial nucleus of the septum, the lateral one is also lesioned. In these cats, in parallel to the development of the septal syndrome, the pre-elaborated discrimination of the conditioned signals was disturbed and we had to reelaborate them, this requiring about the same number of combinations as their elaboration for the first time in the intact animals (Fig. 5, solid line). At the same time, owing to the development of the septal syndrome, the percentage of correct responses in the first relearning session decreases below 50%. A 100% discrimination of conditioned signals is reached only within 4 session of relearning.

Thus, the lesion of only the medial septum, in spite of disappearance of the hippocampal theta rhythm, does not affect the previously elaborated conditioned reactions and discrimination of conditioned signals whereas the lesion of the lateral septum disturbs the discrimination of conditioned signals and relearning is required for its restoration.

3. The effect of septal lesion on the elaboration of instrumental alimentary reflexes. In a subsequent series of experiments, a study was made of the influence of septal lesion on the acquisition of instrumental alimentary reflexes to two feeders. Proceeding from the fact that in response to conditioned signals the hippocampal theta rhythm especially increases in the first learning sessions one might expect a negative effect of the disturbance of the septo-hippocampal system on learning. The effect in this case also was dependent on the extent of the lesion. In cats with a restricted lesion of the medial nucleus, in which the hippocampal theta rhythm had been abolished, instru-

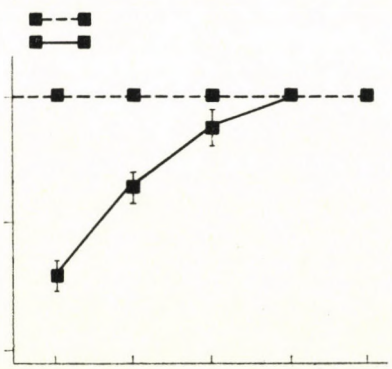

Fig. 5. Effect of septal lesion on previously elaborated instrumental alimentary reflexes. Dotted line — the medial septum lesion, solid line — a massive septal lesion. Ordinate — percentage of correct responses. Abscissa — learning sessions

mental alimentary reflexes were as readily elaborated as in controls (Fig. 6). As it can be seen from the percent of corrected responses, the experimental animals do not differ significantly from the controls in all sessions of learning. If the lesion involved the lateral nucleus too, then the elaboration was considerably delayed (Fig. 7) and a 100% discrimination of conditioned signals became almost impossible.

4. Effect of septal lesion on the extinction of instrumental alimentary reflexes. In this series of experiments, after a 100% discrimination of conditioned signals to two feeders had been achieved, lesions were placed in the septum. As in the previous experiments, the previously elaborated conditioned reflexes were not disturbed if the lesion involved only the medial nucleus, or the discrimination of conditioned sounds was disturbed when the lesions were massive and involved the lateral nucleus as well. In the latter case we had to re-elaborate discrimination, followed by the procedure of extinction. Retardation of the extinction of conditioned reflexes in the

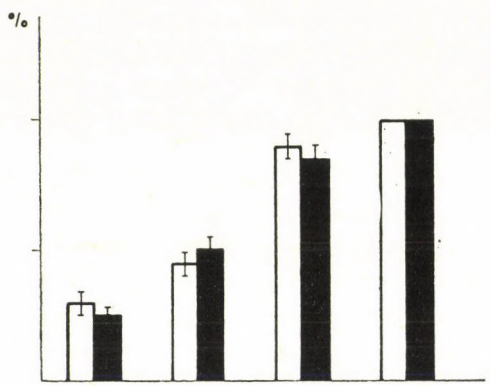

Fig. 6. Effect of lesion of the medial septum on the acquisition of instrumental alimentary reflexes. Light bars — data obtained in control animals, dark bars — in experimental ones. Ordinate — percent of correct responses. Abscissa — learning sessions

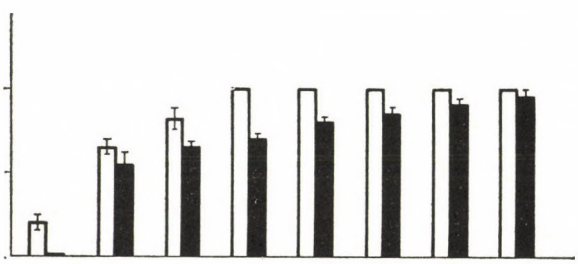

Fig. 7. Effect of a massive lesion of the septum on the acquisition of instrumental alimentary reflexes. Light bars — data obtained in the controls, dark bars — in the experimental ones. Ordinate — percent of correct responses. Abscissa — learning sessions

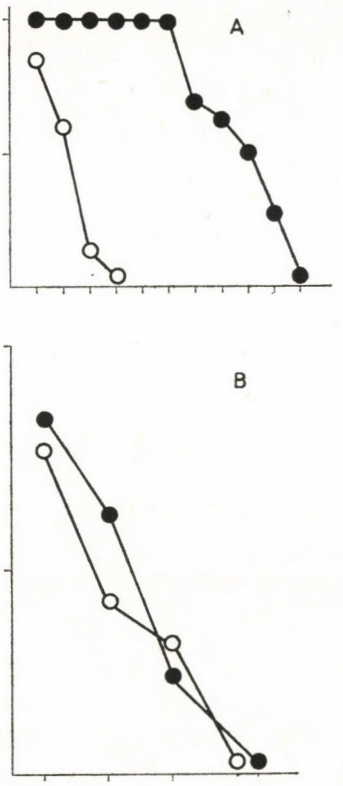

Fig. 8. Effect of septal lesion on the extinction of the instrumental alimentary reflexes. Light circles — data obtained in the control, and dark ones — in the experimental animals. A — the massive lesion of the septum, B — lesion of the medial septum. Ordinate — percent of correct responses. Abscissa — extinction sessions

case of isolated lesion of the medial septum was not observed (Fig. 8B). If, however, the lesion involved also the lateral nucleus of the septum the animal reached an acute extinction after a 100-fold presentation of conditioned sounds without reinforcement, while chronic extinction was attained only on the 13th day of work (Fig. 8A).

The extinction of conditioned reflexes is therefore retarded only in case of the electrocoagulation spreading over the lateral septum, whereas the lesion of the medial septum does not lead to any changes in the course of the extinction.

5. Effect of septal lesion on conditioned delayed reactions. After the maximum of delayed reactions was established, a lesion was placed in the septum, and after recovery the duration of conditioned delayed reactions was again checked. The cats with septal lesion were found to be unable to perform conditioned delayed reactions. It should be emphasized that the delayed reactions were equally disturbed in the cats with restricted lesion

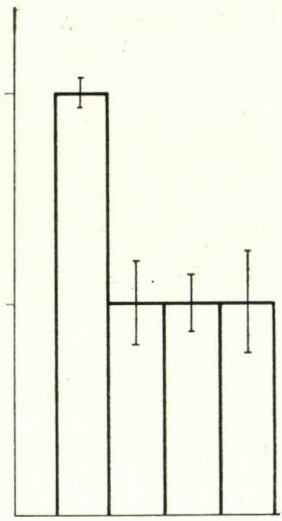

Fig. 9. Effect of septal lesion and section of the entorhinal cortex on conditioned delayed reactions. A — prior to the surgery, B — following the lesion of the medial septum, C — following a massive lesion of the septum, D — after section of the entorhinal cortex. Ordinate — percent of correct responses

of the medial nucleus of the septum (Fig. 9) as well as in those with a lesion involving the lateral nucleus. Prior to the surgery the cats of this experimental group accomplished almost 100% discrimination of conditioned sounds even 5 min delays. Following the lesion of the medial septum in the same cats with 5 min delays one can observe a complete disturbance of the discrimination of conditioned signals. Lesioned cats left the starting section, but their reactions to conditioned signals to the appropriate feeders were no more than 50% correct; which may be considered as an absolute error in the case of two choices. Their choice of feeders was random even when they were released from the starting section 2—5 s after the cessation of conditioned signals. This indicates that even the restricted lesion of the medial nucleus of the septum renders the animals totally incapable of performing conditioned delayed reactions, while discrimination of conditioned signals during their action is well preserved.

As indicated above (Fig. 5), in the case when the lateral nucleus of the septum is also damaged we had to re-elaborate sound discrimination, but restoration of conditioned delayed reactions did not occur.

Effect of section of the entorhinal cortex

1. Effect of section of the entorhinal cortex on the motivational-emotional reactions and on the sleep-wakefulness cycle. The section of the entorhinal

cortex did not considerably change such motivational behaviour as aggression and fear and there were only insignificant changes in food consumption (Fig. 3C). However, a few days after the operation the animal's motility increased and the development of perseverative movements was observed in response to the conditioned signals. About two weeks later the changes of the motor activity passed, perseverative movements ceased, and general behaviour, characteristic of the preoperative period, recovered.

The section of the entorhinal cortex did not noticeably change the structure of the sleep-wakefulness cycle. In spite of the increase in the motor activity during perseverative movements towards the feeders, the operated animals slept as calmly as before the operation and no considerable changes in the percentage of different phases of 24 h sleep-wakefulness cycle occurred.

The section of the entorhinal cortex resulted in noticeable changes in the electrohippocampogram, which found expression in an increase of the hippocampal theta rhythm during wakefulness and paradoxical sleep (Fig. 2) and in the suppression of slow potentials during slow wave sleep.

2. Effect of section of the entorhinal cortex on learning and memory. To elucidate the role of the entorhinal input of the hippocampus in learning and memory 4 series of experiments were carried out. In the first series the effect of the section of the entorhinal cortex on the preelaborated instrumental alimentary reflexes to two feeders were studied. Moreover, the effect depended on the postoperative period. When the test sessions coincided with the period of the development of perseverative movements in animals the discrimination of the conditioned signals was significantly disturbed. If, however, the retention of the conditioned instrumental alimentary reflexes was tested after the cessation of perseverative movements a 100% discrimination was retained. From this fact it follows that the intactness of the entorhinal input is not necessary for the retrieval of a learned response.

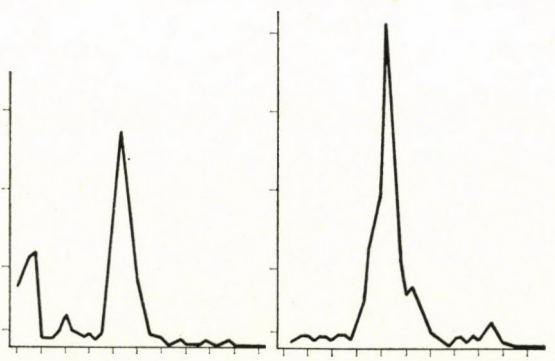

Fig. 10. Effect of the section of the entorhinal cortex on the acquisition of instrumental alimentary reflexes. Light circles — data obtained in the controls, and dark ones — in the experimental animals. Ordinate — percent of correct responses, Abscissa — learning sessions

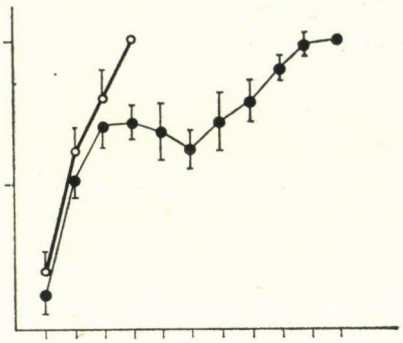

Fig. 11. Effect of section of the entorhinal cortex on the extinction of instrumental alimentary reflexes. Light circles — control animals, dark circles — experimental ones.

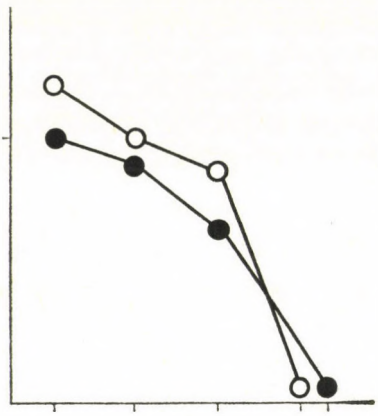

Fig. 12. Effect of section of the entorhinal costex on the extiucion of the instrumental alimentary reflexes. Light circles refer to control animals and dark circles to experimental animals.

In the second series of experiments the effect of the section of the entorhinal cortex on the ecquisition of instrumental alimentary reflexes was studied. In this case retardation of the acquisition of discrimination of conditioned signals was again observed only in connection with the development of perseverative movements. That is why during the first learning sessions the experimental and control animals did not differ from one another in the percentage of correct responses to the conditioned signals (Fig. 10), whereas with the development of perseverative movements experimental animals showed a marked decrease in the level of discrimination. As soon as the perseverative movements ceased, the correct responses approached 100%. The above facts

indicate that the entorhinal input of the hippocampus is not necessary for the acquisition of instrumental alimentary reflexes either.

The next series of experiments was performed to study the effect of the section of the entorhinal cortex on the extinction of instrumental reflexes. The interruption of the entorhinal input of hippocampus was not found to influence the extinction of instrumental alimentary reflexes. Both acute and chronic (Fig. 11) extinction in the experimental animals occurred at the rate identical to that of the controls. Since the procedure of the extinction was started after 100% discrimination of conditioned reflexes had been reached, perseverative movements had already stopped by that time and could not have a negative effect on the extinction.

Thus, the entorhinal input of the hippocampus is not necessary for the organization of negative learning in the animals.

The purpose of the last series of experiments was to study the effect of the section of entorhinal cortex on delayed conditioned reactions (Fig. 9D). Cats capable of performing a rather long (1—5 min) delayed reactions prior to the section of the entorhinal cortex, were selected for the experiments. After the interruption of the entorhinal input, the same cats proved to be unable to choose the signalled feeders correctly even when they were let out of the starting section of the experimental chamber 2—5 s after the termination of the conditioned signals. These findings indicate that the section of the entorhinal cortex leads to a complete disturbance of operative short-term memory underlying the performance of delayed conditioned responses.

Discussion

While considering the question of the effect of septal lesions upon learning and memory special attention is devoted to the behavioural and EEG changes arising thereat. Among them the most prominent is the development in septectomized animals of range, hyperactivity, disturbance of attention [3, 4, 12], disappearance of the hippocampal theta rhythm [30, 31], and other changes subsumed under the name of "septal syndrome". In our opinion the septal syndrome may involve also the disturbance of a normal structure of the sleep-wakefulness cycle, which was also reported by Lena and Parmeggiani [15].

Among these changes particular significance has been lately attached to the disappearance of the hippocampal theta rhythm following the septal lesion. The development of the hippocampal theta rhythm, reflecting the function of the septo-hippocampal system on a high level, has been associated with the organization of memory [1, 17, 35] and the regulation of a normal sleep-wakefulness cycle [15]. This lends support to the conclusion that the

elimination of the hippocampal theta rhythm will result in a deficit of the organization of learning and memory as well as in a disturbance of the normal structure of the sleep-wakefulness cycle. However, as shown above, in the case of a lesion of only the medial part of the septum the signs of the septal syndrome were lacking in spite of a complete disappearance of the hippocampal theta rhythm. The structure of the sleep-wakefulness cycle did not alter either. After the disappearance of the hippocampal theta rhythm cats did not become easily excitable, as described by Lena and Parmeggiani [15] in septal animals. This exclude the regulating influence of the hippocampal theta rhythm upon the excitability of the cortex which is allegedly an important factor of the normal course of sleep.

This is a direct indication that neither the septal syndrome as a whole nor its separate components are causally associated with the lack of the hippocampal theta rhythm. Some components of the septal syndrome in our experiments on cats developed only in the case when the lateral septum was lesioned alongside with its medial part.

Further, our experiments have shown that the lesion of only the medial septum, leading to a complete disappearance of the hippocampal theta rhythm without development of the septal syndrome, has no considerable effect on the acquisition of instrumental alimentary reflexes to approach one of the two feeders in response to an appropriate sound stimulus. Delay with learning of this type is observed only in the case when the lateral septum is also lesioned, resulting in the development of pronounced signs of the septal syndrome. This indicates that the development of the hippocampal theta rhythm is not obligatory either for the recording of information by the brain or for the consolidation of memory traces and conversion of short-term memory into its long-term form. On the other hand, this does not imply that the development of the hippocampal theta rhythm does not reflect definite steps of organization of memory. Ample evidence can be adduced in favour of the fact that the development of the hippocampal theta rhythm is correlated with the process of information recording by the brain. However, the hippocampal theta rhythm serves only as an electrographic correlate of these behavioural acts and is not an obligatory factor in information recording by the brain; still less obligatory is it in the consolidation of memory traces and conversion of short into long-term memory.

Neither did restricted lesion of the medial septum, not resulting in the development of the hippocampal theta rhythm, alter considerably the rate of the extinction of instrumental alimentary reflexes with sound discrimination. Both chronic and acute extinction was as successful in experimental animals as in controls. Bearing in mind the circumstance that extinction of a conditioned reflex is a type of learning [27, 33, 35], the above-described fact may be qualified as an additional argument in favour of the statement

that the hippocampal theta rhythm is not involved in the regulation of learning and memory.

Among the tests used by us, restricted lesion of the medial septum has a drastic influence only upon the conditioned delayed responses. The cats, which before the surgery approached the signalized feeder during 5 min delay, after lesion of the medial septum failed even when they were released from the starting box 1 s after the cessation of a conditioned signal. This fact may be interpreted, on the one hand, from the position of the hypothesis [13, 24] according to which delayed responses to conditioned signals are regulated by short-term memory in "pure form" and, on the other, in terms of the hypothesis considering the septo-hippocampal system to be an important link in the system of mechanisms responsible for the short-term operative memory [22, 23].

The development of the septal syndrome, delay with the process of acquisition and extinction of instrumental conditioned reflexes, disturbance of delayed responses, disturbance of preacquired discrimination of conditioned signals and disruption of the normal structure of the sleep-wakefulness cycle, observable during massive lesions of the septum, are indicative of a more important role of the lateral septum in the regulation of general behaviour as well as of learning and memory, since the lateral septum is a relay link for the hippocampus to the mesodiencephalic structures and thus the above-described changes may be produced by cessation of the regulating descending influence of the hippocampus on the activating and motivative mesencephalic and diencephalic brain structures.

In dealing with the role of the hippocampus in learning and memory organization the study of the effects of the interruption of its cortical (entorhinal) input on the acquisition and extinction of instrumental alimentary reflexes as well as on delayed conditioned responses is of considerable interest. The fact that the study of the dynamics of hippocampal electrical activity have revealed a reciprocal relationship of the septal and entorhinal inputs redoubles the interest [20]. Whereas the former is responsible for the hypersynchronization of the slow electrical activity of the hippocampus in the range of the theta rhythm, the later, on the contrary, leads to the desynchronization of the electrohippocampogram. Moreover, the lesion of the entorhinal input resulted in an increase of the hippocampal theta rhythm [2, 6, 20, 35]. This fact, on the one hand, reveals the inhibitory influence of the cortical input on the septo-hippocampal system, and on the other, it questioned the hypothesis about the significance of the hippocampal theta rhythm for learning and memory trace consolidation, because no facilitation of learning was observed during the increase in this rhythm (Figs 10, 11). Thus, whereas the lesion of these two inputs differently affects the electrogenesis of the hippocampus, its effects on learning and memory are identical. In both cases

only the disturbance of delayed conditioned reactions is observed and no changes in the processes of acquisition and extinction of instrumental alimentary responses occur. These findings allow to conclude that the hippocampus with its inputs participates in the organization of short-term operative memory in "pure form" rather than in the organization of long-term memory through the consolidation of traces.

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SPECIFICITY OF THE CELL-TO-CELL TRANSMISSION OF HORMONAL IMPRINTING IN CELL CULTURES

P. Kovács, G. Csaba and Ottilia Török

Department of Biology, Semmelweis University Medical School, Budapest, Hungary

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Chang liver cells and Chinese hamster ovary (CHO) cells were imprinted either with insulin or with thyrotropin (TSH). Chang liver cells responded to insulin but not to TSH. As an effect of imprinting evoked by insulin administration the binding of insulin administered for the second time was enhanced. In the mixed culture of imprinted and intact cells the extent of the binding was similar to that seen in the cultures of the cells having received imprintatory treatment alone. CHO cells also responded to TSH, imprinting developed and was transmitted to the cells which were not in interaction with the hormone (intact cells). In CHO cells also insulin gave rise to imprinting for insulin, whereas TSH gave rise to moderate binding imprinting for insulin. On the other hand, insulin imprinting did not enhance the binding of TSH. The obtained results indicate that both the imprinting itself and the specificity of the transmission of imprinting depend on the characteristics of the cell-type in question. The extent of the transmission, however, is always proportional to the extent of imprinting.

Keywords: hormonal imprinting, cell lines, cell-to-cell transmission, insulin, thyrotropin

The first interaction of the target cell and the hormone gives rise to hormonal imprinting which accounts for the completion of the maturation of the receptor and the development of the normal response capacity of the cell to the hormone [3, 4]. In mammals the imprinting takes place in the postnatal period [5]. The phenomenon of imprinting, however, can also be observed in unicellulars [6] at the first interaction of a hormone or any molecule which is able to bind to the receptor, and the template of the cell membrane. Cell cultures serve as a good model for the development of mammalian imprinting [5]; though the cells of the culture had been previously exposed to the hormone in the living organism, during the long decades of the cultivation period the template of the cell membrane changes to such an extent that the next interaction with the hormone gives rise to imprinting. Thus, in Chang liver cell cultures interaction with insulin leads to the strengthening of both the receptor binding and the response capacity of the cell [13]. In Chinese

Correspondence should be addressed to
Péter Kovács
Department of Biology, Semmelweis University Medical School,
1445 Budapest, P.O.B. 370, Nagyvarad tér 4, Hungary

hamster ovary (CHO) cell cultures the interaction with either gonadotropin (FSH, LH) or thyrotropin (TSH) hormone leads also to the strengthening of the receptor and to the development of a more marked response capacity of the cell [14]. In the case of CHO cells, such as in the living organism [8, 9], also the phenomenon of hormonal overlap can be demonstrated, i.e., due to the identical alpha and related beta subunits gonadotropins and thyrotropin [24] overlap one another's binding capacity and influence. The overlap manifests itself also in the imprintatory ability of these hormones [12]. Moreover, the binding of TSH to CHO cells is more pronounced and the response evoked is more marked than that of the corresponding gonadotropin hormone.

In the living organism, such as in cell cultures, cell division takes place continuously. Therefore the perinatally imprinted cells are not the same ones which respond to the hormone in the adulthood. In cell cultures, subsequently to imprintatory treatment, only the offsprings of the imprinted cells can be found. These offspring cells, however, also exhibit enhanced response capacity to the hormone, since the relatively short period of cultivation allows neither any change of the formed (strengthened) membrane template to develop, nor any loss of receptor structure to occur. Or to put it in an other way, imprinting is inherited from mother to daughter cell; such inheritance, however, can hardly be explained by genetic mechanisms. It seemed to be probable that imprinting was transmitted by means of cell-to-cell interactions. In recent years several authors [19, 22] reported on the observation that in cell cultures it is only a group of the cells which is actually involved in hormone binding, whereas the utmost majority of the cells respond to the hormone. This means that following the binding of the hormone to the receptor the information is transmitted from cell to cell, and the postreceptor mechanisms are triggered in the recipient cells just as they have been triggerred in the first cell which have actually been in interaction with the hormone. It has been demonstrated in our previous investigations [9] that the above-mentioned phenomenon can be observed also during imprinting, i.e. the information is transmitted from the imprinted cell to those intact cells which have not been in interaction with the hormone. If the time duration is sufficient, very low proportion of the cells, even as low as 1%, is enough to secure the transmission of the information.

The present experiments were aimed at studying the extent of the specificity of the transmission of imprinting, i.e. whether it is really the presence of the given hormone (the imprinting with the hormone) which is necessary for imprinting to develop in an other cell, or also aspecific treatments (with an other hormone) are able to evoke imprinting.

Materials and methods

Chang liver cells and CHO (Chinese hamster ovary) cells were used for the experiments. The cultures were maintained in Parker's solution containing 2% colostrum-deprived calf serum. Insulin (Semilente MC, Novo, Copenhagen) and thyrotropin (TSH, Ambinon, Organon, Oss) were used for the experiments in concentrations of 10^{-6} and 10^{-5}M, respectively.

The cell cultures were incubated with 0.25% trypsin solution (Difco, 1 : 250) for 2 to 5 minutes. The cells were removed by pipetting from the wall of the culturing flasks and centrifuged. After removal of the supernatant the sediment was added to plain medium and the cell suspension thus obtained was distributed into centrifuge tubes. The 1st tube served as control, to the 2nd insulin was added and to the 3rd TSH was given. The cell suspension was kept in revolving tubes at 37 °C for 4 hours. It was centrifuged anew, washed twice with Tyrode solution and after repeated centrifugation plain medium was added to the sediment. The number of the cells was determined (40.000 to 50.000 cells per ml) and the cell suspension was distributed into H tubes (equipped with coverslip). Each tube contained 3 ml of cell suspension and the distribution was as follows: 1st tube: control, 2nd: control+cells treated with insulin, 3rd: cells treated with insulin, 4th: control+cells treated with TSH and 5th: cells treated with TSH.

The experimental groups were cultivated for another 24 hours and fixed. The cells were partly stained according the May-Grünwald-Giemsa method and partly washed following fixation in neutral 4% formol solution (dissolved in PBS) for 5 minutes. The cultures were incubated either with FITC (fluoresceine isothiocyanate, BDH, England)-labeled insulin (protein content: 0.2 mg/ml, FITC/ protein ratio: 0.42) or with FITC-labeled TSH (protein content: 0.2 mg/ml, FITC/ protein ratio: 1.83), washed twice with PBS and in distilled water, and dried. The cultures were tested for the intensity of fluorescence in a Zeiss Fluoval cytofluorimeter. The results were recorded by a Hewlett Packard 41 CX microcomputer. The analogous signs of the fluorimeter were converted into digital ones by means of a digital processor. According to its mathematical program the computer provided the mean values, the standard deviations and the significance levels between the experimental groups (Student's t test and analysis of variance).

The cells of both cell lines received specific as well as aspecific treatment. FITC-insulin and FITC-TSH binding were investigated in both subgroups of both groups.

In each group twenty cells were measured for the intensity of fluorescence. The measurements were performed separately above the cytoplasm and the nucleus. The fluorescence measured above the cytoplasm indicated the hormone binding of the plasma membrane, whilst the fluorescence measured above the nucleus represented the hormone binding of the nuclear membrane. The measurement of this latter binding was also necessary since investigations performed during the last decade (2) have revealed that also the nuclear membrane is able to bind hormones and that this hormone binding may in certain instances be different form the hormone binding of the plasma membrane. The Figure shows the mean values of the consistent results of the experiments repeated three times, i.e. each column represents the results obtained in cells.

Results and discussion

Two cell lines and two hormones were used for the experiments. The target cell for insulin is the liver cell, whereas the target cell for TSH is the thyroid cell. Still, it has been demonstrated in previous investigations [8, 9, 12, 14] that TSH overlaps with the gonadotrop hormones. Moreover, the efficacy of TSH exceeded that of the gonadotropins from the point of view of both binding and the capacity to evoke response, either when TSH was administered in the perinatal period or when it was investigated in the Chinese hamster ovary cell model. In the present experiments Chang liver cells responded well to insulin. Insulin treatment gave rise to imprinting leading to the characteristic and marked increase of insulin binding.

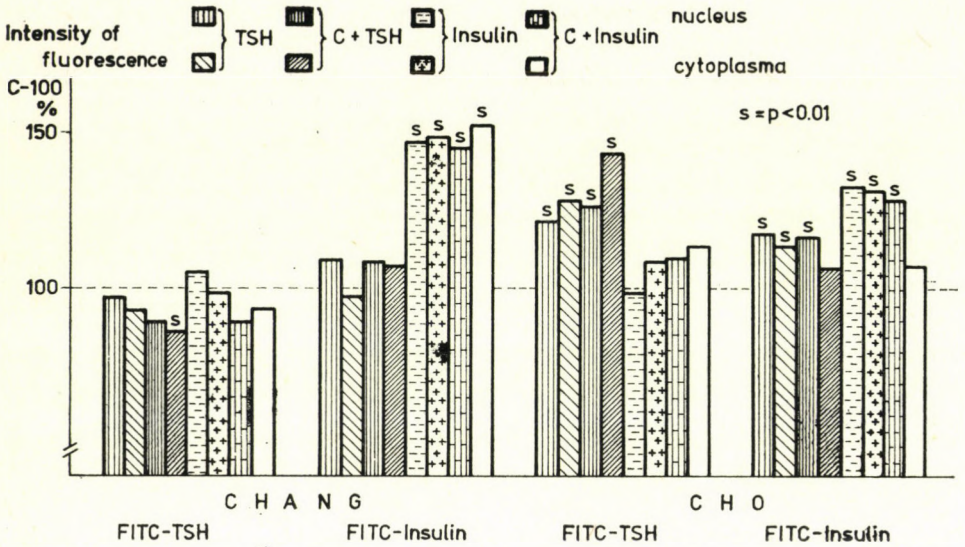

Fig. 1. FITC-TSH and FITC-insulin binding of pure and mixed CHO and Chang cell cultures treated with TSH or insulin. C = control (untreated) cells; TSH, insulin = cells treated with the hormone indicated; C+ = 50% to 50% mixture of untreated and hormone treated cells

The fluorescence of the mixed cultures, i.e., of those cultures where the ratio of the cells imprinted with insulin was equal to the ratio of the intact cells (the determinations were carried out 24 hours after mixing, i.e., at a time when the ratio could not change), proved to be entirely similar to the fluorescence of the cells treated with insulin alone. This finding indicates the cell-to-cell transmission of the information of imprinting (Fig. 1). When the Chang cells were imprinted not with insulin but by TSH administration, the binding of FITC-insulin did not increase. This observation indicates the specificity of insulin imprinting. The binding of FITC-TSH also corroborated this specificity since irrespective of whether insulin or TSH was used for evoking imprinting in the Chang cell model, the final result was invariably negative.

TSH treatment of CHO cells gave also rise to imprinting and the extent of the imprinting of the mixed (imprinted and intact) cells was similar. This means that the imprinting was transmitted from the imprinted CHO cells to the non-imprinted ones (as it had been also previously demonstrated [9]). The imprinting evoked by TSH administration markedly enhanced the binding of FITC-insulin to CHO cells meanwhile the imprinting evoked by insulin administration enhanced the binding to an even greater extent. That is to say, insulin proved to be able to give rise to imprinting in CHO cells. This phenomenon indicates that the CHO cell has either an insulin receptor or such a membrane structure which is able to bind insulin and to give rise to

imprinting. This statement, on its own, seems to be surprising enough; nevertheless, it is well known that also such cells contain insulin receptors which are not definite target cells of insulin [23]. The binding of any hormone evokes some perturbation of the membrane [18] and, thus, may alter the binding of other hormones [15—17]. It is possible that this phenomenon manifested itself in the insulin binding of the CHO cells treated with TSH. On the other hand, it is also possible that the binding to CHO cells can be regarded as being specific, as it has been previously demonstrated in the case of the testis (1). The observation that insulin influences the porcine granulosa cells [23] may support the former assumption. Anyhow, the binding of FITC-labeled TSH was not enhanced in the CHO cells treated with insulin, i.e. insulin did not evoke such changes of the membrane which would result in the enhancement of TSH binding.

On the basis of the experiments also another, presumably important, conclusion can be drawn, namely, that the extent of the imprinting evoked (either specifically or aspecifically) was identical to the extent of the transmission of imprinting from the directly involved cells to the intact ones. This observation indicates that it is the change of the membrane which is being transmitted (by means of direct intercellular contact [10] via some, so far unknown mechanisms), regardless to the specific or aspecific nature of the change in question.

Thus, cell cultures of liver and ovary origin proved to be similar from the point of view that the phenomenon of imprinting could be evoked in both cultures by the administration of the specific hormone and, moreover, the imprinting was transmitted to those cells which were not in direct interaction with the hormone. The cell cultures of different origin, however, proved to be different from the point of view of the extent of the specificity of the hormonal imprinting. High specificity could be observed only in the case of the liver cells.

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BREATHING PATTERN OF SPONTANEOUSLY HYPERTENSIVE RATS

L. A. DEBRECZENI, Krisztina VÉRTES, Erzsébet BARÁT, Éva HUSZÁR*

Experimental Pathophysiological Research Unit and *Clinico-pharmacological Research Unit, National Korányi Institute for TBC and Pulmonology 1529 — Budapest, Hungary

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The trachea of rats anaesthetized with sodium pentobarbitone was cannulated and the air flow velocity and the pressure of the oesophagus were measured. In the spontaneously hypertensive rats the breathing frequency was higher, the tidal volume and the effective lung resistance were smaller than that of the normotensive Wistar rats. It seems that the neurohumoral control of respiration in SHR animals differs from that of normotensive rats.

Keywords: Breathing pattern, spontaneously hypertensive rats, ventilatory drive, breathing frequency, tidal volume, lung resistance

The strain of the spontaneously hypertensive rats (SHR) is accepted as one of the best model of human essential hypertension. The details of the mechanisms of hypertension developing in these rats are not known in certain aspects. There is, however a consensus that increased sympathetic activity plays an important role in pathogenesis of this form of hypertension [5, 6, 7, 9].

The hypothesis tested in the present experiments was whether the increased sympathetic activity in SHR would result in a different breathing pattern compared to normotensive Wistar rats (WR). The central inspiratory activity, the "ventilatory drive" was estimated by means of the ratio of the inspiratory volume and the inspiration time (averaged inspiratory airflow velocity) [2, 4].

Methods

The rats were anaesthetized with sodium pentobarbital (50mg*kg^{-1}, i.p.) and were lying in the supine position. A pneumotachograph was introduced into the trachea and the difference in pressure between the trachea and oesophagus was measured. The signals of airflow velocity and pressure were monitored and stored on an analogue taperecorder (frequency bandwidth, DC-625 Hz per channel).

The analogue signals were fed into a digital signal processing system to estimate the following parameters:

a. in respect to time: respiratory frequency, time of inspiration
b. in respect to volume: tidal volume computed for 100 cm^2 of body surface, inspiratory volume referred to one sec. of inspiration considered as sign of "ventilatory drive", and minute

Correspondence should be addressed to
Lóránd A. DEBRECZENI
Experimental Pathophysiological Research Unit National Korányi Institute for TBC and Pulmonology 1529-Budapest, XII. Pihenő u. 1., P.O.Box 1., Hungary

ventilation expressed for 100 cm^2 of body surface. Body surface (cm^2) = 9.2*body weight $(g)^{2/3}$.

c. effective resistance of lungs

In a signal series selected for analysis generally 4—10 respiratory cycles were analysed and, to improve accuracy, the arithmetic mean value of the parameters calculated for each

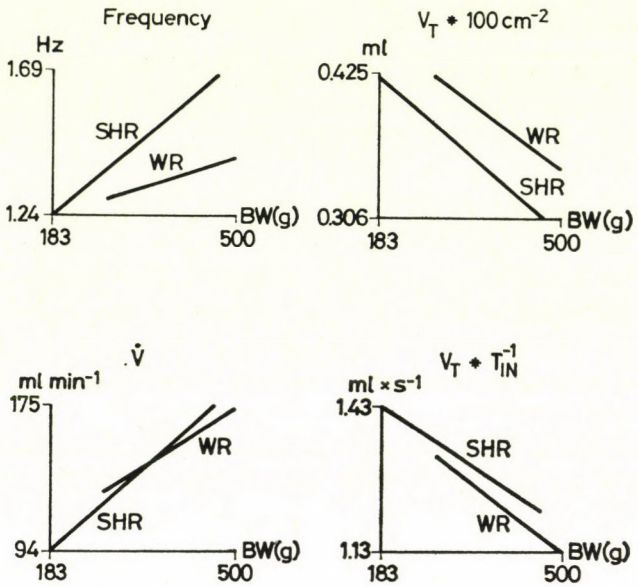

Fig. 1. Breathing frequency (frequency), tidal volume ($VT*100cm^{-2}$), minute ventilation ($\dot{V}$) and ventilatory drive ($VT*TIN^{-1}$) as functions of body weights in spontaneously hypertensive rats (SHR) and in normotensive Wistar rats (WR).

Breathing frequency:

$$SHR: Y = 0.944(\pm 0.078) + 0.0016(0.0002)x;$$
$$WR: Y = 1.12(\pm 0.10) + 0.005(\pm 0.0002)x;$$
$$tb = 4.973 \text{ and } p < 0.001,$$

the lines are different if the BW >231 g

Tidal volume:

$$SHR: Y = 0.496(\pm 0.020) - 0.0005(\pm 0.0001)x;$$
$$WR: Y = 0.526(\pm 0.0308) - 0.0004(\pm 0.0001)x;$$
$$tp = 4.643 \text{ and } p < 0.001,$$

the lines are significantly different and parallel.

Minute ventilation:

$$SHR: Y = 42.0(\pm 9.99) + 0.286(\pm 0.029)x;$$
$$WR: Y = 69.6(\pm 15.8) + 0.207(\pm 0.0397)x,$$

there is no differences between the lines.

Ventilatory drive:

$$SHR: Y = 1.58(0.093) - 0.0008(\pm 0.0002)x;$$
$$WR: Y = 1.59(\pm 0.132) - 0.001(\pm 0.0003)x,$$

there is no difference between the lines.

respiratory cycle was used for further biometrical analysis. (Depending on the experimental conditions, respiratory cycles may very significantly even in a brief time interval.) (For details see (3).)

Biometrical analysis (1, 8): The mean values and their standard errors (SEM) are given in Table I, and the groups were compared by means of the unpaired t-test. The relationships between the parameters and the body weight were estimated by means of linear regression equations. In the regression equations ($Y = a (\pm sa) + b (\pm sb)\ x$) on Fig. 1 a refers to the intercept, sa to the error of the intercept, b to the regression coefficient and sb to the error of the regression coefficient. Only significant regression equations are given. To compare two regression equations at the first step the difference between the regression coefficients was estimated (tb). If the regression coefficients were different, the limit values of x-axis were estimated to indicate a significant distance ($p = 0.05$) between the two regression lines based on the equations solved for the same x limit value. If the regression coefficients did not differ, the significant distance between the two parallel regression lines was estimated (tp).

Results

In the SHR group the body weight, tidal volume and the effective lung resistance were smaller, the respiratory frequency, the inspiratory volume computed for unit of time (the "ventilatory drive") were greater than that of the WR group (Table I).

To eliminate the error due to the differences in body weight of groups, linear regression estimations were performed as a function of body weight. There was a difference between the groups concerning the respiratory frequency and tidal volume (Fig. 1). There was no difference between the groups in respect of minute ventilation and respiratory drive based on the inspiratory volume expressed for unit time (Fig. 1).

Table I

Lung function parameters

	WR	SHR
n	75	95
Body weight (BW, g)	388 ±8.93	324 ±12.7**
Frequency (Hz)	1.35±0.022	1.46± 0.032**
Tidal volume (VT) (ml* 100 cm^{-2})	0.38±0.0074	0.36± 0.0082
"Ventilatory drive" (VT* TINE-1, ml* s^{-1} * 100 cm^{-2})	1.23±0.030	1.32± 0.031*
Resistance (R) (Pa *ml^{-1} *s)	13.0±0.69	10.0± 0.74**
Minute ventilation (V = ml *min^{-1} *100 cm^{-2})	31.0±0.72	31.4± 0.77

* = $p < 0.05$; ** = $p < 0.01$

Discussion

In the present experiments the respiratory frequency was higher in the SHR group than it was in the WR group. According to the literature reviewed by Howes [5] in the brainstem and in the hypothalamus of the SHR animals the adrenergic as well as the nonadrenergic activities and thus inhibition are decreased. This phenomenon may be one of the explanation of increased respiratory frequency in these rats: the cells of the respiratory rhythm generator escaping inhibition.

The tidal volume decreased in the SHR group compared to the WR group, while the regression lines of minute ventilation and that of the ventilatory drive did not differ. The ventilation is determined by the metabolic demand to provide a constant level of the arterial gas tensions and it is modified by numerous reflexes. The rats of the two groups were anaesthetized so the difference in breathing pattern is probably the consequence of different central inspiratory activity. The humoral factors also may alter the respiration in SHR animals: e.g., the increased plasma catecholamine level resulting from the augmented sympathetic activity may decrease lung resistance.

It seems that the neurohumoral control of breathing and that of the airways of SHR animals is different from that of the WR and needs further investigations.

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EFFECTS OF PROPRANOLOL, NALORPHINE AND HALOPERIDOL ON THE BREATHING AND ON THE METABOLISM OF PGI_2 AND TXA_2 OF NORMOTENSIVE AND SPONTANEOUSLY HYPERTENSIVE RATS

Erzsébet BARÁT, Éva HUSZÁR,* Krisztina VÉRTES, L. A. DEBRECZENI

Experimental Pathophysiological Research Unit and *Clinico-pharmacological Research Unit, National Korányi Institute for TBC and Pulmonology 1529 — Budapest, Hungary

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The influence of propranolol, nalorphine and haloperidol on the breathing pattern and on the blood levels of cyclooxygenase products of anaesthetized spontaneously-breathing normotensive Wistar rats (WR) and of spontaneously hypertensive rats (SHR) were investigated. The respiratory rate was higher and the effective lung resistance was smaller in the SHR than in the WR. Breathing frequency decreased after nalorphine in both groups, while only in SHR after haloperidol. Propranolol augmented the dynamic lung resistance in both groups. The blood 6-keto-$PGF_{1\alpha}$ level was higher and the TXB_2 level was lower in the SHR than in the WR. The central inspiratory activity as well as the levels of peripherally acting substances involved in the regulation of respiration and in the control of bronchial smooth muscle tone are different in the SHR and WR.

Keywords: Spontaneously hypertensive rats, breathing pattern, propranolol, nalorphine, haloperidol

In spontaneously hypertensive rats (SHR) respiratory frequency was higher and tidal volume was smaller than in normotensive Wistar rats (WR), but there was no difference in the minute ventilation of the groups. Thus the neuro-humoral control of breathing of SHR is different from that of WR. The aim of the study was to investigate the influence of different drugs (propranolol, nalorphine and haloperidol) acting centrally and/or peripherally on the breathing of WR and SHR as well as to study their effects on prostanoid levels (PGI_2 and TXA_2).

Methods

Lung function parameters

The rats (body weight: 250—350 g) were anaesthetized with sodium pentobarbitone (50 $mg \times kg^{-1}$, i.p.) and were kept supine. A pneumotachograph was attached to the trachea and airflow ($\dot{V}$), and difference in pressure (PTP) between the trachea and oesophagus were

Correspondence should be addressed to
Erzsébet BARÁT
Experimental Pathophysiological Research Unit National Korányi Institute for TBC and Pulmonology 1529-Budapest, XII. Pihenő u. 1., P.O.Box 1., Hungary

monitored and stored in magnetic tape (frequency bandwidth, DC-625 Hz per channel). $\dot{V}$ and PTP were fed into a digital converter to estimate the following parameters: respiratory frequency (Fr), time of inspiration (tI), tidal volume (VT) referred to 100 cm^2 of body surface and minute ventilation ($\dot{V}E$). Central inspiratory activity, the "ventilatory drive", was estimated from the VT/tI ratio, using averaged inspiratory airflow velocity (1, 4). Dynamic lung elastance (Edyn) and resistance (Rdyn) were calculated. Generally 4—10 respiratory cycles were analysed for each period, and the arithmetic means of parameters were calculated for each respiratory cycle [2].

Drugs used were; propranolol (Inderal, ICI, 100 $\mu g \times kg^{-1}$), nalorphine (Nalorphin, Chinoin, $1mg \times kg^{-1}$) and haloperidol (Haloperidol, Richter, $2mg \times kg^{-1}$). The drugs were injected via a catheter inserted into the jugular vein.

Protocol

Airflow and pressure signals were stored before and 15 min. after treatments for two 2-min intervals. At the end of the experiments the animals were killed and blood samples were collected.

6-keto-$PGF_{1\alpha}$ and TXB_2 assays

Having decapitated the rats, blood samples (4—5 ml/rat) were collected into tubes containing 150μl of 6.5% EDTA (Reanal) and 100μl of 0.5 mg/ml indomethacin (Chinoin) (dissolved in ethanol-water, 60/40 v/v). After separation at 4 °C, the plasma samples were stored at —20 °C. 6-keto-$PGF_{1\alpha}$ and TXB_2 levels of the samples were estimated directly from the plasma by 125I-6-keto-$PGF_{1\alpha}$ RIA kit and by 125I-TXB_2 RIA kit (Izinta). Specific activity of the 125I-labelled 6-keto-$PGF_{1\alpha}$-TME was about 1800 $Ci \times mmol^{-1}$ and the 125I-TXB_2 tracer concentrate contained approximately 2μCi radioactivity. Duplicate determinations were performed. Recovery was about 60%. The ranges of standard curves were between 2.5—250 pg/tube. The values were not corrected for recovery percentage.

Statistical analysis

Means $\pm$ s.e.m.s. are given, and the groups were compared by the unpaired t-test. The differences (between before and after treatments) of groups were compared by the paired t-test.

Results

Table I shows the results of lung function tests and the effects of treatments on cyclooxygenase products. After physiological saline there was no difference between the two groups.

In the group of WR, compared to saline, propranolol increased Rdyn, nalorphine decreased Fr, VT/tI, $\dot{V}E$ and haloperidol increased VT/tI. In the WR groups propranolol, nalorphine and haloperidol significantly ($P < 0.05$) decreased the level of TXB_2 (110 ± 17.3; 78 ± 12.9 and 137 ± 32.9 $pg \times ml^{-1}$ respectively compared to 288 ± 33.8 $pg \times ml^{-1}$ for saline) but the level 6-keto-$PGF_{1\alpha}$ was not significantly affected.

For saline-treated rats the SHR had higher Fr and lower Rdyn than the WR, while the level of 6-keto-$PGF_{1\alpha}$ was significantly higher ($P < 0.05$) in the SHR group compared to the WR group (333 ± 33.8 and 209 ± 22.5

Table I

Effect of Propranolol, Nalorphine and Haloperidol on normotensiv and spontaneously hypertensive rats

		Physiol. saline		Propranolol		Nalorphine		Haloperidol	
		A	δ	A	δ	A	δ	A	δ
WR	FR	1.42 ±0.072	−0.013 ±0.019	1.43 ±0.016	−0.058 ±0.027	1.26 ±0.060	−0.20* ±0.041	1.31 ±0.049	0.046 ±0.054
	VT	0.35 ±0.008	0.006 ±0.005	0.32 ±0.015	0.013 ±0.011	0.32 ±0.006	−0.003 ±0.01	0.37 ±0.008	0.013 ±0.004
	VT/Tin	1.14 ±0.035	0.019 ±0.022	1.02 ±0.057	0.014 ±0.048	0.93 ±0.035	−0.199* ±0.041	1.17 ±0.051	0.101* ±0.36
	V	29.5 ±1.48	0.14 ±0.81	27.3 ±1.47	−0.34 ±1.31	23.8 ±1.11	−4.03* ±0.78	28.61 ±1.07	2.23 ±1.25
	E dyn	144.0 ±11.89	13.1 ±5.89	159.0 ±12.0	0.7 ±5.10	142.3 ±9.47	−0.3 ±10.46	167.0 ±12.23	−4.4 ±6.53
	R dyn	16.4 ±2.59	2.52 ±1.29	15.0 ±1.54	3.16 * ±1.38	17.1 ±2.05	4.23 ±3.30	16.3 ±2.26	1.3 ±2.63
SHR	FR	1.65 ±0.051	−0.009 ±0.097	1.57 ±0.096	−0.08 ±0.067	1.68 ±0.056	−0.47*# ±0.051	1.57 ±0.055	−0.15*# ±0.037
	VT	0.35 ±0.007	0 ±0	0.30 ±0.012	0.18 ±0.024	0.33 ±0.009	0.011 ±0.008	0.31 ±0.024	0.033 ±0.012
	VT/Tin	1.31 ±0.054	−0.035 ±0.054	1.18 ±0.076	−0.029 ±0.074	1.26 ±0.044	−0.041*# ±0.031	1.16 ±0.084	0.11* ±0.046
	V	34.3 ±1.59	−0.410 ±1.39	28.4 ±2.04	0.71 ±1.08	32.9 ±1.38	−8.65*# ±0.74	28.70 ±2.05	−0.4 ±0.70
	E dyn	137.0 ±7.1	3.8 ±11.0	185.0 ±13.08	−4.4 ±9.8	130.0 ±9.0	14.3 ±8.90	148.4 ±7.8	−17.7 -±13.0
	R dyn	6.39 ±0.89	1.48 ±0.94	13.7 ±2.67	5.09 ±1.91	10.84 ±0.65	0.99 ±0.085	6.39 ±0.51	4.97* ±1.61

WR	6-keto	20.85±2.25	16.33±1.80	24.58±2.36	26.08±3.27
	TXB	28.83±3.38	10.98±1.73*	7.79±1.29*	13.65±3.29*
SHR	6-keto	33.33±3.38*	33.08±3.62	37.75±5.02	33.66±2.83
	TXB	9.67±1.69*	16.75±2.14*	16.46±2.02*	16.16±1.36*

Lung function parameters after various treatments. Values are means ±s.e.m., N = 10. A = before treatment; δ = change after treatment; Fr = respiratory frequency (Hz); VT = tidal volume (ml/100 cm² body surface); VT/tI = "ventilatory drive" (ml·s^{-1}/100 cm²); $\dot{V}E$ = minute ventilation (ml·min.$^{-1}$/100 cm²); E dyn = dynamic elastance (Pa·ml^{-1}); R dyn = dynamic resistance (Pa·ml^{-1}·s), 6-keto = = 6-keto-PGF$_{1\alpha}$ expressed in pg/100 μl, TXB expressed pg/100 μl.

= P <0.05 for changes caused drugs; * = P <0.05 for differences between WR and SHR groups.

$pg \times ml^{-1}$, respectively) and the level of TXB_2 was significantly smaller (96.7 ± 16.9 and 288 ± 33.8 $pg \times ml^{-1}$, respectively). For the SHR propranolol and nalorphine increased Rdyn and nalorphine decreased Fr, VT/tI and $\dot{V}E$. Haloperidol decreased Fr and increased VT/tI and Rdyn. In the SHR the level of TXB_2 was significantly ($P < 0.05$) increased by propranolol, nalorphine and haloperidol (168 ± 21.4; 165 ± 20.2 and 162 ± 13.6 $pg \times ml^{-1}$, respectively, compared to saline, 97 ± 16.9 $pg \times ml^{-1}$), but the level of 6-keto-$PGF_{1\alpha}$ was not affected.

For SHR compared to WR, nalorphine decreased more markedly Fr, VT/tI and $\dot{V}E$, and haloperidol decreased Fr.

Discussion

Hypertensive rats exhibit hypo-algesia [11] suppressed by naloxone [9]. Hypertension may facilitate the production of endorphins thereby increasing the treshold of pain. In the present experiments nalorphine decreased Fr in both groups but more in the SHR animals. Thus the endorphin level may be elevated in the SHR. Ward and Holaday [10] demonstrated that both μ and δ receptors are involved in morphine-induced respiratory depression in the rat. There may be neurons e.g. in the cat [3], which are stimulated by morphine or endorphins and which are insensitive to nalorphine. A similar pattern was observed concerning the VT/tI and $\dot{V}E$. Nalorphine-induced respiratory depression of the WR may be strain-specific.

Haloperidol decreased Fr in the SHR. In awake mice haloperidol also decreases Fr [8]. Administration of haloperidol intracerebro-ventricularly induces an immediate, long lasting increase in VT and a decrease in Fr while $\dot{V}E$ remains virtually unchanged [6]. These results indicate a tonically active dopamine system in the central regulation of respiration, which seems to be more pronounced in the SHR.

Propranolol increased Rdyn in both groups indicating that peripheral catecholamines may control bronchial smooth muscle tone.

TXA_2 is a very potent vasoconstrictor and bronchoconstrictor, and it induces platelet aggregation [5]. Prostacyclin can modify the bronchial smooth muscle tone [7]. The balance between these cyclooxygenase products may influence bronchial smooth muscle tone.

The stable metabolite of prostacyclin, 6-keto-$PGF_{1\alpha}$, was increased in the SHR compared to the WR, and the employed treatments did not modify its level. The level TXB_2 decreased in WR after treatments, but in the SHR it changed in the opposite direction. There may be a relationship of both the increased level of TXB_2 and the decreased prostacyclin/thromboxane with the augmented Rdyn in SHR animals, observed particularly after propranolol

or haloperidol treatments. The regulation of breathing seems different in the SHR and in the WR; several central and peripheral mechanisms may be involved.

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VANADIUM INDUCED CHANGES IN GLYCOGEN CONTENT OF *CLARIAS BATRACHUS* (LINN) AND *CHANNA PUNCTATUS* (BLOCH)

K. B. JAGADEESH, S. A. SHAFFI, S. JEELANI

Department of Science (Zoology), Regional College of Education (NCERT), Bhopal, India

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Clarias batrachus (Linn) and *Channa punctatus* (Bloch) were exposed to 5, 10 and 15 ppm of vanadium and investigated the effects on tissue glycogen at 3, 6 and 9 hrs. Results indicated the variations in the level of glycogen were highest at 15 ppm after 9 hr exposure in liver than muscle, brain, kidney and heart of *Clarias batrachus* as compared to *Channa punctatus*. The variation recorded in tissue glycogen content was discussed in relation to respiratory distress, formation of mucus on the whole fish and disturbed behaviour of the fish.

Keywords: Vanadium, glycogen, liver, brain

Vanadium a transitional metal is known to its role in cardiac ailments and nutritional processes (Macara, 1980). However, vanadium has yet to find a place in the biochemistry of animal systems. From the literature it appears that the acute and chronic effects of vanadium was investigated in relation to behavioural studies [2, 3, 5, 6, 7, 9, 15].

In the present investigation an attempt was made to find out the effects of vanadium on visceral carbohydrate reserves of two freshwater fishes, i.e., *Clarias batrachus* (Linn) and *Channa punctatus* (Bloch) at 3 hr, 6 hr and 9 hr exposure.

Material and methods

Live, healthy and mature fish species of *Clarias batrachus* and *Channa punctatus* of 18—20 cm in standard length were obtained from the local pond and acclimatized in the laboratory for five days (12½) before they were exposed to various concentrations of vanadium (5, 10 and 15 ppm). The sublethal and lethal concentration of vanadium for *Clarias batrachus* is 5.8 ppm, 11.2 ppm, and for *Channa punctatus* is 3.2 ppm, 6.8 ppm. In this study an attempt was made to investigate the effect of vanadium concentrations below sublethal and lethal to understand the acclimatization abilities of the fish at high toxic concentrations.

Correcpondence should be addressed to
K. B. JAGADEESH
Department of Science, Zoology, Regional College of Education
Bhopal 462 013 (M. P.), India

Time exposure

Actual studies: Both fish species were exposed to short term exposure (9 hrs). The authors made an attempt to find out the exact interfering time of the pollutant on carbohydrate metabolism via measuring glycogen content. Hence, the experiment was terminated at 3, 6, and 9 hrs.

Control studies: (i) Then species of *Clarias batrachus* (Linn) and *Channa punctatus* (Bloch) were sacrified to find out the level of glycogen in liver, muscle, brain, kidney and heart. (ii) Water samples were subjected for the analysis of O_2, CO_2, pH and BOD using the standard methods of Examination of water and Wastewater (1976).

Table I

Vanadium toxicity — tissue glycogen level in Channa punctatus (Bloch)
After 3, 6 and 9 hr. Exposures

Organs	Control	5 ppm	10 ppm	15 ppm	% Fall
		2784.6* ±65.7	2214.7* ±29.3	1948.7* ±37.5	37.64
Liver	3124.80 ±61.35	2633.9○ ±70.3	2189.3○ ±40.2	1788.5○ ±40.9	42.76
		2148.4● ±54.5	2089.0● ±60.0	1690.4● ±50.7	45.90
		2347.8* ±35.8	1804.9* ±33.0	1787.6* ±35.2	12.31
Muscle	2038.86 ±41.88	2250.0○ ±50.9	1756.1○ ±42.0	1504.4○ ±26.5	26.20
		2214.8● ±39.7	1587.5● ±27.0	1352.9● ±38.0	33.64
		894.1* ±40.8	975.8* ±19.5	620.8* ±24.2	25.06
Brain	828.40 +26.85	889 ○ ±49.8	915.6○ ±21.0	575.0○ ±15.0	30.58
		878.0● ±25.7	894.8● ±23.0	475.0● ±19.2	42.66
		683.3* ±20.8	733.9* ±20.2	482.2* ±21.5	10.07
Kidney	536.21 +30.20	636.7○ ±19.8	717.9○ ±15.0	400.5○ ±14.5	25.30
		597.5● ±22.5	693.9● ±11.6	339.7● ±16.3	36.64
		96.8* ±10.3	78.2* ±5.6	70.6* ±8.5	14.24
Heart	82.33 +14.80	88.4○ ±5.8	77.4○ ±7.3	64.8○ ±8.5	21.29
		80.1● ±5.0	65.8● ±2.8	59.5● ±2.1	27.7

Values (μg/g), wet wt. tissue) are mean + SE of 5 replicates. Symbols (*, ○, ●) indicate the exposure at 3, 6 and 9 hr.

Table II

Vanadium toxicity — tissue glycogen level in Clarias Batrachus (Linn) after 3, 6 and 9 hr exposure

Organs	Control	5 p pm	10 ppm	15 ppm	% Fall
		2960.2* ±55.1	2342.9* ±65.3	1148.6* +26.3	56.32
Liver	2632.8 ±73.8	2750.0○ ±62.23	2220.3○ ±54.5	1043.7○ ±30.8	60.35
		2420.3● ±70.8	2242.8● ±50.5	994.9● ±21.9	62.21
		2013.4* ±42.8	1512.1* ±30.4	1156.2* ±29.0	32.5
Muscle	1714.3 ±45.2	1871.8○ ±50.5	1412.0○ ±25.2	1113.2○ ±30.4	35.3
		1704.5● ±29.3	1326.8● ±19.0	886.8● 29.7	48.2
		710.3* ±18.5	942.7* ±33.4	513.9* ±26.0	21.7
Brain	656.7 ±28.5	650.4○ ±32.0	748.6○ ±25.3	404.3○ ±14.8	35.03
		617.9● ±17.5	396.3● 21.0	386.1● 18.3	48.2
		453.5* ±21.0	349.1* ±25.3	294.9* ±26.7	41.2
Kidney	502.2 ±19.5	383.2○ ±15.8	310.5○ ±21.8	250.4○ ±30.5	50.13
		351.2● ±20.0	284.9● 14.8	230.9● ±17.8	54.02
		88.2* ±5.4	62.3* ±8.8	48.7* ±5.9	35.83
Heart	75.9 ±5.8	63.3○ ±8.5	56.7○ ±5.8	39.1○ ±5.8	48.48
		51.9● ±3.6	49.9● ±7.5	32.3● ±4.5	57.7

Values (μg/g wet wt. tissue) are mean ± SEM of 8 replicates. Symbols (*, ○, ●) indicate the exposure at 3, 6 and 9 hr.

Preparation of vanadium concentration

Vanadium of Sarabhai Chemicals (India) was taken and dissolved in the double distilled water to produce the required concentration.

Procedure of the experiment

Ten species of *Clarias batrachus* (Linn) and *Channa punctatus* (Bloch) were kept in six separate truffs (5 litre capacity). The three truffs contained 5, 10 and 15 ppm concentrations of vanadium and the remaining three were the control ones.

Tissue preparation and glycogen assay

After 3, 6 and 9 hr. exposure the fishes were sacrified for liver, muscles, brain, kidney and heart. The preparation of tissue samples and the estimation of glycogen were described elsewhere (11). The experiment was repeated five times.

Results

From the data (Tables I and II) it is evident that significant variation in the amount of glycogen of five organs was recorded in *Clarias batrachus* and *Channa punctatus*. Among these five organs the highest fall was observed in liver at 9 hr exposure with 1.5 ppm vanadium salt followed by brain, kidney, muscle, and heart in *Channa punctatus*. However, the fall in tissue glycogen content was higher in *Clarias batrachus* than in *Channa punctatus*. Out of these three concentrations the 15 ppm was more toxic than 10 and 5 ppm. Fishes exposed to 9 hr. duration had great fall in tissue glycogen content than at 6 hr or 3 hr exposures, respectively.

Table III

Vanadium induced changes in physico-chemical parameters

Parameters	Duration (hours)	Control inater	Eperimental water		
			5 ppm	10 ppm	15 ppm
(2) *C. punctatus*					
	3	7.5 ±0.5	7.2 ±0.2	7.0 ±0.2	6.5 ±0.3
pH	6	7.5 ±0.5	7.0 ±0.1	6.7 ±0.1	6.2 ±0.1
	9	7.5 ±0.5	6.8 ±0.3	6.5 ±0.2	6.0 ±0.2
	3	7.0 ±0.1	6.7 ±0.5	6.4 ±0.5	6.0 ±0.5
DO	6	7.0 ±0.1	6.2 ±0.4	5.5 ±0.4	4.8 ±0.3
	9	7.0 ±0.1	5.3 ±0.4	5.0 ±0.5	4.0 ±0.1
	3	4.5 ±0.3	10.4 ±0.4	19.8 ±0.5	35.7 ±0.4
BOD	6	4.5 ±0.3	18.5 ±0.7	26.3 ±0.4	40.8 ±0.5
	9	4.5 ±0.3	28.5 ±0.5	40.3 ±0.7	48.9 ±0.5
	3	Nil	1.8 ±0.2	3.8 ±0.3	6.0 ±0.5
CO_2	6	Nil	3.0 ±0.2	4.9 ±0.2	8.6 ±0.1
	9	Nil	4.2 ±0.3	5.7 ±0.6	10.3 ±0.2

Table IV

(2) *C. batrachus*					
	3	7.4 ±0.5	7.1 ±0.5	7.0 ±0.5	5.8 ±0.3
pH	6	7.4 ±0.5	6.8 ±0.2	6.6 ±0.3	5.6 ±0.2
	9	7.4 ±0.5	6.2 ±0.2	5.7 ±0.2	5.0 ±0.3
	3	6.5 ±0.4	6.1 ±0.5	5.8 ±0.5	5.1 ±0.2
DO	6	6.5 ±0.4	5.4 ±0.1	5.0 ±0.3	4.8 ±0.4
	9	6.5 ±0.4	4.8 ±0.3	4.3 ±0.2	3.5 ±0.9
	3	4.0 ±0.3	63.2 ±0.5	27.3 ±0.3	40.9 ±0.8
BOD	6	4.0 ±0.3	22.6 ±0.6	35.8 ±0.5	53.8 ±0.2
	9	4.0 ±0.3	30.1 ±0.8	49.2 ±9.9	62.5 ±0.9
	3	Nil	3.1 ±0.2	5.6 ±0.5	8.9 ±0.9
CO_2	6	Nil	4.9 ±0.5	7.8 ±0.5	11.7 ±0.9
	9	Nil	7.3 ±0.3	9.2 ±0.4	14.8 ±0.4

The pH of the water, level of DO, CO_2 and BOD respectively showed significant variations (Tables). The fall in water pH, DO level and BOD were greater at 15 ppm during 9 hr exposure than at 10 ppm and 5 ppm or at 3 and 6 hr exposures.

Discussion

Pollutants may influence the various physiological and biochemical mechanisms through altering several physico-chemical parameters and finally, the death may be due to the metabolic inhibition imposed by the precipitation of pollutants in the animal systems.

The variations in the selected physico-chemical parameters may help to understand the effects of pollutants and the fall in the tissue glycogen level. Due to mucus formation all over the surface of the body, increased opercular movement, respiratory distress and change in the behaviour of the fish might have enhanced the tissue acidosis process which is a result of changed buffering system in the tissues. When the fish is unable to procure the oxygen because of vanadium exposure, hypoxia may deplete carbohydrate energy reserve due to the stress conditions.

Liver being an important organ of various metabolic process. The storage of glycogen showed a maximum fall as compared to the remaining tissues of *Channa punctatus*. Out of these two fish species the changes in tissue glycogen content in *Clarias batrachus* were higher than in *Channa punctatus*. This may indicate a difference in their physiological adaptive mechanisms in a new situation. The changes observed at 9 hr exposure are highest than at 3 and 6 hr exposure. This may be due to a difference in the adaptation abilities. A corollary to our findings, such changes were also observed with other pollutants on tissue glycogen level [11, 12].

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EFFECT OF ATRIAL DILATATION ON THE TENDENCY OF ATRIAL ARRHYTHMIAS

F. Solti, T. Vecsey, Viola Kékesi

Cardiovascular Surgical Clinic, Semmelweis University Medical School, Budapest, Hungary

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The arrhythmogenic effect of atrial dilatation was studied by electrophysiological investigations carried out on 24 dogs. Atrial distension was evoked by increasing the pressure in the right atrium (12 to 14 mm Hg) or by the baloon dilatation of the left atrium. Programmed electrical stimulation of the heart was used for the electrophysiological investigations. In addition to the superficial ECG leads also atrial and ventricular epicardial electrograms were obtained for the ECG recording. Acute atrial dilatation led to shortening of the atrial refractory period, whereas neither impulse conduction of the heart, nor pacemaker activity of the sinus node exhibited any alteration. Atrial dilatation resulted in pathological atrial irritability, and early or frequent atrial stimulation caused atrial tachycardia of shorter (non sustained) or longer (sustained) duration. Repetitive atrial extrasystoles in response to early stimuli could also frequently be observed during atrial dilatation. The obtained results indicate that atrial dilatation is arrhythmogenic and may lead to the development of atrial tachycardia.

Keywords: atrial dilatation, atrial irritability, supraventricular tachycardia, atrial fibrillation

Various factors are involved in the pathogenesis of arrhythmias and the underlying mechanisms have only partially been revealed so far. Several experimental data as well as clinical observations indicate that stretching and dilatation of the atrial wall can frequently underlie arrhythmias of atrial origin. The observation that supraventricular tachycardia, atrial fibrillation and atrial flutter frequently occur in such diseases where the atrium is enlarged (mitral disease, cor pulmonale) deserves special attention.

Our present investigations were aimed at studying the effect of atrial stretching — atrial dilatation on cardiac impulse generation and impulse conduction as well as on the tendency of the heart (and especially of the atrium) to develop arrhythmias.

Experimental design and methods

The experiments were carried out on mongrel dogs of both sexes (body weight: 15 to 25 kg) under pentobarbital sodium anaesthesia (30 mg/kg intravenously). After intratracheal insertion of an inflatable cannula the animals were mechanically ventillated by means of a

Correspondence should be addressed to
Ferenc Solti
Cardiovascular Surgical Clinic, Semmelweis University Medical School,
1122 Budapest, Városmajor u. 68, Hungary

volume controlled respirator. The chest was opened by horizontal sternotomy. For the recording of epicardial electrograms and to carry out the electrical stimulations unipolar electrodes were sewed into the epicardial surface of the right and left atria and ventricles. Indwelling catheters were inserted into the right atrium and into the right and left ventricles for the measurement of atrial and ventricular pressures. Pressures were measured by means of a Statham transducer.

Two methods were used for atrial stretching, i.e., acute atrial dilatation: (1) Constant increase (12 to 15 mm Hg) of the right atrial pressure by means of infusion (administration of physiological saline solution via a catheter inserted into the superior vena cava); (2) Dilatation of the left atrium by means of an inflatable baloon catheter inserted via the pulmonary vein (intraluminal pressure: 12 to 15 mm Hg).

The strain gauge method (resistance gauge) was used for recording the changes of contractility of left ventricle. Programmed electrical heart stimulation (atrial and ventricular pacing) for the purposes of electrophysiological investigations was used. The intensity of the electrical stimulus was twice as high as the diastolic threshold of the heart. The duration of the impulse was 2.0 ms. A Medtronic 5925 impulse generator was used for the programmed heart stimulation (12). The ECG curves, electrograms and pressure curves were recorded by means of a 6-channel Hellige recorder. For studying atrial irritability single and double early atrial stimuli as well as frequent atrial stimuli (with 300 ms cycle length stimulation for 30 s) were used.

After recording the baseline values (control period) the measurements were repeated anew under the circumstances of atrial pressure and atrial baloon dilatation.

Statistical analysis was carried out using Student's one tailed t test.

Results

Neither the impulse conduction of the heart, nor the pacemaker activity of the sinus node exhibited considerable alteration upon the effect of atrial pressure increase. The atrioventricular (PQ time) and atrial (width of the P wave and the difference between the right and left atrial P waves) impulse conduction times somewhat increased. The ventricular impulse conduction time (width of QRS), the corrected reawakening time of the sinoatrial node (CSNRT), the time duration of the cardiac cycle (CL) and the QT time did not change upon the effect of the increase in atrial pressure or atrial dilatation. Similarly, no alteration could be observed in the ventricular refractory period (VERP), meanwhile the atrial refractory period (AERP) was consequently and significally shorter during atrial dilatation.

The results are summarized in Table I.

Table I

Effect of atrial stretching on the electric parameters of the heart

Period	CL ms	P time ms	PQ time ms	QRS time ms	QT time ms	CSNRT ms	AERP ms	VERP ms
Control (n = 24)	550 (±35)	50 (±10)	105 (±20)	64 (±10)	215 (±25)	30 (±10)	135 (±12)	140 (±14)
Atrial pressure increase (n = 24)	560 (±32)	54 (±10)	109 (±22)	63 (±09)	218 (±24)	34 (±12)	110* (±10)	138 (±16)
Atrial baloon dilatation (n = 10)	562 (±30)	54 (±10)	108 (±20)	65 (±10)	220 (±30)	28 (±12)	112* (±12)	141 (±14)

The numbers represent mean ± SD values.
* = Statistically significant difference ($p < 0.05$) compared to the control.

During atrial stretching characteristic increases of atrial irritability, i.e., tendency of the atrium to arrhythmias, could be observed. Under normal circumstances (control period) early single or double atrial stimuli did not lead to repetitive responses and no atrial arrthythmia developed. Similarly, neither did frequent electrical stimulation evoke arrhythmia. After stretching of the atrial wall, however, increased atrial irritability and the development of supraventricular arrhythmia could be observed. Following the combined increase of right atrial pressure and administration of early atrial stimuli (14 cases) repetitive atrial response (repetitive atrial extrasystoles) occurred in 8 cases, while atrial tachycardia developed in 6 instances (4 non sustained and 2 sustained). As a result of frequent atrial stimulation atrial tachycardia developed in 9 cases (3 non sustained and 6 sustained). Transient atrial fibrillation was seen in 3 instances, whereas transient flutter occurred in 2 cases. During acute dilatation of the left atrium and following early atrial stimulation (10 cases) repetitive response (repetitive atrial extrasystoles) occurred in 6 cases, whereas atrial tachycardia could be detected in 4 instances (3 non sustained and 1 sustained). Frequent atrial stimulation led to atrial tachycardia in 7 cases (3 non sustained and 4 sustained). Transient atrial fibrillation could be detected in two cases.

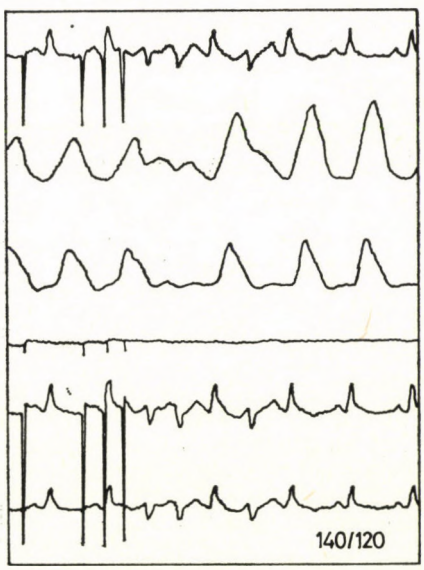

Fig. 1. Repetitive atrial extrasystoles after early atrial stimuli given to dogs in which right atrial pressure was increased
Paper recording speed: 50 mms^{-1}. Upper line: II. limb lead, second line: left ventricular pressure curve, third line: right ventricular pressure curve, fourth line: I. bipolar limb lead, fifth line: II. bipolar limb lead, sixth — bottom — line: II. bipolar limb lead. Two early electrical stimuli (delay period $S_1—S_2$: 160 ms and $S_2—S_3$: 120 ms) evoked repetitive responses (three atrial extrasystoles)

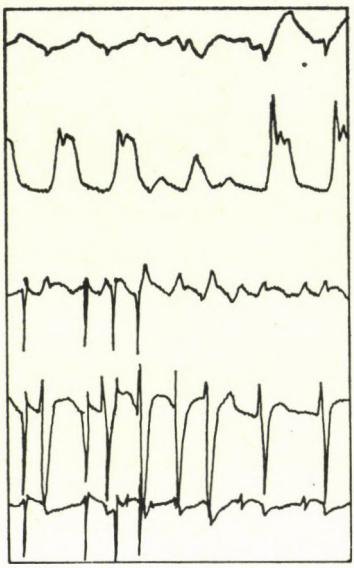

Fig. 2. Short — non sustained — atrial tachycardia following early atrial stimuli in dogs — subjected to left atrial baloon dilatation
ECG recording during acute dilatation of the left atrium. Paper speed: 50 mms^{-1}. Upper line: II. limb lead, second line: left ventricular epicardial electrogram, third line: left auricular epicardial electrogram, fourth line: right atrial epicardial electrogram, fifth line: left atrial epicardial electrogram. Two early stimuli (delay period S_1-S_2: 180 ms and S_2-S_3: 140 ms) led to short (10 sec) atrial tachycardia (ectopic impulse generation partly from the right and partly from the left atrium)

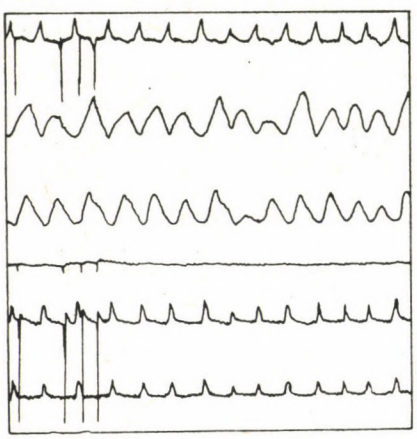

Fig. 3. Continuous — sustained — atrial tachyarrhythmia following early atrial stimuli given to dogs with increased atrial pressure
ECG recording during increased right atrial pressure. Paper speed: 50 mms^{-1}. Upper line: II. limb lead, second line: left ventricular lead, third line: right ventricular lead, fourth lead: I. bipolar limb lead, fifth line: II. bipolar limb lead. Two early atrial stimuli (delay period S_1-S_2: 180 ms and S_2-S_3: 130 ms) led to continuous atrial tachyarrhythmia. After 80 seconds the tachycardia spontaneously resolved and the sinus rhythm was restored

Characteristic types of arrhythmias developing after atrial dilatation are shown on Figures 1—3.

Neither ventricular contractility, nor ventricular pressure exhibited considerable alteration upon the effect of increased right atrial pressure or left atrial dilatation. The ventricular pressure curve did not change considerably during atrial dilatation, i.e., neither systolic pressure, nor end-diastolic pressure changed. The left ventricular strain gauge curve was also unaltered.

Discussion

Elongation and stretching of the cardiac myofibrils increase irritability and may evoke arrhythmia [4, 6, 7, 8, 10]. Stretching of the cardiac wall seems to result more easily in arrhythmia if either the myocardium itself or the cardiac metabolism is not intact [9, 13]. One possible cause of the arrthythmia occuring after stretching may be the phenomenon that as an effect of the stretching of the myocardial fibres the action potentials exhibit pathological change and the delayed after depolarization and ectopic impulse generation may ensue [4, 6]. Another possible cause of the occurence of the stretching-caused arrhythmia may be the stretching-induced local disturbances of impulse conduction (unidirectional block) which may predispose to the development of the reentry type of arrhythmia [10]. From the point of view of the development of atrial — supraventricular — arrhythmias the main question is just how does atrial stretching alter atrial irritability. Boyden et al. reported on the observation that as the result of the enlargement of the left atrium (provoked by artificial mitral insufficiency) in dogs atrial extrasystoles, supraventricular tachycardia and atrial fibrillation could frequently be detected [2, 3]. In this experimental model of mitral insufficiency atrial action potentials were mostly normal; histological analysis, however, revealed atrial fibrosis and myocardial hypertrophy [2, 3]. According to the findings of cardiac biopsies carried out in humans chronic atrial dilatation results in pathological alterations of the atrial action potentials, and slow response type action potentials can frequently be detected [1, 11, 16]. In patients with atrial fibrillation developing as a result of chronic atrial dilatation focal fibrosis and cell necrosis of the atrial wall is a frequent finding [1].

The results obtained in the present investigations indicate that stretching of the atrial wall (increase of atrial pressure, atrial dilatation) increases both atrial irritability and the tendency of the atrium to arrhythmias and leads to the development of supraventricular arrhythmias. Neither the impulse generation, nor the impulse conduction of the heart exhibited considerable alteration upon the effect of atrial dilatation; following stretching of the atrial wall only shortening of the atrial refractory period could be observed.

The pathological increase of atrial irritability which was caused by atrial dilatation manifested itself in the phenomenon that early or frequent atrial stimuli caused supraventricular arrhythmia or tachyarrhythmia. The provoked supraventricular arrhythmias proved to be of different type (reentry atrial tachyarrhythmia, atrial fibrillation and atrial flutter associated with tachycardia), but from the clinical point of view they were similar to the supraventricular tachycardia associated with atrial dilatation. Such tachycardia can relatively often be seen in mitral disease (left atrial dilatation) and, less frequently, in congenital heart malformations which are associated with atrial dilatation (atrial septal defect, Ebstein's anomaly). Dilatation associated atrial arrhythmia may develop without ischaemic damage to the myocardium; hypoxic and ischaemic damage, however, increases the probability of such arrthythmias to develop. According to our pilot investigations the tendency to arrhythmias caused by atrial dilatation is more pronounced under the conditions of cardiac ischaemia (resulting from strangulation of the coronaries). It is also of importance that following coronary surgery — aortocoronary bypass — supraventricular tachycardia frequently develops, especially in patients with enlarged atrium [5]. Atrial natriuretic peptide (ANP) may also be involved in the development of dilatation associated atrial arrhythmias. Atrial stretching enhances ANP secretion [14, 15] and the attacks of paroxysmal supraventricular tachycardia are sometimes accompanied by polyuria and polydipsia. Still, it seems reasonable to assume that ANP does not play a major role in the pathomechanism of supraventricular tachycardias. The substance itself (ANP) is without arrhythmogenic effect and does not alter considerably the tendency of the heart to arrhythmias. Thus it is the stretching of the atrial wall which, on the one hand, has arrhythmogenic influence on its own and, on the other, enhances ANP secretion.

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DDT TOXICITY: VARIATIONS IN TISSUE NON-SPECIFIC PHOSPHOMONOESTERASES AND GLUCONEOGENIC ENZYMES IN THREE TELEOSTS

S. A. SHAFFI, R. P. DUBEY

Department of Science Regional College of Education NCERT, Bhopal, India

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Three fish species were exposed to a sublethal dose (0.35 mg/l) of DDT continuously for a period of 50 days and the effect of hepatic and renal acid and alkaline phosphatases, glucose-6-phosphatase and fructose-1,6-diphosphatase activities was observed at 15, 30 and 45 days. Exposure to DDT at 15 days led to the fall and increase thereafter (at 30 and 45 days) in the activities of acid phosphatase, glucose-6-phosphatase and fructose-1,6-diphosphatase in hepatic tissue, where as alkaline phosphatase in liver registered an increase at 15, 30 and 45 days DDT exposure. In renal tissue the trend of 4 phosphatases was same as that of alkaline phosphatase in the liver. The changes in these 4 phosphatases were more pronounced in C. punctatus than in G. batrachus and L. rohita.

Keywords: DDT, toxicity, phosphomonoesterase, gluconeogenetic enzymes, teleost, alcaline phosphatase

Pollutants act as physiological stressers for the exposed animals, as do the environmental parameters, and interfere with various physiological processes at the molecular level [6, 17, 18]. Reduction in dissolved oxygen content is the common effect of many pollutants and may result from a physiological reaction between the organism and its environment [7, 14, 15].

An increase in oxygen intake of the animal results in a decrease of their life span through increased toxicity of the solution [17, 20]. It is also known that DDT exposed animals consume more oxygen by increasing the secretion of oxygen consumption enhancing hormone [13].

In an earlier communication [19] from this laboratory the author reported the effect of sublethal dose of DDT (0.035 mg/l) at 50 days on tissue phosphatases in three teleosts. To understand further about the toxic nature, the author's investigated the chronic effect of DDT at 15, 30 and 45 days on hepatic and renal acid phosphatase (orthophosphoric monoester phosphohydrolase E.C.3.1.3.2) alkaline phosphatase (orthophosphoric monoester phospho-

Correspondence should be addressed to
S. A. SHAFFI
Department of Science, Regional College of Education NCERT
Bhopal-13 M. P., India

hydrolase E.C.3.1.3.1) glucose-6-phosphatase (D-glucose-6-phosphate phosphohydrolase E.C.3.1.3.9) and fructose-1,6-diphosphatase (D-fructose-1,6-biphosphate 1-phosphohydrolase E.C.3.1.3.11) activities in Labeo rohita (Ham), Clarias batrachus (Linn) and Channa punctatus (Bloch).

Material and methods

L. rohita, C. batrachus and C. punctatus (18—20 cm standard length) were obtained locally, acclimatized in the laboratory for a couple of days before six of each species were sacrified for hepatic and renal acid and alkaline phosphatase, glucose-6-phosphatase and fructose-1,6-diphosphatase activities. Twenty five fish of each species were exposed to sublethal dose (0.035 mg/l) of DDT for a period of 50 days. Equal number of fish were kept subsequently in tap water as control for the same period. After 15, 30 and 45 days exposure both groups of control and experimental fish species were dissected for liver and kidney tissues.

ENZYME ASSAYS:

Glucose-6-phosphatase

To 0.6 ml of cacodylate buffer (0.1 M, pH 6.5), 0.1 ml of EDTA (10 mM in buffer, pH 6.5) and 0.2 ml of glucose-6-phosphate (50 mM in buffer) was added and incubated at 30 °C. After 10 min incubation, 0.1 ml of 10% tissue homogenate (buffer 0.1 M, pH 6.5) was added and incubated at the same temperature for 15 min. The reaction was stopped by adding 1 ml of ice cold TCA (10%). The other details were described earlier [11].

Fructose-1,6-diphosphatase

0.4 ml of borate buffer (0.05 M boric acid pH 9.5) was added to 0.1 ml of fructose-1,6-diphosphate (0.05 M, pH 7.4), 0.1 ml of $MgSO_4$ (0.05 M), 0.1 ml of $MnCl_2$ (0.005 M) and 0.1 ml of cysteine (0.05 M, pH 9.5, fresh solutions were prepared regularly). After 5 min incubation at 38 °C, 0.2 ml of 10% tissue homogenate was added to the above reaction mixture and incubated at 38 °C for 20 min. The reaction was terminated by adding 1 ml of 0.1 M TCA. The other details of the procedure were same as desribed by McGilvery [9].

Alkaline phosphatase

To 1.0 ml of substrate (disodium-P-nitrophenyl-phosphate tetrahydrate) 0.5 ml of buffer (pH 10.0, .75 g) glycine and 21 mg $MgCl_2$ dissolved in 70 ml of water. 8.5 ml of M NaOH was added and made up to 100 ml) was added and incubated at 37 °C for 5 min. 0.5 ml of 10% tissue homogenate (prepared in buffer) was added and incubated again at 37 °C for 15 min. The reaction was stopped by adding 4 ml of 0.04 M NaOH. A control was also maintained side by side without tissue homogenate. The other details of the enzyme assay were same as those described by Frais [5].

Acid Phosphatase

Citrate buffer (pH 5.0, .1 M) was used in acid phosphatase estimation. The reaction mixture contained 0.1 ml of 1% (W/V) Triton-×-100. The other details of the assay is same as that of alkaline phosphatase.

Stock solution

DDT (Parry, Madras India) stock solution (1mg/ml) was prepared in acetone (BDH) and the required amount was added to have the desired concentration.

The experiment was repeated for 6 times and the data were subjected to test of variance by Fisher method [4].

Results

DDT exposure for 15, 30 and 45 days led to marked changes in the activities of hepatic and renal acid and alkaline phosphatase, glucose-6-phosphatase and fructose-1,6-diphosphatase in L. rohita, C. batrachus and C. punctatus (Tables I—III). Exposure for 15 days to DDT resulted in the inhibition of acid phosphatase, glucose-6-phosphatase and fructose 1,6-diphosphatase levels in the hepatic tissues of the three fish species.

Table I

Variations in tissue phosphatases due to DDT toxicity in L. Rohita (ham)

Enzymes	DDT exposure in days				% of Rise
	control	15	30	45	
(A) Kidney					
Acid phosphatase	0.294 ±0.041	0.312 ±0.057 (+)	0.330 ±0.042 (+)	0.366 ±0.056 (+)	24.48
Alkaline phosphatase	0.724 ±0.132	0.780 ±0.112 (+)	0.789 ±0.134 (+)	0.952 ±0.084 (+)	31.49
Glucose-6-phosphatase	0.222 ±0.092	0.264 ±0.090 (+)	0.295 ±0.060 (+)	0.345 ±0.078 (+)	55.40
Fructose 1,6-diphosphatase	0.305 ±0.075	0.338 ±0.081 (+)	0.374 ±0.092 (+)	0.392 ±0.096 (+)	28.52
(B) Liver					
Acid phosphatase	0.382 ±0.097	0.345 ±0.114 (+)	0.464 ±0.091 (+)	0.585 ±0.118 (+)	53.14
Alkaline phosphatase	0.248 ±0.016	0.276 ±0.092 (+)	0.305 ±0.093 (+)	0.324 ±0.122 (+)	30.64
Glucose-6-phosphatase	0.368 ±0.119	0.294 ±0.099 (+)	0.376 ±0.111 (+)	0.589 ±0.125 (+)	60.05
Fructose 1,6-diphosphatase	0.494 ±0.124	0.415 ±0.212 (+)	0.502 ±0.120 (+)	0.695 ±0.131 (+)	40.68

Values (acid and alkaline phosphatase = μ moles pf P-nitrophenol/mg protein at 37 °C G-6-ase and F-D-ase = μ moles of Pi/mg protein at 30 °C and 38 °C, respectively) are mean ± SDM of 6 replicates. Test of variance was performed between control and treated values of respective tissues. Side script (+) indicates that $P < 0.001$.

This inhibition was reversed at 30 and 45 days exposures. Whereas, alkaline phosphatase registered an increase at 15, 30 and 45 days in liver tissue. In contrast to liver, an increase in the activities of the four enzymes at 15, 30 and 45 days in renal tissue was recorded.

Among these four enzymes the highest rise was recorded in renal alkaline phosphatase level (C. punctatus, Table III). It was followed by hepatic acid phosphatase (C. punctatus, Table III), glucose-6-phosphatase (C. batrachus, Table II) fructose-1,6-diphosphatase (L. rohita, Table I).

Table II

Variations in tissue phosphatases due to DDT toxicity C. batrachus (linn)

Enzymes	DDT exposure in days				% of rise
	Control	15	30	45	
(A) Kidney					
Acid phosphatase	0.224 ±0.069	0.247 ±0.111 (+)	0.283 ±0.059 (+)	0.324 ±0.061 (+)	44.64
Alkaline phosphatase	0.520 ±0.215	0.546 ±0.221 (+)	0.584 ±0.064 (+)	0.769 ±0.218 (+)	47.80
Glucose-6-phosphatase	0.268 ±0.074	0.292 ±0.092 (+)	0.338 ±0.070 (+)	0.391 ±0.070 (+)	45.89
Fructose 1,6-diphosphatase	0.409 ±0.130	0.445 ±0.917 (+)	0.472 ±0.00 (+)	0.542 ±0.099 (+)	32.51
(B) Liver					
Acid phosphatase	0.306 ±0.051	0.264 ±0.041 (+)	0.379 ±0.080	0.491 ±0.021 (+)	60.45
Alkaline phosphatase	0.262 ±0.051	0.285 ±0.059 (+)	0.322 ±0.065 (+)	0.362 ±0.018 (+)	38.16
Glucose-6-phosphatase	0.486 ±0.124	0.407 ±0.064 (+)	0.572 ±0.120 (+)	0.842 ±0.219 (+)	73.25
Fructose 1,6-diphosphatase	0.606 ±0.227	0.524 ±0.091 (+)	0.646 ±0.230 (+)	0.810 ±0.121 (+)	33.66

Values: (acid and alkaline phosphatase = μ moles of p-nitrophenol/mg protein at 37 °C, G-6-ase and F-D-ase = μ moles of Pi/mg protein at 30 °C and 38 °C, respectively) are mean ± SDM of 6 replicates. Test of variance was performed between control and treated values of respective tissues. Side script (+) indicate that $P < 0.001$.

Table III

Variations in tissue phosphatases due to DDT toxicity in c. punctatus (Bloch)

Enzymes	DDT exposure in days				% of rise
	Control	15	30	45	
(A) Kidney					
Acid phosphatase	0.144 ±0.042	0.188 ±0.039 (+)	0.212 ±0.060 (+)	0.243 ±0.049 (+)	62.00
Alkaline phosphatase	0.280 ±0.032	0.321 ±0.042 (+)	0.386 ±0.077 (+)	0.520 ±0.061 (+)	85.71
Glucose-6-phosphatase	0.362 ±0.089	0.429 ±0.059 (+)	0.489 ±0.081 (+)	0.586 ±0.069 (+)	61.87
Fructose 1,6-diphosphatase	0.532 ±0.097	0.586 ±0.089 (+)	0.636 ±0.096 (+)	0.729 ±0.124 (+)	37.03
(B) Liver					
Acid phosphatase	0.214 ±0.043	0.272 ±0.039 (+)	0.330 ±0.069 (+)	0.392 ±0.040 (+)	63.17
Alkaline phosphatase	0.162 ±0.039	0.187 ±0.021 (+)	0.240 ±0.074 (+)	0.272 ±0.055 (+)	67.90
Glucose-6-phosphatase	0.674 ±0.215	0.572 ±0.049 (+)	0.706 ±0.083 (+)	0.974 ±0.139 (+)	44.51
Fructose 1,6-diphosphatase	0.832 ±0.121	0.718 ±0.084 (+)	0.846 ±0.071	0.965 ±0.229 (+)	15.98

Values (acid and alkaline phosphatase = μ moles of P-nitrophenol/mg protein at 37 °C' G-6-ase and F-D-ase = μ moles of Pi/mg protein at 30 °C and 38 °C, respectively) are mean ± SDM of 6 replicates. Test of variance was performed between control and treated values of respective tissues. Side script (+) indicate that $P < 0.001$

Discussion

Pollutants influence various metabolic processes. Increase in the secretion of oxygen consumption enhancing hormone disturbs the adjusting capabilities and compel the animal to consume more oxygen under contaminated situation. These effects are attributed to the physiological stressing nature of the toxicants [6, 14, 16, 17].

It is known that organochlorine compounds bring about various alterations by influencing quite a number of biochemical parameters. It is also known that they interfere seriously with the hepatic, renal and neural metabolism and can depress secondary sexual characteristics [1, 2].

In the present investigation the author observed that the nonspecific phosphomonoesterases and gluconeogenic enzymes in renal and hepatic tissue registered a linear increase for 45 days DDT exposure, except that the two gluconeogenic enzymes observed a fall at 15 days exposure in hepatic tissue and the rise was higher in a carnivore then herbivore or piscivore fish.

The rise of these phosphatases may be discussed on the following sequence: Increase in hepatic and renal acid phosphatase, an enzyme that plays an important role in intermediary metabolism may be related to tissue damage and pre-necrotic changes [3, 8, 10, 12]. However, the rise in alkaline phosphatase may be presumed owing to an increase in transphosphorylation mechanism as the animals are under physiological stress. Increase in liver and renal G-6 ase and F-D-ase can be attributed to stimulated gluconeogenic process which is because of organochlorine intoxication as the latter is reponsible for the depletion of energy reserve [19].

Elevation in hepatic and renal phosphatase levels may reflect physiological adjustments to cope with continued DDT stress and to function for the survival of the fish in poisoned environment. It is known that DDT exposed animals would consume more oxygen and may drag the animal to a new steady state of metabolism as the increased oxygen consumption is required to support enhanced physiological activities in metabolizing and eliminating the pollutants by the exposed animals. Such a mechanism might have occurred in the present study and the above alterations in four phosphatases might be due to the above changes. Moog [10] and Shaffi [19] while discussing the physiological significance of non-specific phosphomonoesterases said that the elevation in serum and tissue phosphatases might be associated with the pathological condition of the tissues. Chronic DDT exposure might have led to the above situation and increase in the level of two phosphatases might be related to the above phenomena.

The highest rise in hepatic and alkaline phosphatase, G-6-ase and F-D-ase in these three fish species indicate the degree of pathological condition of the tissue owing to chronic DDT exposure and vice versa.

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INFLUENCE OF SEX ON GASTRIC ACID SECRETION AND PARIETAL CELL MASS IN THE RAT

Kayode O. ADENIYI, Michael O. OLOWOOKORUN*

Department of Human Physiology, University of Jos, Jos, Nigeria and *Department of Veterinary Physiology and Pharmacology, University of Ibadan, Ibadan, Nigeria

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The role of sex on the development and function of parietal cell was investigated in the rat. Basal gastric acid secretion and parietal cell mass were found to be significantly lower in female rats than in male. The results suggest that sex is an important factor influencing the growth and function of the gastric mucosa.

Keywords: rat stomach; acid; parietal cell mass; gastric mucosa; growth

Amure and Omole [2] showed that female rats secrete less gastric acid than male and that the administration of oestrogen to rats caused a reduced gastric acid secretion. There is however no consensus in the literature on the reasons for these observations.

The present study was designed to investigate the influence of sex on gastric acid secretion and parietal cell mass in the rat.

Materials and methods

Animals

Adult Wistar strain rats weighing 110—140 g were divided into two groups: male and female. The two groups of rats were kept in separate cages where rat chow and tap water were provided ad-libitum for a period of 35 days prior to the determination of gastric acid secretion and parietal cell mass.

Preparation of animals for gastric acid secretion study

Rats were fasted overnight with free access to water so as to give a reasonably clean stomach at the time of the experiment. They were then anaesthetized with an intraperitoneal injection of urethane solution (25% w/v) at 0.6 ml/100 g body weight and prepared according to the technique described by Ghosh and Schild [7].

Correspondence should be addressed to
Kayode O. ADENIYI
Department of Human Physiology
Faculty of Medical Sciences University of Jos
Jos, Plateau State, P.B.M. 2084, Nigeria

The stomach lumen was perfused at a rate of 1±0.1 ml/min with distilled water at 37 °C and the effluent collected at 10 min intervals and assayed for titratable acid against 0.01 N sodium hydroxide using phenolphthalein as an indicator.*

Preparation of stomach for parietal cell count

The stomach of the rat was removed under general anaesthesia induced with an intraperitoneal injection of urethane solution. They were opened along the greater curvature and contents removed by gentle washing. The empty stomach was pinned with serosal surface downward on a cork sheet immersed in a tray containing normal saline to stretch out all mucosal folds as described by Card and Marks [5]. The flattened specimens were removed and trimmed carefully to separate the antrum from the fundus. The fundic region was traced carefully on white paper and the surface area determined. The specimens were then fixed for 24 hours in 10% formol saline and embedded in paraffin.

From every specimen, six strips of glandular mucosa were excised approximately perpendicular to the antral margin rather than parallel to the curvatures as described by Cox and Barnes [6]. Care was however taken to exclude the transitional type mucosa which separates the fundus from the antrum and the cardiac type mucosa adjacent to the transverse ridge. The histological observations were confined to true fundic mucosa.

The sections were stained according to the technique described by Asojo [3]. The different gastric mucosal secretory cells are well differentiated with this technique the mucous neck cells were pale green, zymogenic granules of chief cells dark green and parietal cells appeared yellow. Nuclei were stained black.**

Histological observations and calculations

Mucosal thickness were determined from 10 readings on each section with a calibrated ocular micrometer (magnification 90x). Final calculations were made according to the method of Card and Marks [5].

Parietal cell counts were done using calibrated ocular grid (magnification 900x). A single 'count' represented all parietal cell nuclei or nuclear fragments seen in a column of tissue 0.1 mm broad and extending from the base to the surface of the mucosa. Ten counts were made on each section and the average (designated subsequently as the average count per unit area) was calculated. PCM is derived from the following determinations [4].

1. Mean parietal cell count per unit area of each stomach.
2. Surface area of the acid bearing region of the stomach.
3. Correction factor for shrinkage of the mucosal strips during fixation and staining.

Table I

Basal gastric acid secretion

Type of rats	Initial body weight (g)	Body weight of rats after 35 days (g)	Basal gastric acid secretion in μeq/10 minutes
Male (20)	138.5 ±4.13	248.1 ±6.90	3.69±0.08
Female (20)	138.5 ±4.06	247.9 ±5.74	3.49±0.06*

Values are means ± S. E.; no. of rats is shown in parentheses. * $P < 0.05$ compared with male rats.

* The measurement of gastric acid secretion usually commenced by 8.00 a.m. and concluded by 2.00 p.m.

** All dyes used were obtained from G. T. Gurr Ltd., London.

4. The actual thickness of the sections and the cross-sectional diameter of the cells.
5. Correction factor for nuclei over-estimation.

Shrinkage factor was determined according to the method described by Card et al. [5], while the thickness of the sections and the diameter of the cells were derived from direct measurements using the method of Morengo [8]. The correction factor for nuclei over-estimation was calculated according to a formula derived by Abercombie [1]. Parietal cell mass was estimated using the formula of Naik et al [9].

Statistics

All results are expressed as means ± S.E. Statistical comparisons between the experimental groups were performed using the Student's t test.

Results

Basal gastric acid secretion

The mean basal gastric acid secretion for the male rats (Table I) was 3.69 ± 0.08 μeq/10 minutes and this was significantly ($P < 0.05$) higher than the value obtained for the female rats (3.49 ± 0.06 μeq/10 minutes).

Parietal cell mass

The results (Table II) show that the average count per unit area for male rats was 17.50 ± 0.16 and the corrected count per unit area was 12.23 ± ± 0.11. When these values were compared with the corresponding values for female rats (17.16 ± 0.18 and 11.89 ± 0.19) the differences were not significant. The surface area of the acid-bearing region of the stomach of female rats (7.68 ± 0.30 cm^2) was however significantly ($P < 0.05$) lower than the observed value for normal male (8.66 ± 0.42 cm^2). As a consequence of this, the parietal cell mass recorded for the female rats (18.26 ± 0.16 million) was significantly ($P < 0.001$) lower than the observed value for the male (21.18 ± ± 0.13 million).

Discussion

The higher mean basal gastric acid secretion rate in the male rats than in the female rats, observed in the present work, tend to support the contention of Amure and Omole [2] that female rats secrete less gastric acid than male.

It was revealed by this work that the value for the fundic surface area in the female rats was significantly lower than the observed value for normal male rats. As a result of this, there was a statistically significant decrease in the parietal cell mass in the female rats. This result shows that sex is an important factor influencing the growth and development of the gastric

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Table II

Parietal cell mass

Type of rat	Average count per unit area X	Correction factors			Corrected count per unit area (Z) $Z = X \times Y$	Surface area of fundus (cm^2)	Parietal cell mass ($X10^6$)	Height of fundic mucosa (MM)	Volume of fundic mucosa (mm^3)
		S.F.	F.N.O.	T.C.F. Y					
Male	17.50 ±0.16	0.953 ±0.004	0.733 ±0.002	0.699 ±0.003	12.33 ±0.11	8.66 ±0.42	21.18 ±0.13	0.431 ±0.012	373.25 ±26.50
Female	17.16 ±0.16	0.939 ±0.011	0.730 ±0.015	0.686 ±0.011	11.89 ±0.19	7.68* ±0.30	18.26** ±0.16	0.428 ±0.024	328.70 ±18.86

Each value represents mean ± S.E. from 360 counts in 6 rats. S.F., Shrinkage factor. F. N. O. Correction factor for nuclear overestimation. T.C.F., Total correction factor. * $P < 0.05$, ** $P < 0.001$ compared with the value for male rats.

mucosa. Furthermore, the low parietal cell mass in the female rats could have accounted for the low basal gastric acid secretion recorded for these animals during the gastric acid secretion study.

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NITRATE INDUCED CHANGES IN SENSORO-MOTOR DEVELOPMENT AND LEARNING BEHAVIOUR IN RATS

Éva MARKEL, C. NYAKAS, S. ORMAI

Central Research Division, Postgraduate Medical University
Budapest, Hungary

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Development of sensoro-motor functions and adult learning behaviour was studied in rats subjected to nitrate exposure. Pregnant and lactating dams and their offspring were supplied with drinking water containing nitrate, 1.12 or 2.24 mM KNO_3, and compared with nitrate-free controls. Postnatal maturation of reflexes, that of sensory and somatic parameters and motor activity, furthermore, the acquisition of one-way avoidance and rewarded discriminative learning behaviour in adulthood were examined. Reflexes (righting, cliff-avoidance) and hearing startle reaction maturated earlier in nitrate treated groups. No difference was found in olfactory homing behaviour, in the time of eye opening and in body weight growth. Open-field motor activity was higher at days 5, 7, 10 after birth, but hypoactivity ensued after day 20. A marked learning deficit was observed both in punished and in rewarded learning paradigms. The results indicate a nitrate induced deviation in behavioural development, and an impairment in learning behaviour, particularly of discriminative type.

Keywords: nitrate contamination, drinking water, cerebral functions, learning deficit

As a consequence of intensified agriculture the nitrate contamination of drinking water shows a gradual increase in Hungary [5]. It is particularly important in the provinces where aproximately 38.9 percent of the population consume their drinking water from individual wells. Numerous studies have demonstrated that nitrate induces methemoglobinaemia in newborn and small children [4, 11, 17]. It was also demonstrated that nitrate and its reduced form nitrite, can also cause digestive disturbances [18], their metabolites may induce malignant tumors in the stomach and intestines [7, 16]. Various lesions were detected also in the central nervous system, mainly in acute experiments [8, 9].

The present study was aimed to clarify whether chronic nitrate consumption by pregnant rats and their offspring has any deleterious effect on the development of sensoro-motor functions and the adult learning behaviour.

Correspondence should be addressed to
Éva MARKEL
Central Research Division, Postgraduate Medical University
H-1389 Budapest, Szabolcs u. 35. P.O.Box 112. Hungary

Material and methods

Animals and nitrate treatment

Pregnant Wistar rats were provided with water and standard food pellets (LATI. Gödöllő, Hungary) ad libitum. The nitrate was given in drinking water in doses of 1.12 or 2.24 mM potassium nitrate (120 mg/l or 240 mg/l respectively). The control animals received nitrate-free water. The daily water intake of mothers was measured during pregnancy and lactation. After weaning at day 21 post partum the mothers were excluded from the experiments, and the offspring received the same water containing 1.12 or 2.24 mM nitrate, or nitrate-free, as their mothers had previously. The young animals were separated according to sex and were kept in groups of 5 per cage at room temperature and under controlled schedule of light and dark periods. All the tests were performed between 8.00 and 12.00 a.m. For behavioural studies in adulthood only the male animals were used.

Sensoro-motor development

The maturation of reflexes i.e. righting on a surface and cliff avoidance was studied as described by others [2]. The reaction times, i.e. latency of turns from the back to normal position in the case of righting reflex, and the time to the onset of withdrawal from the edge of a cliff were measured daily in seconds. The maturation of reflexes were expressed in reciprocal reaction time. The daily observations of reflex performances were continued until the criterion was fulfilled, i.e. the reflexes had to be performed within 2 seconds.

Open-field locomotor behaviour was measured on days 5, 7, 10, 20 post partum and closed-field activity was measured at day 42 by using the time-sampling method of 10 seconds as described earlier [12]. To assess sensory development olfactory homing behaviour was analysed from day 6 to 11 and the date of eye opening and hearing startle was recorded [2]. The body weight of the animals was measured weekly.

Adult learning behaviour

One-way active avoidance (pole jumping, [6]) and water rewarded discriminative learning tests were applied. In the discrimination test animals deprivated of water for 23 hours were trained to push a swing-door for water supplied by an automatic distributor. After the establishment of the positive conditioned response to a continuous sound of 70 dB, 1000 c/s for 5 seconds, a differential stimulus was introduced (interrupted sound of 70 dB, 2000 c/s for 5 seconds), which was not reinforced by water. Evaluation of the learned response was based on the observation of performing correct responses. Correct responses were scored if the rat pushed the door for water in response to the reinforced sound or failed to respond to the nonreinforced signal.

Results

Nitrate intake

The nitrate content of drinking water did not influence the volume of water consumption significantly (Table I). The amount of daily nitrate intake is also shown in the table which was calculated according to the nitrate concentration of the drinking water.

Sensoro-motor development

The maturation of reflexes and sensory function was affected in the nitrate exposed animals (Fig. 1). Both nitrate doses accelerated the appearance

Table I

Average daily water intake of nitrate exposed and control rats during pregnancy and lactation

	Control	KNO_3 doses in mM	
		1.12	2.24
Gestation			
water volume+	157±21	160±11	165±8
KNO_3 amount++	0.0	19.8	39.6
Lactation			
Water volume	250±16	273±18	289±15
KNO_3 amount	0.0	32.4	69.4

+ Intake = mean ± S.E.M. ml/kg·day^{-1},
++ Calculated uptake of KNO_3 = mg/kg·day^{-1}.

of the fully organized motor reflexes, i.e. righting and cliff-avoidance. The criterion of reflex performance was reached earlier in the treated rats. Concerning the development of sensory functions it was seen that maturation of the hearing startle reaction was moderatly accelerated in nitrate exposed

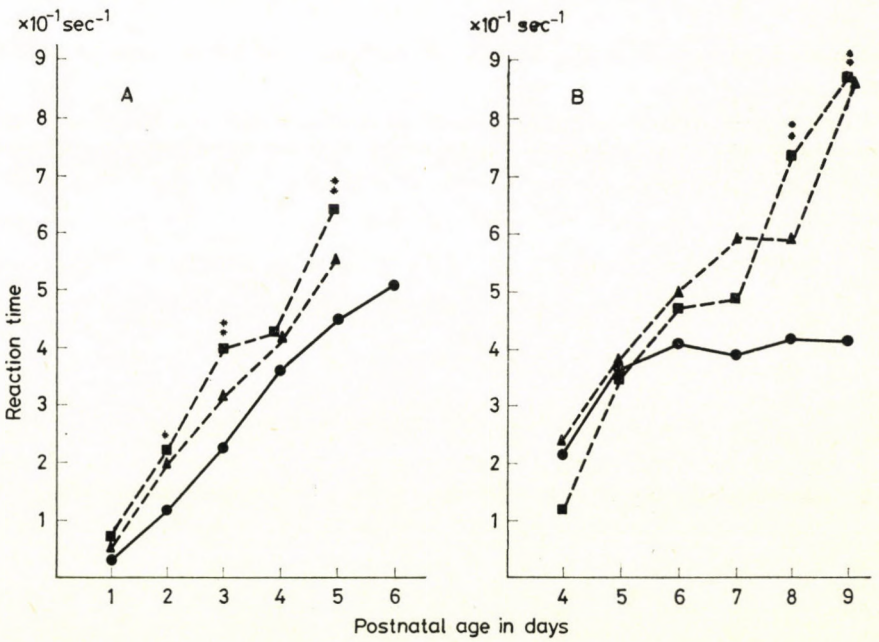

Fig. 1. Maturation of righting reflex (part A) and cliff avoidance reaction (part B). Mean reciprocal values of reflex time (sec) are shown. ● = control group (n = 49), ■ = 1.12 mM KNO_3 exposed group (n = 52), ▼ = 2.24 mM KNO_3 exposed group (n = 31), * = $p < 0.05$, ** = $p < 0.01$ *vs* control, Student's test

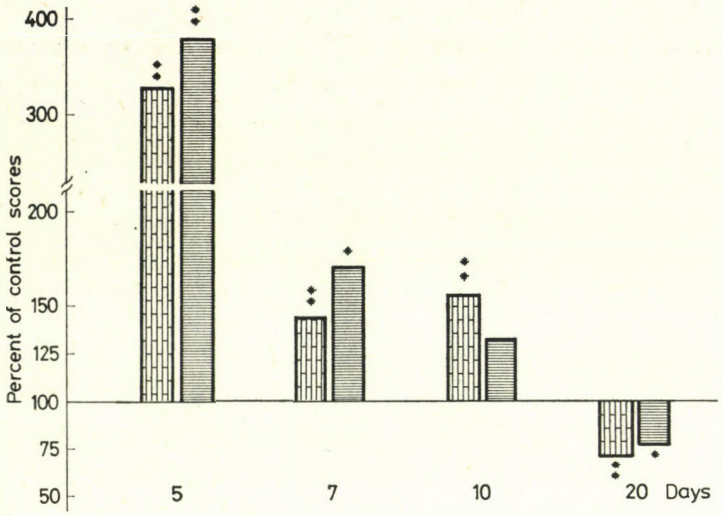

Fig. 2. Effect of nitrate contamined drinking water consumption on the novelty induced motor activity in rats of different ages. Motor activity is expressed in percent of control (n = 51) scores. Stippled columns represent 1.12 mM KNO_3 dose (n = 49), hatched columns represent 2.24 mM KNO_3 dose (n = 31). * = $p < 0.05$, ** = $p < 0.02$ *vs* control Students' t test

groups as compared to control ($F > 3.4$, $p < 0.03$; data are not shown). Nitrate did not influence the time of eye opening. The maturation of olfactory homing behaviour, i.e. the ability of an isolated pup to return to its littermates, was also not influenced. No changes was found in the body weight.

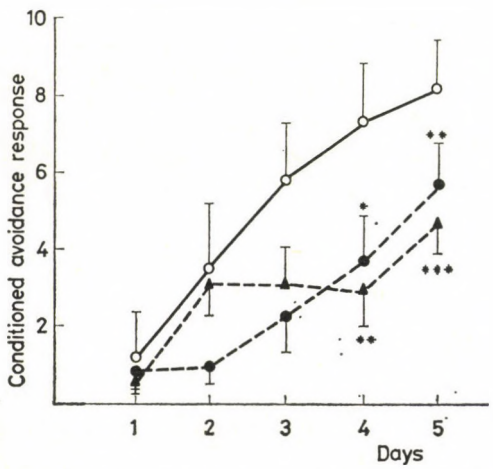

Fig. 3. Effect of nitrate contaminated drinking water consumption on the punished learning behaviour in adult rats. Each point represents mean ± S.E.M. perf ormance· ○ = conrol (n = 10), ● = 1.12 mM KNO_3 (n = 8), ▼ = 2.24 mM KNO_3 (n = 6). * = $p < 0.05$, ** = $p < 0.02$, *** = $p < 0.01$ *vs* control, Mann-Whitney U-test

Novelty induced locomotor activity

These studies showed that the locomotor activity of nitrate treated rats was increased in the early postnatal period and later it was reversed gradually by day 20 (Fig. 2). In accordance with the observed hypoactivity on day 20, treated animals displayed lower locomotor activity also in adulthood in a closed-field test ($p < 0.05$ Student's t test, data are not shown).

Adult learning behaviour

A deficit in the acquisition of the pole jumping avoidance response was found in rats supplied with nitrate (Fig. 3). The learning deficit became significant by the 4th day of training. The two nitrate doses influenced the learning performance in a comparable manner. Water-rewarded discriminative learning was altered markedly as a result of nitrate exposure (Fig. 4). Practically no sign of conditioned supression of nonreinforced responses could be observed in the nitrate-exposed animals while in the controls the number of cor-

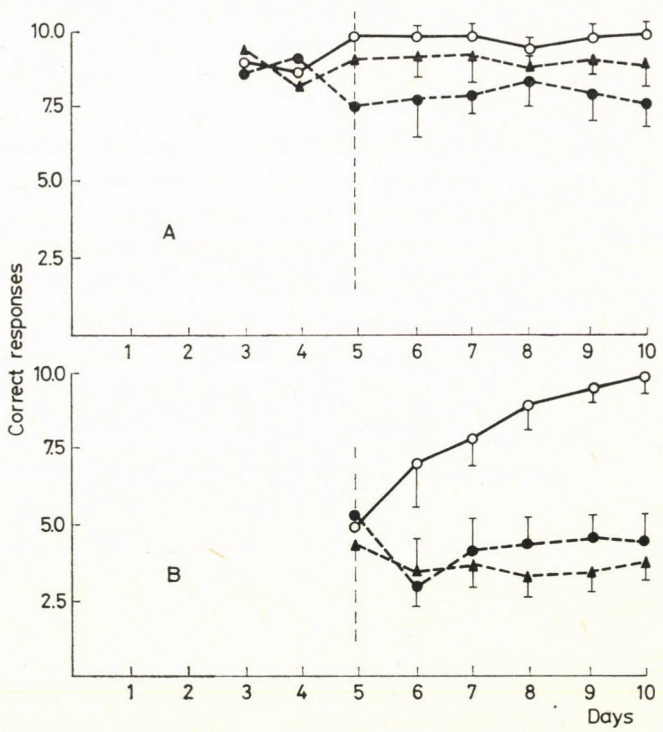

Fig. 4. Effect of nitrate contaminated drinking water consumption on the rewarded discriminative learning behaviour in adult rats. A = performances to reinforced conditioned signal, B = performances to non-reinforced, discriminative signal. ○ = control (n = 10), ● = 1.12 mM KNO_3 (n = 8), ▼ = 24 mM KNO_3 (n = 6)

rect responses to the discriminative signal reached 90 percent on days 9 and 10 (Graph B of Fig. 4). For statistical evaluation the discriminative performance from days 6 to 10 was pooled and analysed using Mann-Whitney's U-test. Either the lower ($U_{10\cdot8} = 0$, $p < 0.001$) or higher nitrate dose ($U_{10\cdot6} = 0$, $p < 0.001$) caused significant learning deficit. There was no change in the number of correct responses to the reinforced conditioned signal during the acquisition, but after the introduction of the discriminative conditioned signal from days 5 to 8 some deficit could be seen in the retention of reinforced learned responses in the nitrate groups (Graph A of Fig. 4).

Discussion

The main finding in these experiments was that the offspring of nitrate consuming dams showed remarkable hyperactivity and abnormal maturation in some behavioural tests. Furthermore, our results also showed a highly significant learning deficit both in punished and in rewarded conditioning. The learning deficit was most pronounced in the discriminative task.

Other studies have shown that nitrate or nitrite may cause various forms of injuries such as methemoglobinaemia [5, 13], hypothyreoidism [10, 14], or behavioural deficit [3, 9, 15]. Since nitrite as the reduced form of nitrate oxidizes not only ferro-haemoglobin but also other ferrous haemoproteins as myoglobin and cytochrome P-450 [13], it might be assumed that the fundamental mechanism of action is a generalized cellular hypoxia.

While studying the effects of nitrate contamined drinking water on the amount of water intake it became clear that there was no a significant treatment effect. Based on the measurement of water consumption the daily amount of nitrate intake could be calculated which showed that it was 6 or more times higher than the tolerance value (5 mg/kg b.w./day) declared for the human by WHO cited [1]. However, the dose used in this experiment are comparable to a moderate nitrate contamination often found in drinking water of numerous Hungarian settlements leading to methemoglobinaemia in children [5].

Two doses of nitrate were used in these experiments and both led to a marked hyperactivity measured in openfield in early postnatal age. The maturation of two motor reflexes was followed throughout the early postnatal period. Both righting and cliff avoidance reflexes showed an apparent accelerated maturation, but this could be the consequence of hyperactivity. The hyperactivity was most pronounced in the very early postnatal age, then gradually decreased and reversed already by day 20. These results suggest that nitrate neurotoxicity may interfere with the normal development of those stimulatory and inhibitory processes which regulate motor activity.

In adulthood two behavioural test were used to assess the learning ability of animals subjected to lifelong nitrate exposure. Both training paradigms of the avoidance and approach types, have revealed deficient learning capacity. The acquisition of the pole jumping avoidance response was impaired and the learned suppression of an approach response was almost absent in the course of discriminative conditioning. Especially this later observation might suggest a relatively well circumscribed damage in the neocortex. Since nitrate treatment was continued even during the learning tests a number of factors may be considered to explain the learning deficit. Such factors could be deficient brain development due to hypoxia or a direct damage to certain brain areas, eventually the ensuing hypothyreoidism due to chronic nitrate consumption [10]. Further studies are necessary to clarify the contribution of these unwanted consequences of chronic nitrate consumption.

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CONDITIONED TASTE AVERSION ELICITED BY INTRACEREBRAL ADMINISTRATION OF DRUGS

(REVIEW)

J. Bureš, O. Burešová

Institute of Physiology, Czechoslovak Academy of Sciences, Prague, Czechoslovakia

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Conditioned taste aversion (CTA) is a vital adaptive reaction governed by highly reliable but poorly understood central mechanisms. In an attempt to elucidate the site of action of various CTA eliciting drugs, equipotent dosages were applied by the systemic (i.p.) and intracerebral (i.c.) route. Rats were offered water on days 1 and 2. On day 3 they received 0.1% sodium saccharin (CS) followed by pentobarbital anaesthesia and i.p. or i.c. injection of the drug (US). After water on day 4, the rats were allowed to choose between water and saccharin on day 5. The putative central action of amphetamine was not confirmed by this experimental arrangement, since CTA was evoked by only moderately (about 10 times) lower i.c. than i.p. dosages. Similar ratio of the i.c. to i.p. effective dosages was obtained with carbachol. On the other hand, CTA of clearly central origin was caused by harmaline and by other monoamine oxidase inhibitors, pargyline and clorgyline, which elicited comparable aversion using 500, 400 and 250 times lower i.c. than i.p. dosages, respectively. The intracerebral gradient of the effect pointed to the lower medulla (inferior olive, raphe nuclei) as the critical brain region and to serotonin as the transmitter participating in the aversive labeling of the gustatory stimulus. The CTA-forming mechanism can also be studied by analysing the action of drugs, e.g. convulsants, which do not produce CTA even when applied at highly toxic dosages (LD 50) eliciting long lasting convulsions (picrotoxin, 5 mg/kg; bicuculline, 5 mg/kg). It is concluded that comparison of brain events elicited by drugs which can or cannot serve as unconditioned stimuli in the CTA paradigm may substantially contribute to the exploration of the underlying neural mechanisms.

Keywords: conditioned taste aversion, adaptive reactions, amphetamine, carbachol, monoamine oxidase inhibitors, convulsants

Conditioned taste aversion (CTA) has become, during the past 10 years, one of the most thoroughly studied areas of memory research, the results of which are extensively covered in monographs [5, 58], review articles [3, 15—17, 33, 35, 77, 78], and bibliographies [72]. Whereas concentrated effort has established the fundamental properties of CTA at the behavioural level, analysis of the relevant brain mechanisms has progressed more slowly. The neurophysiological investigation of CTA is hampered by the multistage organization of the acquisition process and by the poorly defined unconditioned stimulus. At least 5 different phases can be distinguished in CTA acquisition: perception of the taste stimulus, formation of the short term

Correspondence should be addressed to
J. Bureš
Institute of Physiology,
Czechoslovak Academy of Sciences
142 20 Prague 4.— Krč, Videňská 1083

gustatory trace, perception of the aversive stimulus, association of the aversive stimulus with the gustatory trace, formation and consolidation of the long term CTA engram. Functional ablation procedures, allowing transient interference with selected phases of CTA acquisition, showed that formation of the gustatory trace is an extremely fragile process requiring the participation of the cerebral cortex and intact motivational and attentional mechanisms. On the other hand, persistence of the gustatory trace, its association with the symptoms of poisoning, and formation of the long term CTA engram are processes highly resistant to interference which proceed even during functional decortication, anaesthesia or comatose states. The above phases of CTA acquisition cannot be differentiated by brain lesions which block CTA learning by disrupting the weakest link of the chain. The failure of lesioned animals to acquire CTA may indicate that the lesion has disrupted the gustatory trace formation rather than its association with the aversive stimulus. It is obvious that the latter phase cannot be isolated by the lesion technique.

There are several possibilities of circumventing the above difficulties. In the ideal case, the experimental interference should be limited to only one component of the process. The long delay between the CS and US makes it possible to use spreading depression, electrical brain stimulation, systemic or intracerebral injection of drugs, hypothermia, hypoxia and other interventions for this purpose, but the results of these studies have not so far led to a reliable establishment of the neural substrate of different CTA components. Another possibility is to analyze CTA acquisition by examining the signals generated by the respective CS and US inputs. Since poisoning is a pivotal element of the CTA mechanism, analysis of the circuits activated by the various CTA-eliciting stimuli can hopefully specify the structures responsible for the aversive labelling of the gustatory trace. The aim of the present paper is to review several recent attempts to determine the basic properties of the US and to find a common system ultimately activated by the wide range of CTA eliciting stimuli.

The nature of the US

X-radiation used in the first CTA studies [32] elicits an internal malaise comparable to that induced by emetic drugs. Gastrointestinal distress is certainly a common, but not indispensable, cause of CTA. Many drugs elicit CTA without inducing any obvious signs of sickness. On the other hand, there are instances of almost lethal poisoning which do not elicit CTA [44, 63]. Comparison of various stimuli which elicit or fail to elicit CTA can help to characterize the effective US and to determine the pathways mediating its effect.

A CTA eliciting drug may affect the gastrointestinal system and its nociceptive receptor which are connected to the CNS through vagal and splanchnic afferents. Other drugs may act upon the area postrema, a chemoreceptive zone at the ground of the fourth ventricle, sensitive to blood-borne toxins. Finally, the drug may directly affect the brain centers mediating the US. The different routes can be distinguished by appropriate experimental procedures. The role of area postrema was demonstrated by Berger et al. [9] who reported that a lesion of this structure abolished CTA produced by methylscopolamine, a drug not penetrating the blood-brain barrier, but did not prevent CTA elicited by amphetamine which readily passes into the

brain. Area postrema lesions also attenuate CTA induced by LiCl [55, 73], intravenous copper sulfate [24] and gamma radiation [67]. The significance of vagal afferents was demonstrated in experiments [25], showing that subdiaphragmatic vagotomy prevented CTA elicited by intragastric administration of $CuSO_4$ but did not reduce CTA induced by intraperitoneal or intravenous injection of this poison. Vagal deafferentation did not diminish the CTA-inducing potency of intraperitoneal LiCl [54] or of intragastrically or intraperitoneally administered alcohol [46].

The central effect of a drug can be confirmed by intracerebral administration of the substance which should elicit CTA at dosages at least 100 times lower than the systemic application. It can also be expected that the effect of intracerebral injection will be inversely related to the distance between the injection site and the critical brain locus.

Although central action has been surmised for a number of CTA eliciting agents, the assumption has been only rarely tested by intracerebral administration of drugs. Amit et al. [2] reported that Δ^9-tetrahydrocannabinol elicits comparable CTA with either a 5 mg/kg intraperitoneal injection or a 30 μg intrahippocampal injection. The drug was about 30 times more effective with intrahippocampal than with systemic application. No CTA was induced by intracerebral injection of morphine (30 μg), although i.p. injection of 9 mg/kg morphine elicited marked CTA. Since only one intracerebral and one intraperitoneal dosage was used, the above study does not allow a reliable comparison of the efficacy of centrally and peripherally applied drugs. We have employed a similar approach for ascertaining the central effects of other CTA--eliciting drugs.

Amphetamine

The belief that amphetamine-induced CTA is centrally mediated is largely based on the observation that amphetamine fails to elicit CTA in rats whose brain contents of norepinephrine and dopamine have been reduced to 16 and 10%, respectively, by intraventricular injection of 6-hydroxydopamine [74]. The finding that a 6-hydroxydopamine lesion of the dorsal tegmental noradrenalinergic pathway [75] did not prevent amphetamine-induced CTA supported the view that the aversive effect is mediated by dopamine rather than by noradrenaline [21]. Consonant with this hypothesis is the attenuation of amphetamine-induced CTA by the dopamine antagonist pimozide [39]. A central amphetamine effect is also inferred from the failure of the peripherally acting hydroxyamphetamine to elicit CTA [12]. In a direct test of the central mediation of the amphetamine effect [38], rats received either an intracerebral amphetamine injection through a cannula chronically implanted in the third ventricle or an intraperitoneal dose 15 min after saccharin drinking. A choice between saccharin and water on the next day revealed CTA of similar intensity in cannulated animals who had received 5 mg/kg of d,l-amphetamine intraperitoneally or 250 μg intracerebroventricularly. Available pharmacokinetic data [70] indicate that intraperitoneal injection of amphetamine increases brain level of that drug to a maximum of 10 μg/g 10 min after the injection and to 6.5 and 2.5 μg/g after 1 and 2 h, respectively. If we assume that the intracerebrally administered drug is uniformly distributed in the approximately 1 g weight of rat brain, than the drug level attained with the ineffective intracerebral injection of 160 μg amphetamine exceeds by at least an order of magnitude the concentration attained after intraperitoneal injection of 5 mg/kg amphetamine. In fact,

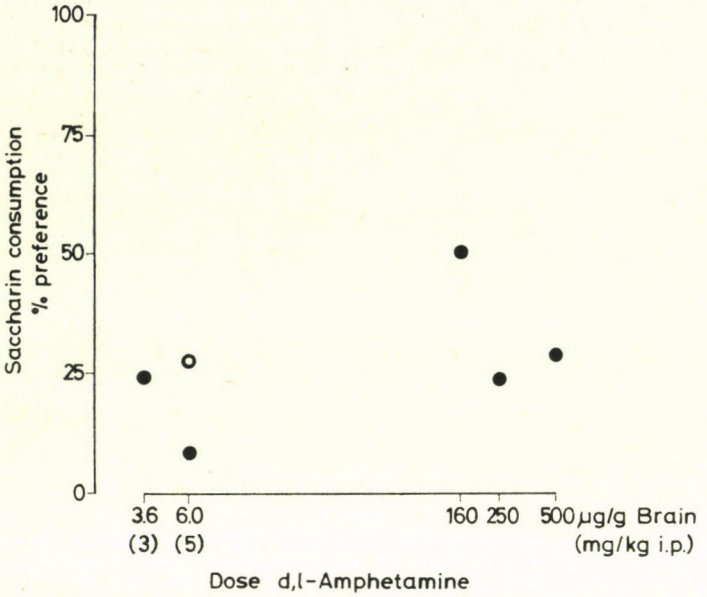

Fig. 1. Mean saccharin preference on the first retention day for each group receiving peripheral or central amphetamine on the first conditioning day, as a log function of estimated (IP) or applied (ICV) brain amphetamine levels. The dose-response function is markedly shifted to the right in animals receiving ICV amphetamine The open circle denotes the effect of 5 mg/kg amphetamine in the cannulated animals. From Greenshaw and Burešová [38]

with the 200 g rats used in this study, 250 μg per rat corresponds to 1.25 mg/kg, i.e. to the range of effective systemic dosages (Fig. 1). CTA elicited by intracerebral application of amphetamine may possibly be due to the penetration of the drug across the blood-brain barrier into the circulation. The peripheral effect can be stronger in this case than it is with intraperitoneal application, since by-passing the liver may slow down amphetamine detoxication.

The above results indicate that, contrary to the prevailing view, centrally applied amphetamine is a relatively ineffective US in the CTA paradigm. However, a contribution of a central effect to the CTA inducing properties of amphetamine cannot be ruled out. It is possible that the central effects of amphetamine are a necessary but not sufficient condition for the efficacy of this drug in CTA learning and that a combination of central and peripheral effects is required with either route of drug administration.

Carbachol

Carbachol is another drug which has been claimed to elicit CTA in rats if administered intracerebrally. According to Myers and de Castro [62] application of carbachol into the medial septum elicited CTA by activation of the

central cholinergic system. Carbachol injection into the ventral hippocampus was ineffective, while intraventricular carbachol caused CTA mediated by a peripheral cholinergic effect, which could be blocked by methyl atropine. Unfortunately, the authors did not specify the amount of intracerebrally applied carbachol (2 or 3 "tamps") and did not determine the carbachol dosage eliciting CTA with systemic application. The relative efficacy of intracerebrally and systemically applied carbachol was examined, therefore, in a series of experiments performed in 160 male hooded rats. The animals were first adapted to drink their daily ration of water during a 15-min stay in the drinking box (days 1 and 2) and were exposed on day 3 to the novel taste (0.1% sodium saccharin) followed by intraperitoneal or intracerebral injection of carbachol. After receiving water on day 4, the rats were allowed to choose between saccharin or water on day 5. The drug was dissolved in physiological saline and applied in volumes which did not exceed 2 μl. The procedure took advantage of the fact that the gustatory trace — poisoning association proceeds under anaesthesia [16]. The animal was anaesthetized with pentobarbital (40 mg/kg) immediately after saccharin drinking, the skull was exposed and a small trephine opening was made above the intracerebral injection site. The rat was then fixed in the stereotaxic instrument, and the injection cannula (0.4 mm external diameter) was inserted into the brain (nucleus of the solitary tract, lateral hypothalamus, dorsal hippocampus, mesencephalic reticular formation). Intraperitoneal carbachol injections were applied to unanaesthe-

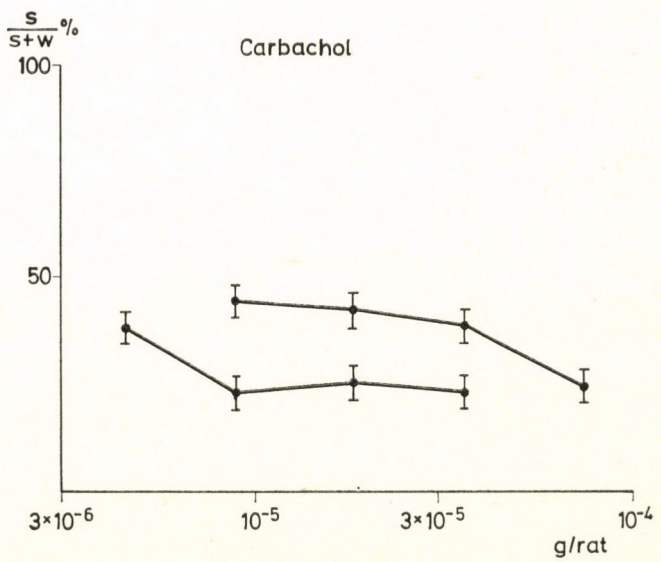

Fig. 2. CTA-inducing properties of intracerebrally (lower curve) and intraperitoneally (upper curve) applied carbachol. Ordinate: saccharin-water preference in the multiple bottle retention test on day 5. Abscissa: carbachol dosage per rat (average body weight 200 g)

tized animals, but the effect of anaesthesia was determined for the critical dosages.

The results are illustrated on Fig. 2 which shows that injection of 5 μg carbachol into the region of the solitary tract elicits a CTA of similar intensity as an intraperitoneal injection of 30 μg carbachol (i.e. 0.15 mg/kg to rats weighing 200 g on the average). Since the intracerebral threshold dosage is only slightly lower than the systemic one, the assumption seems to be justified that, with both routes of application, the critical site of the carbachol effect is peripheral. Consonant with this conclusion is the finding that the intracerebral carbachol effect was not substantially changed with different injection sites (lateral hypothalamus, hippocampus, midbrain reticular formation) or by dividing the dosage into equal parts injected into symmetrical brain areas. As in the case of amphetamine, failure of carbachol to elicit CTA with low central dosage does not exclude the possibility of a combination of central and peripheral effects in the CTA-inducing action of the drug. The interpretation of the intracerebral injection of carbachol is further complicated by the possibility that the drug does not elicit CTA directly but that it produces visceral reactions constituting an internal aversive state perceived as the US. Intracerebral carbachol disrupts homeostasis of body fluids [42, 79], elicits pulmonary oedema [71] and produces a state of internal malaise [64].

Harmaline

The conventional CTA eliciting stimuli act through visceral afferents or through the chemoreceptors of the area postrema, on the emetic center in the lateral reticular formation. The emetic center probably plays an important role in the neural circuits of CTA. CTA can also be elicited by activation of the emetic center through channels by-passing the above inputs, e.g., in the case of motion sickness induced by vestibular stimulation [14, 37, 40, 47]. Burešová et al. [19] succeeded in eliciting CTA by electrical stimulation of the vestibular nuclei or by their unilateral functional ablation with a local injection of KCl. Area postrema lesions may even facilitate acquisition of CTA induced by systemic injection of apomorphine [13] or by rotation [67]. On the other hand, the emetic center is not indispensable for CTA acquisition. The claim that antiemetics attenuate a previously acquired LiCl-induced CTA [23] was not confirmed by Rabin and Hunt [69] who failed to prevent acquisition of radiation, or LiCl-induced CTA by antiemetics. Similar observation was reported earlier by Levy et al. [50].

In spite of these negative results, the assumption seems to be justified that the CTA eliciting properties of centrally administered drugs can be most productively investigated with substances exerting a specific influence on the lower brainstem circuits, presumably activated by the US. We examined

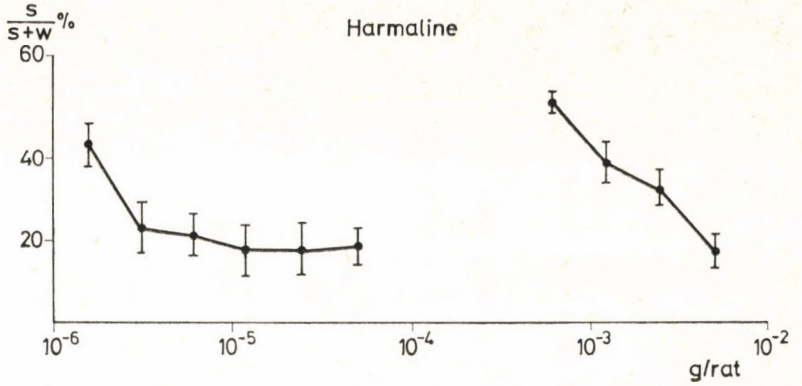

Fig. 3. CTA-inducing properties of intracerebrally (inferior olive) (left) and intraperitoneally (right) applied harmaline hydrochloride. Other legends as in Fig. 2

one of such drugs, harmaline, an indolaminergic compound and monoamine-oxidase inhibitor [80], which induces a generalized tremor in laboratory animals by activation of the olivo-cerebellar system [29, 49]. The harmaline tremor is due to suppression of a tonic inhibitory influence exerted on the inferior olive by serotoninergic fibres originating in the raphe nuclei [41, 80]. Since harmaline affects also the lateral reticular nucleus [6, 7], i.e. the centres presumably affected by other CTA-eliciting stimuli, examination of harmaline in the CTA paradigm seemed to be warranted [18].

The procedure described in the carbachol experiments was followed. Intraperitoneally injected harmaline elicited significant CTA at 5 mg/kg, and this effect was not diminished when the drug was applied to rats anaesthetized with pentobarbital. Intracerebral injection of harmaline into the inferior oliva of anaesthetized rats elicited CTA at the dosage of 3.1 μg. The results summarized in Fig. 3 indicate that the intracerebral injection was about 500 times more effective than the intraperitoneal one. This ratio is consonant with a central action of the drug, the intraperitoneal application of which does not elicit any overt signs of gastrointestinal distress. Assuming that the intracerebrally injected harmaline diffuses into a volume of about 100 μl of surrounding brain tissue, the effective concentration approaches the 35 μmol/l level attained at the start of harmaline tremor [76].

The central effect of harmaline was confirmed in another series of experiments comparing CTA after harmaline injection (6 μg or 50 μg) into the inferior olive, bulbar reticular formation, mesencephalic reticular formation, medial hypothalamus, cerebral cortex or cerebellum (Fig. 4). CTA was most pronounced with harmaline injection into the inferior olive. It was almost absent with neocortical, hypothalamic and mesencephalic injections. Intermediate results were obtained at the remaining sites. The spatial gradient of the intracerebrally applied harmaline points towards medulla as the site of

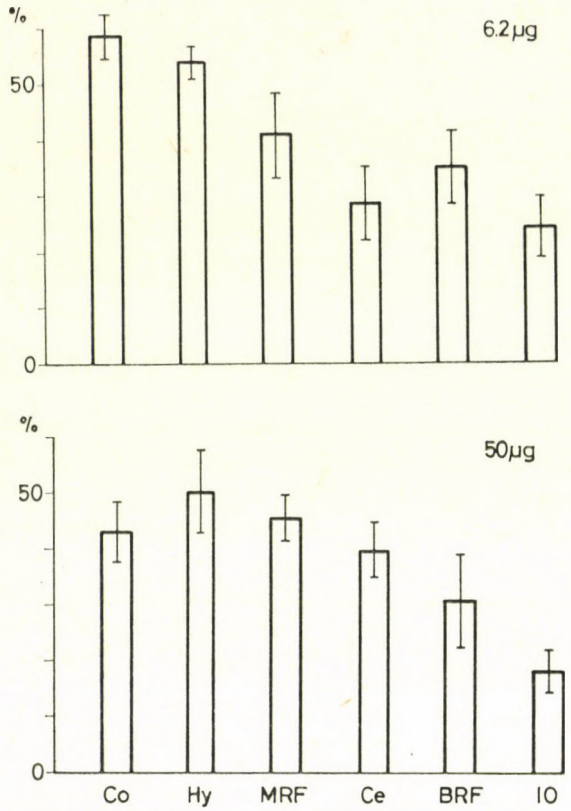

Fig. 4. Conditioned taste aversion elicited by intracerebral injections of 6.2 μg (above) and 50 μg (below) harmaline into cerebral cortex (Co), hypothalamus (Hy), mesencephalic reticular formation (MRF), cerebellum (Ce), bulbar reticular formation (BRF) and inferior olive (IO). Ordinate: percentage of saccharin from the total fluid intake during the retention test. From Burešová and Bureš [18]

the drug action but does not imply that activation of the olivocerebellar circuit is the critical event. Knowles and Phillips [48] showed that the harmaline rhythm in the EEG and neuronal activity proceeds during pentobarbital anaesthesia which completely blocks the tremor. More relevant than the tremor is probably the harmaline activation of the reticular formation and the effect of the drug on the serotonergic neurones contained in the raphe nuclei.

Monoamine oxidase inhibitors

Since harmaline acts as a reversible inhibitor of the monoamine oxidase A, responsible for the metabolic degradation of serotonin [47], it was necessary to examine the possibility that the harmaline elicited CTA is due to this prop-

erty of the drug. The next series of experiments tested the CTA eliciting potency of two monoamine oxidase inhibitors, pargyline and clorgyline, both with systemic and intracerebral administration.

Pargyline is an irreversible monoamineoxidase inhibitor affecting both A and B types of the enzyme [30]. When tested in the standard CTA paradigm, significant aversion was elicited by intraperitoneal injection of 20 mg/kg pargyline. A similar effect was obtained with pargyline applied to rats which had been anaesthetized immediately after saccharin drinking on day 3. Pargyline injection into the nucleus raphe magnus at the level of the inferior olive elicited CTA at the dosage of 2.5 μg administered in 1 μl saline. The results are summarized in Fig. 5 showing that pargyline is about 400 times more effective with intracerebral than with systemic administration.

The selective inhibitor of the A form of monoamine oxidase, clorgyline [20, 61], elicited CTA at still lower concentrations. The results illustrated by Fig. 6 indicate a similar ratio of intracerebrally (2.5 μg/rat) and intraperi-

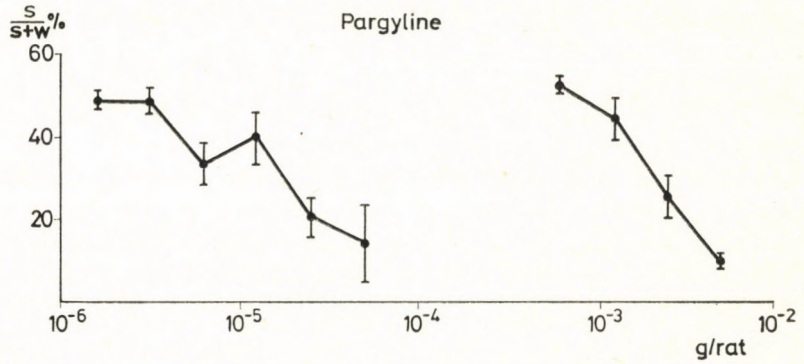

Fig. 5. CTA-inducing properties of intracerebrally (medullary raphe nuclei) or intraperitoneally applied pargyline hydrochloride. Other legends as in Figs 2 and 3

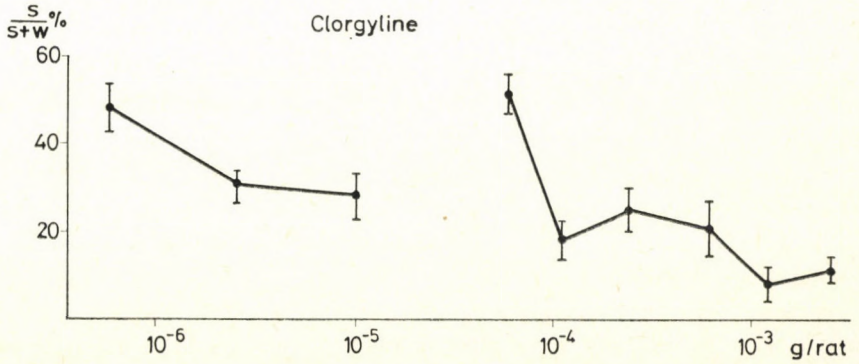

Fig. 6. CTA-inducing properties of intracerebrally (medullary raphe nuclei) or intraperitoneally applied clorgyline hydrochloride. Other legends as in Figs 2 and 3

toneally (2.5 mg/kg) applied effective drug dosages as in the case of pargyline and harmaline. The clearly central effect offers an excellent opportunity for determining the site of the drug action by injections into various brain regions. Experiments attempting to establish the spatial gradients of pargyline and clorgyline effects are now in progress. It should be pointed out that both monoamine oxidase inhibitors elicited CTA at systemic dosages considerably lower than those used in other behavioural studies. For example, Luine and Paden [53] reported that depression of progesterone-facilitated lordosis in ovariectomized female rats required 30 to 50 mg/kg pargyline, while 40 mg/kg clorgyline was ineffective.

Serotonin

Inhibition of the monoamine oxidase by pargyline or clorgyline considerably increases brain serotonin [53]. Since the monoamine oxidase inhibitors interfere with metabolic degradation of other catecholamines as well, the role of serotoninergic circuits in the CTA mechanism remains unclear. The available experimental evidence is rather controversial. Lorden and Margules [51] reported that electrolytic or 5,7-dihydroxytraptamine lesions of the dorsal and median raphe nuclei (providing serotoninergic innervation of most forebrain structures) facilitated CTA acquisition. Conversely, systemic administration of the serotonin precursor 5-hydroxytryptophan attenuated subsequent acquisition of the LiCl-induced CTA to saccharin [52]. The latter finding contrasts with the report by Zabik and Roache [85] that 5-hydroxytryptophan (100 mg/kg) induces moderate CTA to saccharin when administered immediately after the drinking session and that this effect is strongly potentiated when ethanol (12%) is used as the novel taste. Imipramine, a tricyclic antidepressant which increases serotonin availability by blocking its reuptake into presynaptic terminals [22], was reported to elicit mild CTA [8]. We were not able to confirm this when using 5 mg/kg imipramine in the standard CTA paradigm.

Further experiments are needed to resolve the above controversies. It is conceivable that a lesion of the midbrain raphe nuclei can activate the raphe nuclei in the lower medulla, which may play a more important role in CTA acquisition. Similarly, CTA attenuation in animals pretreated with 5-hydroxytryptophan may be an example of the US preexposure effect [31]. Indirectly related to CTA research is the evidence that increased serotonergic activity suppresses the mouse killing behaviour of rats. Onodera et al. [66] reported dose related inhibition of muricide by intraventricular injection of 10 to 50 μg serotonin and potentiation of this effect by tricyclic antidepressants and monoamine oxidase inhibitors. These results also await reinterpretation in the CTA context. In an attempt to directly test the role of serotonin in CTA

acquisition, 10, 20 or 40 μg serotonin creatinine sulfate were injected into the medullary raphe nuclei of rats who had been anaesthetized immediately after drinking saccharin on day 3 in the standard CTA procedure. The 40 μg dose elicited strong CTA while the other dosages were ineffective. The relatively high dosage required to induce CTA is not surprising since in the absence of pharmacologically reduced catabolism and intracellular transport the serotonin effect is rather short lasting. Further experiments are needed to verify the specificity of the effect by blockade with serotonin antagonists, duplication with serotonin agonists and facilitation with subthreshold dosages of monoamine oxidase inhibitors and tricyclic antidepressants.

Convulsants

The diversity of drugs employed as US in the CTA paradigm makes it difficult to find a common physiological system mediating their effects. Since both the agonist and the antagonist may be effective in eliciting CTA (e.g. eserine and methylscopolamine, apomorphine and chlorpromazine), CTA can not be due to simple activation or inhibition of the cholinergic or dopaminergic systems. Similar findings have led to the conclusion that any change of the internal state can serve as a US [31]. This is obviously not the case, however, since many lethal poisons do not elicit CTA, even when administered at dosages causing death of a percentage of the animals [44, 63]. These negative findings are not less important than the positive ones: they contribute to the knowledge of the CTA mechanism by specifying the metabolic and functional changes which fail to induce aversive labelling of the gustatory trace. Unfortunately, negative findings are rarely published so that the list of ineffective drugs is much less impressive than the list of CTA-inducing agents.

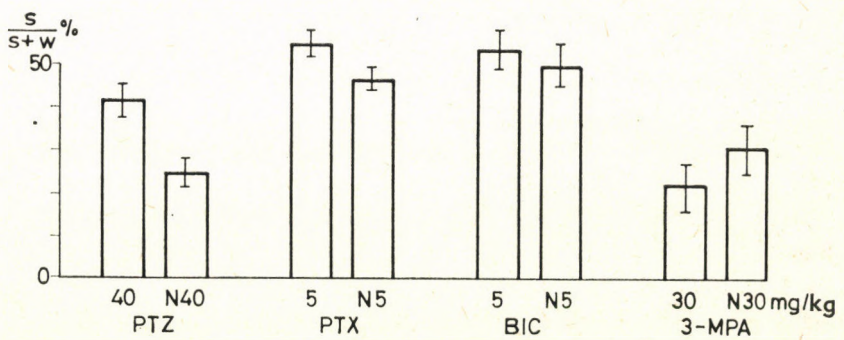

Fig. 7. CTA-inducing properties of pentylenetetrazol (PTZ), picrotoxin (PTX), bicuculline (BIC) and 3-mercaptopropionic acid (3-MPA) applied to intact rats (left columns) or to rats anaesthetized immediately after saccharin drinking with pentobarbital (N, 40 mg/kg). Ordinate: saccharin-water preference in the multiple bottle retention test on day 5. The numbers below the columns indicate the drug dosages in mg/kg

Drugs deserving particular attention in this respect are convulsants: they elicit strong activation of the central nervous system which is often accompanied by striking visceral symptoms, but the seizure may at the same time disrupt the later phases of the CTA acquisition process. The limited experimental evidence is rather ambiguous. Nachman and Hartley [63] found strychnine sulfate to be a relatively ineffective US and Ahlers and Best [1] and Millner and Palfai [59] reported similar negative results with metrazol. According to Golus and McGee [34] and Shaw and Webster [81], metrazol induces mild CTA when a two-bottle technique is used for aversion testing. In view of this scarce and controversial evidence, several well known convulsants were tested in the standard CTA procedure. In order to separate the effect of the convulsions and convulsion-related peripheral changes from the primary central effect of the drug, the same dosages were tested in unanaesthetized and anaesthetized (40 mg/kg pentobarbital) rats.

The results are illustrated in Fig. 7 which shows saccharin preference in a multiple choice test on day 5. Metrazol (40 mg/kg) elicited a weak but statistically significant CTA in unanaesthetized rats and the effect was considerably increased when injecting metrazol into anaesthetized animals. Picrotoxin and bicuculline elicited CTA neither in unanaesthetized nor in anaesthetized rats. 3-mercaptopropionic acid produced stronger CTA in unanaesthetized than in anaesthetized animals.

Since all drugs produced convulsions in about 50% of the animals, seizures cannot acount for the observed positive effects. In fact, most severe convulsions were seen after bicuculline which induced no signs of CTA, although the spasms and jerking movements were probably noxious enough to activate aversive motivation.

The possibility must be considered that the CTA failure is due to seizure induced disruption of the association and/or consolidation processes. This view is supported by the amnesic effects of metrazol administered in the interval between CS (saccharin) and US (LiCl) or shortly after US [1, 59, 81]. The difference between the strength of CTA induced by metrazol injection to unanaesthetized and anaesthetized rats may result from the reduction of the disruptive effect produced by pentobarbital blockade of the epileptic discharge. This mechanism is not universal, however, since pentobarbital influenced CTA elicited by 3-mercaptopropionic acid acts in an opposite way. It seems, therefore, that explanation of the differential effects of the tested drugs must be sought in more subtle differences in their mechanisms of action.

Picrotoxin and bicuculline act as specific GABA antagonists [27], picrotoxin at the level of GABA activated membrane Cl^- conductance [65] and bicuculline by inhibiting GABA binding to the receptor site [84], but both drugs have the capacity to block the GABA-mediated inhibition in

a number of invertebrate and vertebrate neurons. In this context it is understandable that CTA is not elicited by strychnine [63], which blocks the glycine and beta-alanine mediated inhibition [4]. Pentylenetetrazol acts as a weak GABA antagonist [65], but also produces changes in non-synaptic membrane conductance, which may contribute to the convulsant effect [83]. It is probable that this GABA-independent action accounts for the metrazol induced CTA. The 3-mercaptopropionic acid impairs GABA-mediated inhibition by blocking the synthesis of GABA [57], but it also enhances the release of the excitatory aspartic acid [28].

Evidence for the possible involvement of GABA in the central regulation of food intake is limited. Intracerebroventricular injection of the GABA analogue muscimol induces feeding in satiated rats [60], and a similar effect was observed after muscimol injections into the ventromedial hypothalamus [45] and the dorsal raphe nucleus [68]. On the other hand, intraperitoneally applied muscimol has an anorexic effect [26] which is still more pronounced with systemic application of THIP (4,5,6,7-tetrahydroisoxazolo(5,4-c)-pyridin-3-ol), a potent GABA agonist which penetrates the blood brain barrier [11]. The anorexic action of the latter drug can be antagonized by bicuculline but not by strychnine, pentylenetetrazol or picrotoxin [10]. Since THIP exerts marked analgesic and sedative effects, the anorexia can be a non-specific concomitant. The possibility of using muscimol or THIP as the US for producing CTA has yet to be tested.

Conclusion

In spite of the diversity of CTA evoking stimuli, the final result is fairly uniform: a recently formed gustatory trace receives an aversive label. It is highly probable that this effect of the US is implemented by common mechanisms which can be activated through a number of converging routes. Nucleus of the solitary tract, area postrema and emetic centre are links relatively close to, but not identical with, the point of convergence. The latter is probably not a definite centre but rather a pharmacologically identifiable circuit. Two experimental strategies seem to be particularly promising in this research: a) intracerebral administration of drugs which elicit CTA by central action may show a spatial gradient of effectiveness, pointing to the critical brain regions or circuits. b) Changes of brain metabolism elicited by the multitude of drugs, which do or do not serve as US in the CTA paradigm, form a multidimensional space in which each drug is represented by a single point. A hyperplane of this space, giving the best separation of the clusters corresponding to effective and ineffective stimuli, can disclose the relevant metabolic events. Both approaches are straight forward and should lead in the near future to a deeper understanding of the functional anatomy and neurochemistry of CTA.

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ENDOCRINOLOGICAL ASPECTS OF POTASSIUM METABOLISM

J. P. RADÓ

Department of Medicine, Emil Weil Hospital, Budapest, Hungary

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After reviewing the anatomy and physiology of potassium the endocrine regulation of the "external" and "internal" potassium balance is summarized. As the plasma potassium concentration has an extraordinary role in the maintenance of the activity of the living cell membrane, the review focuses on endocrine factors inducing hypokalaemia and hyperkalaemia.

Modern research has revealed many renal and extrarenal influences and we have learned a lot concerning the development of plasma potassium changes. The renal and extrarenal mechanisms governing potassium homeostasis are regulated by precisely coordinated endocrine mediators.

The most important hormones involved in the regulation of internal potassium balance are the catecholamines, insulin and aldosterone. Several drugs causing changes in the plasma potassium level exert their effect through the above mentioned hormones.

Keywords: potassium metabolism, endocrine regulations, hormones, hypokalaemia, hyperkalaemia

In the past decade a number of new concepts have emerged concerning the regulation of potassium metabolism. Our knowledge has been extended also through the use of the new therapeutic means influencing directly or indirectly the endocrine mediators of potassium turnover [4, 5, 7, 16, 20, 21, 27, 40, 41, 56, 60, 65]. From a clinical point of view both hypokalaemia and hyperkalaemia are extremely important, though it is not decided at present which one of the disorders occur more frequently and which one is the more hazardous. At any rate, hypokalaemia is the more extensively studied and, therefore, hyperkalaemia probably requires more attention.

Chemical anatomy and physiology of potassium

The amount of potassium in the 70 kg average man is 55 mmol per kg of body weight, altogether 3850 mmol in the body [27]. From this amount 98% can be found in the intracellular fluid compartment (at a concentration

Correspondence should be addressed to
János P. RADÓ
Department of Medicine,
Emil Weil Hospital,
Budapest, XIV. Uzsoki u. 29. 1426 Pf. 6, Hungary

of 150 mmol/l) and 2% in the extracellular fluid compartment (at a concentration of 4—5 mmol/l). An increase in the extracellular potassium concentration to 9—10 mmol/l could result in fatal cardiotoxicity. Plasma potassium levels would be increased to such an extent by the daily potassium ingestion of 70 mmol provided that it would be distributed exclusively in the extracellular space [64]. Under normal circumstances the maintenance of the large potassium gradient between the intracellular and extracellular space is ensured to 90% by the Na-K-ATP-ase pump of the cell membrane. The potential of the cellular membrane is determined chiefly by the potassium gradient [27, 64]. It is known that the membrane potential is indispensable for the vital activity of the cells and in its maintenance the logarithm of the ratio of internal to external potassium concentrations *(practically the plasma potassium level)* has a decisive role. Hypokalaemia and hyperkalaemia may originate from the disorders of the "external balance" (intake-output) and "internal balance" (distribution of potassium between the intracellular and extracellular space). Nevertheless, hypokalaemia is more frequently the disorder of the external balance and is mostly a chronic abnormality. On the other hand, hyperkalaemia is mostly (but certainly not exclusively) the result of a defect in the function of the internal balance and develops acutely. The regulation of the external balance [2, 3] is provided first of all by the kidney, but the intestines are participating as well. Potassium excretion in the distal and collecting tubules are enhanced by a series of factors [12]. The regulation of the internal balance is carried out by the above mentioned Na-K-ATP-ase as well as by certain hormones. It is of particular importance that from these hormones not only insulin [2, 3, 7, 9] but also catecholamines [56, 64, 65] are activited by feeding. These mechanisms enhance cellular potassium uptake for the prevention of cardiotoxicity and neurotoxicity [16] that would be induced by the ingested potassium load.

Catecholamines

Catecholamines are one of the most important regulators of the distribution of potassium between the intracellular and extracellular fluid compartments [2—5, 10, 21, 27, 56, 61, 63—65]. In healthy subjects the beta-adrenergic epinephrine disposes the potassium surplus during acute potassium loading from the extracellular space [4, 56]. Beta-adrenergic antagonists are acting in the opposite way [63]. The plasma potassium decreasing action of the non selective beta-agonist isoprenaline is strongly dose-dependent, and this effect is abolished by the beta-1-2-adrenergic antagonist propranolol but unaffected by the beta-1-antagonist atenolol. On the basis of the afore mentioned arguments, it is highly probable that the beta-2-receptor is responsible

for the plasma potassium decreasing effect. This was further supported by the plasma potassium decrease induced by the beta-2-agonist salbutamol characterized also by a strong dose dependency [21]. On the other hand the beta-1-agonist prenarterol was without effect while the alpha agonist phenylephrine had an opposite effect: it potentiated the increase of plasma potassium induced by potassium loading [63]. This action could be neutralized by the concurrent administration of the alpha antagonist phentolamine [65]. It should be stressed that beta-antagonists(propranolol) enhance the exercise-induced plasma potassium increase even to a life-threatening level [5, 21]. To summarize, we can say that the adrenergic compounds are important modulators of extrarenal potassium homeostasis. Beta-2-agonists and alpha-antagonists cause hypokalaemia, while hyperkalaemia develops in response to beta-receptor blocking and alpha agonist agents.

Insulin

Hyperkalaemia is well known in diabetic ketoacidosis and high plasma potassium is not rare in insulin deficient conditions even without any disturbance of the acid-base equilibrium [1, 9—11, 13, 14, 16, 24, 28, 34—36]. De Fronzo and his co-workers have demonstrated that the normal basal insulin — despite its relatively low level — is of critical importance in the normal regulation of plasma potassium level [7]. They infused intravenously very small quantities of potassium, elevating serum level only by 0.5 mmol/l. When basal insulin levels were reduced by somatostatin infusion, serum potassium concentration was doubled by the same rat of potassium chloride infusion. However, restoring normal basal insulin concentration resulted in a prompt return of serum potassium to the previous level [7]. In view of this study it is obvious that insulin has an eminent role in the protection against hyperkalaemia [2, 3, 6—10, 13, 14, 24, 33—36].

In hyperkalaemia insulin enhances the entry potassium into the cells. In contrast to previous beliefs potassium enters into the cell without glucose [61]. Nevertheless, it is necessary to infuse glucose intravenously together with insulin white treating hyperkalaemia to prevent of hypoglycemia. Of course, isotonic glucose solution should be used for this purpose because hypertonic glucose may produce hyperkalaemia by itself (see later). In the context of the relationship between plasma potassium and insulin, it should be mentioned that sustained hypokalaemia may lead to deficient insulin production while hyperkalaemia is a stimulus of insulin secretion (if plasma potassium concentration is elevated to the value of 8—9 mmol/l) [6, 10].

Aldosterone

This hormone is perhaps the most important in the regulation of potassium metabolism [1—3, 6—8, 10—20, 23, 25—37, 44, 48, 51, 57, 60—62]. It influences "external potassium balance" through its effect on the kidney and intestine, furthermore it is important also in the regulation of "internal potassium balance" [12, 15, 16, 57]. Aldosterone stimulates the sodium-potassium pump in the distal tubules (in the peritubular membrane) and increases the permeability of the luminar membrane, elevating the blood-urine potassium gradient from 10 : 1 to 50 : 1 [61]. Aldosterone enhances potassium secretion especially in the presence of high "distal volume flow" and sodium supply as well as non-reabsorbable anions associated with sodium [12]. Aldosterone influences also internal potassium balance. It is well known that acute potassium loading is several times more toxic in adrenalectomized animals than in the intact controls and not only because of the difference in potassium excretion [17]. Adrenalectomized animals maintained on glucocorticoids handle the acute potassium load almost normally (some defect in handling is due to the lack of catecholamines owing to the absence of the adrenal medulla [61]).

Endocrine response to the intravenous infusion of hypertonic solutions

Seldin and Tarail demonstrated several years ago [58], confirmed by Mokoff and his co-workers [22], that intravenous infusion of hypertonic sodium chloride and mannitol induces a transitory increase in plasma potassium concentration in healthy animals. Primum movens of this phenomenon is hyperosmolality in the extracellular fluid compartment deriving water from the intracellular space, thereby increasing the potassium concentration within the cells by favouring the outward diffusion of potassium. Nevertheless there are hormones (insulin and aldosterone) in the healthy body which counteract (or at least mitigate) the above process.

If hypertonicity is elicited by glucose, the consecutive increase in insulin levels reverses the above events resulting in decreased plasma potassium. In the presence of combined insulin-aldosterone deficiency glucose may induce so-called paradoxical hyperkalaemia [13, 14]. A similar tendency can be demonstrated when there is only a selective lack of insulin or aldosterone. The concept of lack of aldosterone is, however relative since paradoxical hyperkalaemia can occur in patients with normal aldosterone secretion when the aldosterone requirement of the body is increased [1, 15, 57]. This occurs in some cases of renal insufficiency [57] and diabetes [15]. In "normaldosteronaemic" diabetic and renal patients [35, 37] the paradoxical hyperkalaemia induced by hypertonic glucose or saline [30], could be prevented by pharmacologic doses of a mineralocorticoid.

In certain renal patients with "physiologic suppression" [23, 26] of aldosterone production (due to sodium and water retention) glucose-induced hyperkalaemia could be overcome by aldosterone production stimulated by low salt intake [31—36, 47, 48, 51]. Hypoaldosteronaemia associated with thyreotoxicosis in a diabetic patient could be prevented either by low sodium intake or by adequate antithyroid treatment, preventing by both procedures the paradoxical hyperkalaemic effect of glucose [34]. In hypoaldosteronaemic patients with chronic renal failure and diabetes, a paradoxically increased response in growth hormone after glucose promoted the normal serum potassium decrease [38, 39].

It has been recognized long ago that certain compounds (spironolactone, triamterone, amiloride) antagonizing the effects of aldosterone may cause dangerous hyperkalaemia without and with glucose loading [16]. A similar response may be observed after the administration of the converting enzyme inhibitor captopril which induces "pharmacological selective hypoaldosteronism" [20, 40, 42, 54, 55, 60]. We have seen an analogous effect in response to the beta-blocking agent metoprolol [41, 43, 54]. Upright posture enhanced the glucose-induced hyperkalaemia after the latter compounds. The serum potassium elevating effect of the upright posture [44—47, 59], as well as its mitigating effect on the glucose-induced decrease in serum potassium in healthy subjects have been repeatedly confirmed [53]. In diabetic patients the upright posture favours the development of glucose induced hyperkalaemia [37].

Drugs influencing potassium metabolism through endocrine mediators

Diuretic drugs are responsible for hypokalaemia through enhancing the activity of the renin-angiotensin-aldosterone system. Recently, several beta-2-stimulants (Salbutamol) have been blamed for the development of hypokalaemia [21]. A number of "endocrine" drugs are causing hyperkalaemia [27]. We have already mentioned the alpha-receptor stimulants, potassium sparing compounds and glucagon. Most drugs inducing hyperkalaemia through endocrine mediators suppress the activity of the renin-angiotensin-aldosterone system: beta-blocking agents [5, 41—43, 54], converting enzyme inhibitors [20, 40, 42, 50, 54, 55, 60] non-steroidal anti-inflammatory-antiprostaglandin compounds as well as heparin, cyclosporine and several other drugs [27, 64].

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AMYGDALA, DEPRESSION AND DRUG TREATMENT*

Elzbieta FONBERG

Department of Neurophysiology, Nencki Institute of Experimental Biology, Poland

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The problem of the mechanisms of depression and action of the depressive drugs needs theoretical analysis and multichannel investigations. Animal models of depression are important for such investigations. Various neurochemical behavioral and psychological models were analysed in the present paper. Amygdalar model of depression seems to be one of the most important models. The effects of the treatment by amphetamine, imipramine and chlorpromazine on the amygdalar depressive syndrome in dogs were described. The individual differences were stressed, both in respect to forms of particular depression and to the sensitivity to drug treatment.

Keywords: depression, antidepressive drugs, amygdala.

The problem of the mechanisms of depressive states is very important for the theory of motivation. Depression is characterized by the lack of motivation to perform any behavioral act, by apathy, inactivity and withdrawal. Although, the depressive states are very often observed in humans in their grave or milder form, and they seem even to be a sign of our present civilization, the mechanisms underlying depression are not fully elucidated. Below will be described and analysed theories of various authors, concerning mechanisms of depression. Also our own attempts to elucidate this problem will be reviewed.

Theories and models of depression

There are many theories and models of depression (see McKinney and Bunney [35], Harlow et al. [23] and others). One of the more recent is Seligman's theory of helplessness (produced by unpredictability of rewards) as the main cause of depression [44]. This theory has become very popular lately. According to my mind the most attractive is however Stein's [45, 46, 47] theory of ahe-

Correspondence should be addressed to
Elzbieta FONBERG
Department of Neurophysiology, Nencki Institute of
Experimental Biology
02—093 Warsaw, Pasteur Street 3, Poland

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donia. It was formulated on behavioral, neuroanatomical and neurochemical bases. Stein attributed the depressive states to the impairment of the positive reward circuit within the brain. Such impairment causes the prevalence of the negative punishing system. As in the state of ahedonia all stimuli from the outside and inner world become aversive and reinforcements lost their rewarding properties, therefore all actions are somehow punished. Such state in consequence leads to inactivity, apathy, disgust, sadness. Stein assumed that schizophrenia is also produced by the impairment of the rewarding brain mechanisms. Somehow similar hypothesis was presented by Gray [22], who put stress on the prevalence of the inhibitory and punishment systems in schizophrenics. According to Stein [48] noradrenergic mechanisms are essential for the reward system. Stein and Wise [49] have shown that stimulation by the electrodes implanted into the reward system produced release of norepinephrine in the brain.

Stein assumed that in depressive states adrenergic system is impaired. The role of catecholamine system in reward mechanisms was supported by data of other authors, who beginning from Olds [38, 39, 40] have shown that both systemic and intracranial noradrenaline (or amphetamine) injections increase the rate of selfstimulation. More recently numerous authors demonstrated that rats would press the bar to self inject the amphetamine or noradrenaline into the reward system. For example Cytawa and coworkers [2, 3] have shown that rats would self inject the adrenaline into the hypothalamus and even may learn T-meze to obtain noradrenaline injection. On the other hand Hoebel et al [26] have demonstrated that rats would press the lever to selfadminister d-amphetamine into the nucleus accumbens. The nucleus accumbens is an important station for the dopaminergic system. These results gave the incentive to create neurochemical models of depression. Beside noradrenaline depletion changes in the level of other monoamines were postulated to be essential in depression (see Himvich and Alpers [25], Sabelli and Giardina [42]).

Many investigators used the reserpine model of depression. Both reserpine and also the widely used tetrabenzedine produce a decrease in the level of noradrenaline, dopamine and serotonin, by preventing their storage at the synaptic cleft. It was shown that these drugs may also induce depression in humans. In monkeys and rats reserpine produces a state of apathy, neurotic like responses (huddling) social withdrawal up to some kind of lethargic state [36, 37]. These symptoms were considered as similar to human depression [1]. Reserpine produces however various side-effects like parkinsonian type motor disturbances, which may in turn produce changes in motivation. According to many authors (see Ellison 4) serotonin depletion may be essential in depressive states. Ellison's results indicated that disturbed serotonin — noradrenaline balance led to depressive symptoms in animals.

Another neurochemical model stimulated by the pioneer work of Ungerstedt [50] on catecholamine mapping and then widely used by many ivestigators was the 6-hydroxydopamine model. Initially it was assumed that 6-hydroxydopamine selectively impairs noradrenergic system if it is injected into the locus coeruleus and ventral tegmentum sites from where NE neurons originate. However, later on it was shown that either the global destruction of both dopaminergic and adrenergic systems or the selective damage of the dopaminergic pathways are done. Inevitably 6-hydroxydopamine injection would produce degeneration of DA neurons. As such procedure produces a state similar to depression it is possible that not the adrenergic, but the dopaminergic system is essential in depressive states. Such hypothesis was proposed by Wise [51]. He assumed that neuroleptics which blocked dopamine receptors [43] reduced or abolished the rewarding properties of stimuli and therefore would produce the decrease in motivation and learning and as follows, were the cause of depression. This however seems controversive to the clinical experience that chlorpromazine, which blocks dopaminergic receptors, applied for treatment of schizophrenic patients produces improvement, also concerning depressive symptoms (see discussion after Wise paper [51]). It is also suggested that noradrenergic mechanisms are involved in hedonic i. e. reward mechanisms and dopaminergic in motivational or reinforcement mechanisms [24].

Amygdalar model of depression

Another approach to the problem of depression is to produce depressive state by impairing brain structures involved in rewarding circuit and important for positive hedonic state. As it was shown by our previous work damage of dorsomedial part of amygdaloid complex (DMA) produced a depressive syndrome [7, 12] which may serve as a good model for the investigation of the mechanisms of depressive states and to study the effect of antidepressive drugs. Amygdalar dogs were, as described previously [6, 7, 12], apathetic, atonic, sad, uninterested in the environmental stimuli, as well as they were indifferent toward humans, with whom they had been friendly before the operation.

DMA dogs were also aphagic or hypophagic and showed reduced motivation to perform natural spontaneous acts (like jumping, running, rolling on the grass, playing with the experimenter and other dogs etc). Impairment of learned tasks observed in these dogs may be also produced by low level of motivation and by decreased hedonic values of reinforcement [6, 7, 8, 15]. Our amygdalar model of depression seems to support Stein's hypothesis of ahedonia [9, 15]. Beside the above described symptoms of sadness and apathy

the dogs also seem to stop to enjoy feeding, which before the operation evoked positive arousal. They not only were aphagic or hypophagic, they actively opposed feeding, as if the food had become aversive. When food was introduced into the mouth, they spat it out, vomited and showed other signs of disgust. Later on when food intake was at least partly restored, they were finicky and consumed only the preferred kind of food. It seems that because of impairment of the positive hedonic system amygdalar dogs need higher value of reward for sufficient reinforcement. This assumption is also supported by the fact that introduction of more tasty food in amygdalar dogs not only increased food intake, but also produced improvement of instrumental alimentary performance [32]. Sensory-social reinforcement (petting by experimenter) also lost its previously rewarding value in amygdalar depressed dogs. Time of lying without petting was elongated, and time of lying for petting shortened [17], indicating that social-sensory stimuli were not so pleasant as before operation.

Imipramine treatment of amygdalar depression

Considering that amygdalar model of depression is produced by similar mechanisms as human depressive states, it seemed logical to try the effect of antidepressive drugs used in human clinic. Imipramine (tricyclic antidepressant) has been administered chronically for 3 weeks and it was injected intramuscularly in doses of 2 mg/kg during the first and third week and of 4 mg/kg during the second week. Imipramine treatment produced only slight increase of the instrumental performance in dogs depressed as the result of DMA lesions. The dogs became in general more mobile during treatment. As the number of the intertrial responses evidently increased, a parallel increase of instrumental reactions to conditioned stimuli may be accidental. We did not obtain therefore the direct proof that imipramine restored the motivational and rewarding mechanisms. Strong individual differences were found concerning the effect of drug and in respect to different tasks. The dogs were less apathetic, but their unfriendly attitude towards humans, observed after operation, has not changed. Some dogs displayed aggressive signs, like growling and showing teeth [10, 11, 12, 13, 16, 17, 30, 32, 33, 34].

Responses based on social-sensory reinforcement were slightly improved by imipramine, but mostly those which need physical effort like running and jumping [11]. It is very interesting that the responses such as "give paw" and "sit", which were based on social attachement were the least improved by imipramine treatment [11, 12, 34]. Individual differences among dogs suggested that differences in the extend of damage may differently affect the neurochemical balance. Dorsomedial amygdala is involved in noradrenergic, dopaminergic and serotoninergic transmission, and lateral amygdala mostly in

cholinergic mechanisms. Imipramine acts on one hand anticholinergic and on the other hand potentiating monoaminergic mechanisms, by increasing production or reducing reuptake at the synaptic cleft. The partial or total impairment of different monoaminergic or cholinergic amygdalar systems may destroy in different extent neuronal substrates of action of particular transmitters, mediated by imipramine and thus it may produce different mosaic of disturbances in neurochemical balance.

The effect of pharmacological treatment on normal dogs

The next approach to the mechanisms of depression is to study the effect of drugs, hypothetically involved in depression, on motivated behavior of normal subjects. In our experiments we applied such classical antidepressive drug as imipramine, which has as mentioned above anticholinergic, adrenergic, serotoninergic as well as dopaminergic effect. We also used chlorpromazine — to verify Wise's [51] hypothesis that neuroleptics, because of blocking the dopaminergic system, (which according to this author is essential in reward mechanisms) produce ahedonia. The next drug we applied was amphetamine — an excitant, widely used by students as considered to improve the mental processes and at the same time possibly essential in reward mechanisms [47, 40, 3]. These drugs are also often applied in the human clinic as antidepressant (imipramine), stimulant (amphetamine) and neuroleptic (chlorpromazine).

According to the adrenergic theory of depression amphetamine should improve hedonic and motivational properties of reward, and chlorpromazine — should produce decrease of hedonic or motivational values. Imipramine as antidepressant should improve mood and prevent depression. We tested the effects of the above substances on responses reinforced by various rewards with different hedonic values. The procedures of differentiation between avoidance of shock and food i. e. between negative and positive motivations [5] and also between food and sensory-social reinforcement (petting by the experimenter) i. e. two positive motivations [14, 21] elaborated in our laboratory open the possibility to compare complex effects of particular drugs. (For detailed description see Fonberg [5, 6], Fonberg et al [19], Kostarczyk and Fonberg [33, 31], Kostarczyk, Fonberg and Prechtl [34], Fonberg, Kostarczyk and Kołakowska [20].

Avoidance—alimentary differentiation. Amphetamine injected intramuscularly (in doses of 0.5—2.0 mg/kg in different series of experiments) produced the decrease of both alimentary and avoidance responses [27, 20]. Whenever the decrease of alimentary performance might be produced by the anorexic action of amphetamine, the deterioration of avoidance cannot be explained in such way. Although strong individual differences were found,

in all dogs amphetamine treatment deteriorated conditioned performance. The amphetamine however preserved its excitatory effect concerning the general behavior. The dogs were more mobile, running around, sniffing, exploring and seeking for social contact. In the reflex-chamber, however, they remained standing quietly.

The heart-rate decreased in all dogs in comparison to the pretreatment level. In particular, the increase of heart rate following conditioned stimuli was attenuated, which may indicate the lowered level of motivation for both food and avoidance of shock. As the effect was similar (although not identical) for positively and negatively reinforced responses it would be difficult to say that amphetamine affects stronger either the negative or the positive system. Heart rate deceleration might be produced by a sort of relaxation. According to some authors amphetamine produces the state of "well-being". The relaxation produced by this state may result in heart rate deceleration and also it may interfere with motivated behavior and in particular affect avoidance reactions. However, we noticed that during amphetamine treatment the number of errors increased and that they almost exclusively concerned the avoidance (incorrect) responses to alimentary stimuli. This may indicate an increase of the fear level i. e. activation of punishment system, opposite to reward system. This is also controversial to the relaxation hypothesis.

Imipramine injected chronically as described above, produced either no effect or slight deterioration of performance [28, 29]. The comparison of the effect of imipramine and amphetamine on the avoidance-alimentary differentiation of the same dogs has shown that deteriorating effect of imipramine is less pronounced than the effect of amphetamine. The effect of imipramine concerns only the prolonged latency of response. Errors were almost not observed during imipramine treatment. On the other hand also the excitatory effect of imipramine treatment outside the reflex chamber was less obvious than in case of amphetamine.

Chlorpromazine applied intramuscularly for five days in doses of 1 mg/kg in general produced also deterioration in the response of normal, well balanced dogs. However, in a neurotic dog which was all the time fearful and excited to such extent that he was not able to perform proper avoidance responses, simultaneously with general appeasement, the avoidance performance improved. In this dog the previous treatments by amphetamine and imipramine had been without beneficial effect.

Alimentary — social differentiation. The general behavior of the dogs gave the impression that they became more social under amphetamine treatment. Therefore, it was interesting to investigate how such treatment would affect socially reinforced responses. Also it was interesting to verify whether the various drugs (the same as used in avoidance — alimentary series) would in a similar way affect two positively motivated responses. In this series so-

cial—alimentary differentiation was performed. Two acoustic stimuli (similar as in avoidance — alimentary procedure) were applied in a random order. To one acoustic stimulus the dog had to put one forepaw on the bar for food reinforcement, to another acoustic stimulus the other forepaw in order to obtain social — sensory reinforcement (petting by the experimenter). Opposite to our assumption amphetamine produced deterioration of both responses, and as in the previous series the decrease of heart rate was observed [14]. Such results do not support the hypothesis that amphetamine enhances positive reward system. Anorexic effect of amphetamine may only interfere with food reward, but not with sensory — social reward.

Chlorpromazine also produced obvious deterioration of both social and alimentary responses in all dogs [21]. As in previous series strong individual differences in the susceptibility to drug treatment were observed. Performance of most of the dogs after the chlorpromazine treatment reached the pretreatment level or even was ameliorated, but for example in one dog the strong deterioration of performance overlasted the treatment and the pretreatment level was restituted only later on, after 2 months of rest.

Conclusion and discussion

In spite of various the oretical analyses of the mechanisms of depression and numerous experimental data we are still far from the full elucidation of this problem. Our experiments put however some light on the problem concerning the effects of antidepressive drugs. First, strong individual differences were observed in the reactivity of the dogs to the particular drug, and concerning all three investigated motivations. Our results indicate that the changes in instrumental performance are not due to the general excitatory or sedative effect of the particular drug's action. The performance of most normal dogs under the effect of all the three drugs investigated (acting through different neurochemical mechanisms) was deteriorated. Contrary to our assumption, the amphetamine, which was supposed to have excitatory effect and to improve the mental processes and, what follows, to improve the performance of learned tasks and to activate the reward system, produced lower level of the performance, and increase in the number of errors. The deterioration of instrumental performance concerned three different motivations: fear, food and social reinforcement, although the dogs were in general more excited and showed increased motility. Therefore such deterioration would be difficult to explain by the hypothesis of relaxation produced by positive hedonic state of well-being. These results seem to be controversial also compared to the results of Predy and Kokkinidis [41] and to the data of Olds [39], who found an increase of the selfstimulation rate after amphetamine injection. Similar effect

i. e. increase of selfstimulation under amphetamine was observed by myself while working on rats in Neal Miller's laboratory (unpublished). The mer chanisms of instrumental performance to CS is more complicated than bat pressing (which may be activated by general motility). In addition in mos series we used differentiation of two responses motivated by different motivaf tions. In such procedures the learned tasks were difficult not only from the point of view of discrimination between different acoustic stimuli, but because of the shift in motivations. It must be stressed that the method of differentiation of two responses based on two motivations creates more emotional conflicts than the usually applied stimulus-discrimination. On the other hand the deterioration of performance could not be assessed with the disturbances in discriminatory or differentiation processes as also the simpler tasks with the use of a single response to one acoustic signal were deteriorated. The deterioration of performance could not be due to increased mobility during amphetamine treatment, as in the reflex chamber the normal dogs remained quiet. Therefore it may be concluded that amphetamine exerts a deteriorating effect on the motivational or rewarding processes.

Imipramine, which was assumed to produce rather improvement of the mood than increase of arousal, in amygdalar dogs produced augmented motility and a rather slight improvement of instrumental motor performance, while it left emotional signs of depression unchanged. In normal dogs performance was slightly deteriorated. The fact that imipramine produced a different effect on normal and lesioned animals as well as the observed marked individual differences in sensitivity to treatment seem to prove that the effect of imipramine depends on the initial neurochemical and behavioral pretreatment state. The above results on the effect of amphetamine and imipramine on dogs seem to provide proof against adrenergic as well as dopaminergic hypotheses of reward.

However, the effect of treatment depends also on the task and on the animal species. For example, in cats imipramine produced stimulatory effect on the predatory behavior of normal non-killers or on cats which lost predatory abilities as the result of damage of either lateral hypothalamus or medial amygdala. Moreover, previous submissive cats (both normal and with amygdala damage) under imipramine effect became dominant [13, 52, 54]. If we consider submissive state as sort of depression then, reduction of the depressive state could produce increase of the general activation as well as increase of the hedonic values of the predatory act. Also it could elevate the confidence in efficacy to conquer the partner.

Chlorpromazine produced a general sedative effect on dogs. At the same time it resulted in obvious deterioration in the instrumental performance. These data may be considered in favor of Wise's hypothesis [51]. In some cases however, chlorpromazine improved the performance. For example, in the

neurotic dog chlorpromazine reduced the generalized fear reactions and therefore helped to perform the proper avoidance response. Such effect could not be produced by ahedonia caused by chlorpromazine's blocking of dopaminergic reward system.

The reaction of normal subjects i. e. with well balanced neurochemical homeostasis to drugs (which obviously disturb this homeostasis) might be quite different from the effect of the same drugs on subjects who are neurotic, depressed or with homeostasis disturbed by different causes. For example, imipramine produces deterioration of responses in normal dogs, but improvement in amygdalar or neurotic, depressed animals [10, 11, 29, 30, 34, 52, 53]. Imipramine may produce restoration of amine reuptake or amine production i. e. proper concentration only when it is disturbed, but in normal subjects, on the opposite it may disturb the balance and in consequence produce behavioral deterioration. Similarly, the effect of amphetamine on normal subject may lead to neurochemical disturbances, which in turn deteriorate performance. According to such a point of view, experiments on the effect of neuroleptics performed on normal rats, which were the basis of Wise's hypothesis are not convincing. Neuroleptics which in Wise's experiment did not produce any overt motor, parkinsonian-like changes, might however disturb the neurochemical balance globally not limited to antidopaminergic action. It seems that only the comparison of the effect of drugs on normal and depressive subjects may be useful to verify various hypotheses concerning the neurochemical background of motivation, depression and ahedonia. Our results suggest that the effect of drug depends on a complicated neurochemical balance, which may vary in different pathological states as well as in individual normal subjects. Without such an assumption it would be difficult to understand why amphetamine, which potentiates dopamine action and chlorpromazine, which blocks dopamine receptors, have similar deteriorating effect on instrumental performance with different positive and negative motivations. Stress should belayed once more on the strong individual differences, concerning the reactivity to a particular drug in normal dogs. The same dose may produce in different dogs greater or smaller effect. The results may depend on the individual neurochemical differences, but also on genetic or acquired characteristic of the higher nervous activity and motivational level of the particular animal as well as on the origin of the emotional disturbances.

In normal dogs the effect of all the drugs investigated were mostly deteriorating, but in a different extent. In neurotic, depressed or lesioned dogs imipramine or chlorpromazine may improve performance.

Our results proved once more that drugs like neuroleptics, sedatives, antidepressants and stimulants should be applied with great precaution and precision. Each case should be tested individually and these drugs should never be applied in normal human subjects. Also in depressed and psychotic

patients the efficiency of the particular doses should be carefully determined and not in a way as it is in common use in many hospitals: to apply "three pills every day". Such stereotyped routine may produce deterioration, instead of improvement as it was demonstrated by experiments in animals. On the other hand the results obtained in animals should be taken with great precaution because of the above mentioned species differences. In the tests made by pharmacological industrial laboratories on their products for treatment of neurotic and emotional disturbances not only neurochemical and metabolic features of the animals tested should be taken into account, but also their behavioral and emotional nature.

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EFFECT OF GONADOTROPIN (FSH + LH) AND THYROTROPIN (TSH) ADMINISTERED WITH OR WITHOUT ENDOTOXIN AT THE AGE OF THREE WEEKS ON THE RESPONSE CAPACITY OF THE THYROID GLAND IN ADULT RATS

G. Csaba, Susanna U. Nagy

Department of Biology, Semmelweis University Medical School, Budapest, Hungary

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At the age of three weeks the experimental animals received either thyrotropin (TSH), or gonadotropin (FSH + LH), or endotoxin (LPS) alone or in combination. The effectivity of the treatments was evaluated at the age of two months (with or without further hormone treatment). Contrastingly to neonatal TSH treatment, TSH treatment at the age of three weeks did not give rise to imprinting. In female animals, however, TSH treatment increased the sensitivity to the related gonadotropin hormone. At the age of three weeks gonadotropin treatment — on its own — did not cause damages to the TSH receptors of the thyroid gland. While in previous experiments neonatal endotoxin treatment damaged considerably the thyroxin production of the adult thyroid gland, after treatments at the age of three weeks no similar effect could be observed. The treatment, however, decreased the sensitivity of the receptors to TSH. In female animals simultaneous administration of endotoxin and TSH led, even without further hormone treatment, to constant increase in T_4 level (the increase could also be detected in the adult animal). Imprinting, however, did not develop. In male animals simultaneous administration of endotoxin and gonadotrop hormone decreased considerably the T_4 baseline level, and further TSH or gonadotropin treatment was unable to enhance T_4 production.

Keywords: Hormonal imprinting, gonadotropin, thyrotropin, endotoxin.

The glycoproteid hormones of the pituitary gland belong to the same family. The alpha subunits of follicle stimulating hormone (FSH), luteinizing hormone (LH) and thyrotropin (TSH) are identical and it is the beta subunit which is responsible for the specificity of the hormone. Nevertheless, numerous overlapping amino acid sequences can be found in the beta subunit, too [9, 12]. The above-mentioned hormones are able to bind to some extent to the receptors of each other even in the mature animal. This binding, however, does not evoke functional response. In the newborn animal the hormones bind to similar extent to the receptors of each other and also functionally overlap, i. e. the hormones which have different effect in the adult life are able to evoke

Correspondence should be addressed to
György Csaba
Department of Biology, Semmelweis University Medical School
1445 Budapest, POB 370, Nagyvárad tér 4., Hungary

the same effect in the neonatal period [5]. This characteristic manifests itself also in hormonal imprinting [6].

The first, usually perinatal contact of the target cell and the hormone gives rise to hormonal imprinting and results in the completion of the maturation of the receptor, i. e. both the binding capacity of the receptor and the response capacity of the target cell reach the values characteristic of adulthood [2, 3]. The binding of not the adequate hormone but a structurally different molecule which is able to bind to the receptor gives rise to faulty imprinting which can manifest itself either in increased or, more frequently, decreased response capacity of the cell in adulthood [4]. The effect depends considerably on the quality of the target cell, too. For instance, in the gonads neonatal TSH treatment is as effective in evoking imprinting as FSH administration; more over, as an effect of TSH imprinting the response capacity will be increased in adulthood [6]. On the other hand, if the rat receives gonadotropin treatment in the neonatal period, in adulthood the response capacity of the thyroid to both endogenous and exogenous TSH will be decreased, the thyroxin level as well as the response capacity to TSH will be lower [7]. As an effect of neonatal treatment, however, neither hormone is able to adapt the receptor for itself, the response of the receptor is not more bound to that hormone with which the neonatal treatment has been carried out. An exception is the situation when the imprinting is going on during the perturbation or restitution of the membrane [8].

The lypopolysaccharide endotoxin (LPS) extracted from E. coli [14] damages the plasma membrane [11]. If newborn rats are treated with LPS, in adulthood the receptors of the thyroid gland of the animals will not be able to differentiate TSH from the gonadotropin [10]. If 24 hours after neonatal endotoxin treatment either TSH or gonadotropin is administered the adult thyroid gland will exhibit stronger response to that hormone with which the neonatal treatment after the endotoxin administration has been carried out [8]. This observation indicates that no matter how stable the receptor seems to be, it can be altered. The present investigations were aimed at studying the extent how far combined endotoxin and hormone treatment, administered tollowing the neonatal period (at the age of three weeks), can alter, i. e. adapt fhe receptor for itself.

Materials and methods

CFY rats of both sexes were treated at the age of three weeks and two months. The animals serving as absolute controls were not given any treatment. At the age of three weeks the treated animals received treatments as follows: TSH (Ambinon, Organon-Oss, 3 IU), or FSH + LH (Pergonal, Serono, Rome, 50 IU), or the former hormones plus LPS (OSSKI/Bertók, LD_{50} : 5 mg/kg, in a dose of 150 μg/animal) administered simultaneously, or LPS alone. At the age of two months the animals belonging to the different groups (including the absolute control group) were either not given any treatment or were given TSH or gona-

dotropin in doses similar to those mentioned above. The animals were exsanguinated one hour after the treatment. Serum thyroxin (T_4) level was measured by radioimmunoassay (Izinta, Budapest). Six animals were investigated in each subgroup. The significance levels compared to the absolute control values were evaluated by Student's t-test.

Results and discussion

It is well-known that a single hormone treatment leads to hormonal imprinting [2, 3, 4]. It is not known, however, whether later on — in this experiments on the 2lst day of life — would the cells also respond with an imprinting-like phenomenon. Therefore in the first part of the present experiments we studied this problem.

On the basis of the results (Fig. 1) it can be concluded that the direction of the response was the same in males and in females, whereas quantitative differences could be demonstrated. These differences manifested themselves also in the response to TSH, i. e. the T_4 production was considerably higher in females than in males, and the response to TSH was significant only in females. The female animals responded also to FSH and the increase in T_4 level was at the margin of significance. This finding indicates that the gonadotropin was not neutral for the receptors of the thyroid gland even at the age of three weeks.

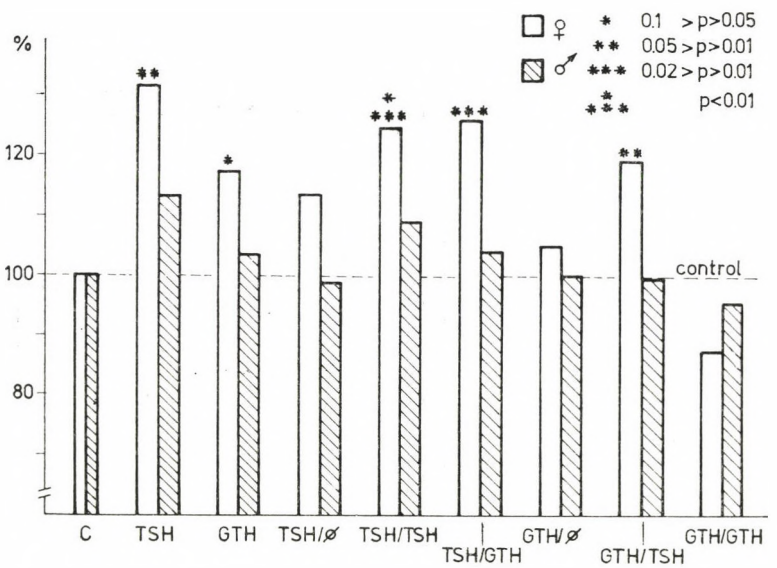

Fig. 1. Serum T_4 levels (compared to the absolute control taken as 100%) in rats treated with thyrotropin (TSH) and/or gonadotropin (GTH) at the ages of three weeks and/or two months. The treatment at the age of three weeks is shown above the sign, whereas the treatment at the age of two months is shown under the sign. Where only one treatment is shown it represents the treatment at the age of two months

In females the effect of TSH administered at the age of three weeks and not repeated anew could be detected in the two-month-old animal (the T_4 level was higher by 13 per cent); the difference, however, was not statistically significant. If the animals pretreated with TSH received TSH treatment repeatedly, the T_4 level became significantly higher than that of the absolute controls. The actual value, however, was not higher than that seen in the animals treated with TSH for the first time at the age of two months. This finding indicates that TSH treatment administered at the age of three weeks did not give rise to imprinting; from the point of view of imprining the treatment proved to be practically ineffective.

Gonadotropin administration at the age of two months — after TSH treatment at the age of three weeks — gave rise to significant increase of serum T_4 levels. This increase (when compared to the results of animals who had received TSH treatment at the age of three weeks but remained untreated at the age of two months) indicates that TSH treatment strengthened the response capacity of the receptor also to FSH. Similarly, gonadotropin administered at the age of three weeks increased the sensitivity to TSH, whereas gonadotropin given at the age of three weeks did not influence at all, on its own, the T_4 levels measured later on. Gonadotropin treatment at the age of three weeks did not influence the response capacity of the receptor to gonadotropins.

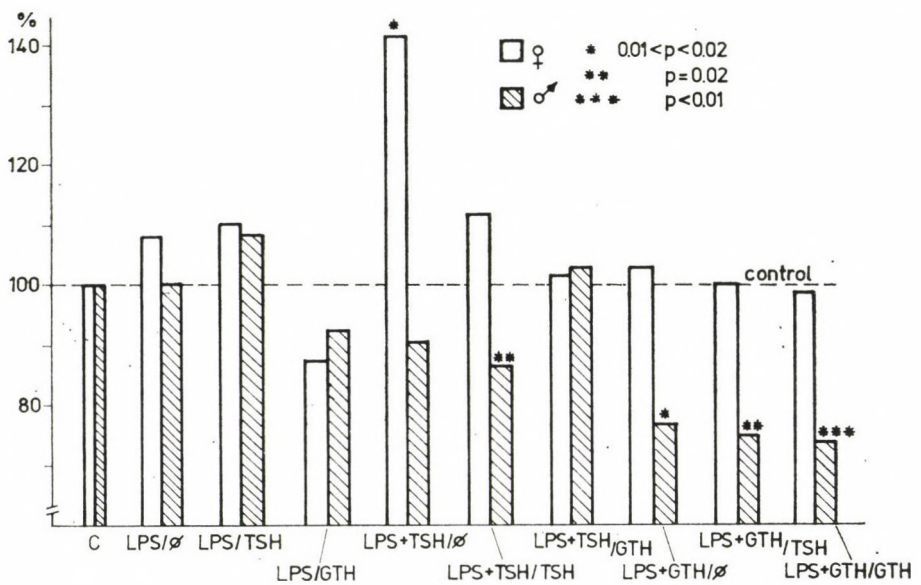

Fig. 2. Serum T_4 levels (compared to the absolute control taken as 100%) in rats treated with endotoxin (LPS) at the age of three weeks and/or with thyrotropin (TSH) or gonadotropin (GTH) at the age of two months. The treatment at the age of three weeks is shown above the sign, whereas the treatment at the age of two months is shown under the sign

In males no significant difference could be obtained pointing to sex-dependent sensitivity. LPS treatment at the age of three weeks did not influence T_4 baseline level; nevertheless, it can be assumed that damages to the receptor occurred, since neither in males nor in females was TSH able to evoke significant increase in T_4 level. However, when LPS and TSH were administered simultaneously, striking increase in T_4 level in the females could be detected at the age of two months even without further hormone treatment (Fig. 2.). This observation renders probable that the combined treatment sensitized the receptors to endogenous TSH. On the other hand, TSH treatment repeated at the age of two months deteriorated this effect, no significant increase could be observed any more. Gonadotropin treatment administered at the age of two months following LPS + TSH treatment proved to be entirely ineffective.

The most striking results were seen following LPS + gonadotropin treatment at the age of three weeks. The thyroxin level of the male animals was lower by 20 per cent than the control level, either with or without further administration at the age of two months. On the one hand, the thyroxin level was considerably lower than the control values and, on the other, neither TSH nor gonadotropin was able to stimulate the receptor. In female animals, however, if at the age of two months no further hormone treatment was carried out, the T_4 level corresponded to the control values and could be stimulated neither by TSH nor by gonadotropin administration.

In previous experiments [10] endotoxin administered to newborn rats in a dose of 1 μg led to considerable decrease in the T_4 level measured in adulthood. In the present experiments not even the subtoxic dose of 150 μg altered the baseline T_4 level. On the other hand, the observation that neither in males nor in females did the administration of the specific hormone, i. e. TSH increase the T_4 level significantly indicates that the receptor was really damaged. The labile condition of the membrane which developed as an effect of LPS treatment administered at the age of three weeks proved to be favourable for simultaneous TSH treatment. Though no imprinting evolved (in the female animals) but by adulthood extreme extent of sensitivity to endogenous TSH developed. In the same system the inadequate hormone which was able to bind to the receptor, i. e. gonadotropin, damaged the receptors both in male and in female animals. The receptors lost their response capacity to their own hormone, whilst no response capacity developed to the adequate hormone (i. e. to the hormone which was able to give rise to imprinting). So, the experiments unequivocally demonstrated that the receptors can be altered even at the age of three weeks, after the completion of the maturation of the receptor, but during destruction — and subsequent restitution — of the membrane. Moreover, the alterations evoked will be constant changes of the receptor. This phenomenon has also practical importance in such situations when imprinting develops

as an effect of hormone treatment, and, at the same time, receptor damages caused by pathogenic organisms or adverse effect of drugs are also present.

It is of interest, and can hardly be explained solely on the basis of the present experiments, that when compared to the controls the significant positive effects could always be observed in the female while the significant negative effects were seen in the male animals.

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THE MESENCEPHALIC RETICULAR FORMATION AS A LINK IN THE CORTICAL CONTROL OF EXPLORATORY AND GOAL-DIRECTED BEHAVIOUR*

F. KLINGBERG, Petra MAGER, R. MAGER

Paul Flechsig Institute for Brain Research, Karl Marx University, DDR

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The general accepted concept about the MRF as an unspecific ascending activating system concerns only one of its multiple functions. Investigations on more than 100 hooded rats of the Long-Evens strain with small bilateral symmetric lesions in dorsal, central and ventral subnuclei of the MRF brought out the following results pointing to further important functions:
1. Each lesion type produced a different syndrome of parameter changes of the spontaneous open field behavior with some common tendencies of reduced ambulatory and exploratory activities.
2. Visual placing responses were strongly reduced or totally abolished after lesions without tendencies of recovery.
3. Changes of locomotion and muscular tonus were quite different or even opposite in dorsal, central and ventral types of lesions.
4. In four tasks of postoperative active avoidance acquisition or retention and performance of preoperatively learned tasks the impairments were different related to the lesion type including a different loss in brightness discrimination.

The results support the hypothesis that MRF subdivisions participate differently in information selection, tuning and coupling information with goal directed movements of different type. Lesions severely disturb the proper use of some information for a cue, especially visual cues when they are in the anterior part of the mesencephalon.

Keywords: mesencephalic reticular formation, behavior, locomotion, muscular tonus.

Motivations induce goal-directed behaviour. In this paper we comprehend goal-directed behaviour as the adjustment of eyes, ears, head, body and limbs to objects of the environment, as movements directed to such objects or even to withdraw from them or to avoid contact with such objects. We do not include in this definition purposeful behaviour in the broadest teleonomic sense. Programs of goal-directed behaviour are closely connected with informa-

Correspondence should be addressed to
Fritz KLINGBERG,
Paul Flechsig Institute for Brain Research,
Karl Marx University
DDR-701 Leipzig

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tion selection and tuning processes, with coupling of sensory informations to motor performance and with concentration (focussing) of attention. It is well known that the tectum and the cerebellum are involved in these processes and in the control of motor performance of goal-directed behaviour. Several nuclei of the ponto-mesencephalic region regulate the directed movements and the muscular tension in postures (for review see [13]). The complex and dynamic motor coordination comes out being the result of regulating interactions between neocortex, teetum, cerebellum and the ponto-mesencephalic region. The latter works under multiple forebrain control and receives descending inputs directly from the different fields of the sensory neocortex, the frontal cortex [2, 18, 30, 36] from the neocortex via basalganglia [5, 6, 9, 31] and from the subpallidal limbic region [4, 17]. We suppose that the mesencephalic reticular formation (MRF) and the brain stem reticular formation in general plays an important role in the precise regulation of goal-directed behaviour and exploratory behaviour.

Concepts about the functional significance of the brainstem RF and the MRF are still controversial. There are a lot of reasons to suggest that the generally accepted concept about the MRF as an unspecific ascending activating system [28, 39, 40] concerns only one of its multiple functions. The polymodal character of MRF neurons was believed to be related to this function (for review see [38]). There is evidence, however, that different parts of the MRF are involved in analytic and integrative processes on the segmental level and exert descending control upon several coordinated motor and vegetative functions. We recently studied changes, syndromes and deficits of spontaneous and of active avoidance behaviour on rats after various mesencephalic lesions.

Methods

The investigations were performed on more than 100 male hooded rats of the Long-Evans strain. Bilateral symmetric lesions were produced by stereotaxical applications of constant anodal currents of 1mA and 15 s duration. For larger lesions we applied two subsequent coagulations with 1 mA. In sham-operated controls the current was not switched on. Localizations and sizes of lesions were histologically checked in Nissl stains at the end of the experiments. Typical representatives of the lesions of each group are demonstrated in Fig. 1.

Results and discussion

1. Visual and tactile placing responses were tested before and 14 and 45 days after surgery [19]. Every rat was suspended by hand with its front feet and head hanging freely down. Then it was moved slowly to a wooden shelf with its eyes on the shelf level and was stopped 3 cm distant from the shelf for 5 s. This test was repeated 5 times on the investigation day. Preoperative

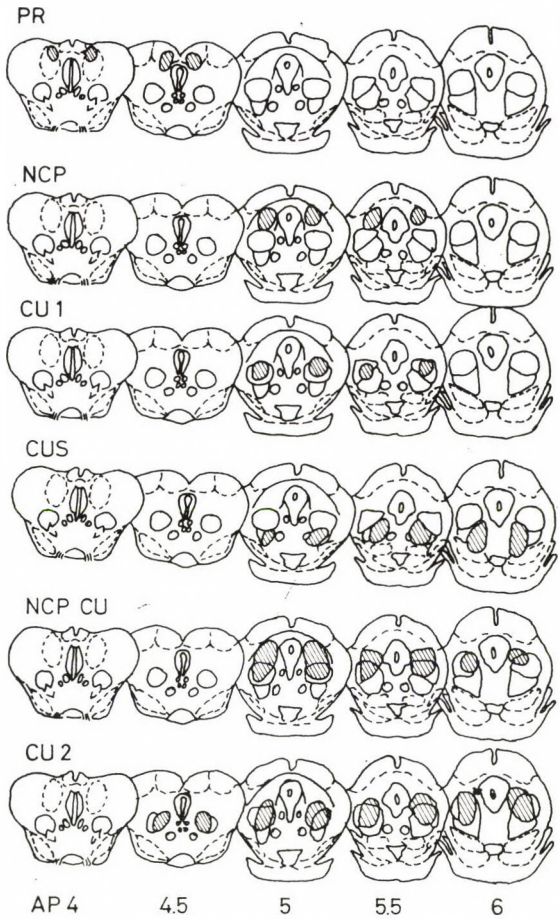

Fig. 1. Typical lesion localizations of all six lesion groups PR — pretectum, NCP — nucleus commissurae posterioris, CU_1 and CU_2 — area cuneiformis, CUS — area subcuneiformis. AP in mm posterior to bregma

and sham-operated (postoperative) control rats performed visual placing responses very quickly with short latencies even before reaching the 3 cm distance by upward elevation of the head and placing their forepaws on the platform. This response occurred in 100% of the trials. This reflectory movement significantly decreased after all lesion types ($p < 0.001$), when movements occurred, then with long delay and rather slowed. After extended lesions of the area cuneiformis (CU_2) or combined lesions of the very anterior part of CU and the nucleus commissurae posterioris (NCP) both visual and tactile placing responses were totally abolished. In these cases rats made no attempt to change their slackly hanging position or to orientate in vertical direction. From the 14th to 45th postoperative day no improvement could be observed,

whereas Braun [4] had found such a recovery after large substantial damage of the neocortex. We suggest that the impairment of the visual placing response after different lesion types may come from different reasons. After lesions of the posteroventral pretectum (PR) this may be strongly impaired brightness discrimination [24]. In NCP rats vertical orientation and eye movements are strongly reduced. But it remained unclear why the deficit was the strongest in the most ventrally lesioned rats (anterior part of the area subcuneiformis, CUS).

2. When we analysed the lesion syndromes we found typical differences depending on the lesion type. All CU_2 rats, 50% of the NCP—CU rats and 21% of the CU_1 rats displayed a syndrome of disturbed locomotion. When they were set on the ground they immediately started with stereotypely performed hyperkinetic locomotoric movements. They ran against walls or any other obstacles without previous orientation. Most of them did not stop at the end of a table but fell or jumped down and continued this typical locomotion on the ground. A similar "locomotor syndrome" was described in cats with extended lesions in the mesencephalic and pontine tegmentum. We suggest that the lesion causes disinhibition of the locomotor region in the caudal pontine part of the CU [7, 8, 37]. The special curious form of the locomotion may result from increased extensor tonus in forelimbs, neck and trunk after dorsolateral or central tegmental lesions which was also observed in cats by Hess [11]. Ogawa [32] called this type "Paradeschritt" and Muskens [29] reported that the distribution of the muscle tonus changed with the lesions' site in dorsoventral direction from extension to flexion. We also found flexion after CUS lesions [23]. Contrary to controls, CU_2 and NCP—CU rats did not resist when pushed foreward (no so called "negativism"). Another symptom of possible disinhibition was found by measuring the time of leaving a round field of 40 cm diameter in the center of a great room. CU_2 rats left this after 3.7 s, CU_1 rats after 6.4 s and NCP—CU rats after 7.8 s, which is a significantly shorter time than that of the sham-operated controls which left it after 12.3 s ($p < 0.001$ for CU_2 and CU_1, $p < 0.05$ for NCP—CU).

PR lesions were typically characterized by mydriasis and reduced or abolished pupillary consensory reflexes.

3. Depending on the lesion type we got special information about changed behaviour from careful investigations in open field which consisted of a 60×75 cm plastic floor and 22 cm high walls. It was subdivied into 20 small fields (15×15 cm) which were controlled by 9 infra-red beams. Figure 2 demonstrates that the lesions of different MRF parts differently influenced the parameters of ambulatory behaviour of the rats (from [20]). The total amount of locomotion in the open field was significantly diminished in all lesion groups (upper left diagram in Fig. 2). This was evident from the first 2 min period and in all the further periods compared to the controls. Their sequential reduction during

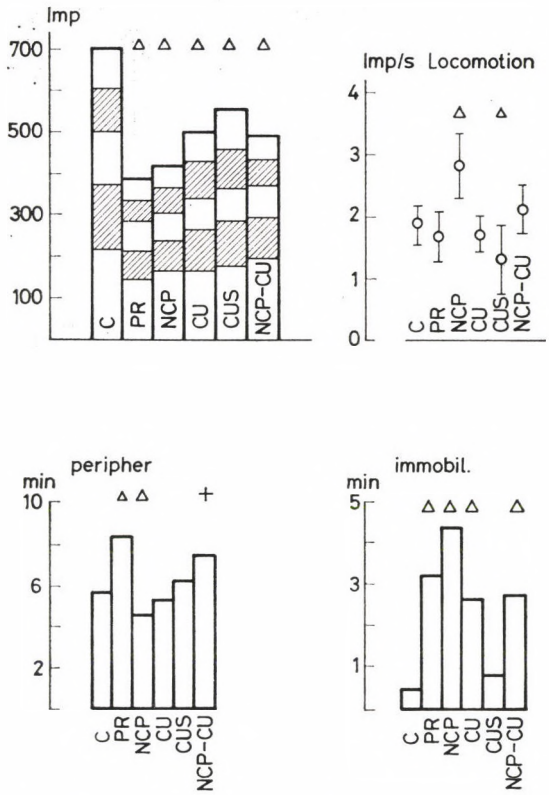

Fig. 2. Ambulatory behaviour of rats in the open field during 10 min exposure. Group mean values (PR,n = 10; NCP, n = 14; CU, n = 10; CUS, n = 11; NCP—CU, n = 6; C, n = 8). Left upper diagram: number of infrared beam crossings (Imp), columns subdivided in 2 min periods (first on the basis). Right upper d.: Velocity of locomotion indicated by Imp/s and standard deviations. Left lower d.: stay on peripheral fields in min. Right lower d.: summated duration of immobility. In Fig. 2—4 triangles indicate $p < 0.01$, + $p < 0.05$

the 10 min exposure demonstrates that the habituation to the open field situation remained undisturbed. The total duration of immobility reveals rather great differences between the groups (lower right diagram) showing a maximum in NCP rats. On the other hand, the quotient of infra-red beam crossings and total duration of locomotion shows that NCP rats moved quicker whenever they did it, whereas CUS rats moved slower than controls (upper right diagram). Pretectal rats showed enhanced thigmotaxis found from the stay on peripheral fields near the walls (lower left diagram).

Exploratory behaviour was strongly reduced in all lesioned groups (Fig. 3). Most evident was the decrease in the number of rearings and reduced vertical orientation is also indicated by the reduction of wall-climbings. The exceptions in the latter case are the PR rats which also express the enhanced thigmotaxis.

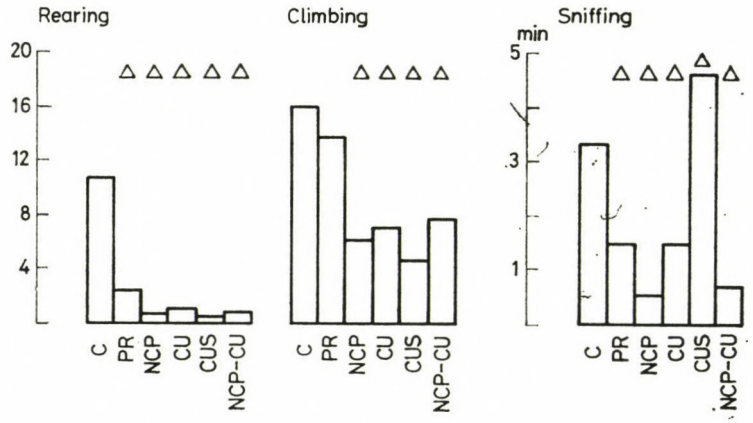

Fig. 3. Exploratory behaviour of rats during 10 min exposures in the open field. Group mean values of number or duration

Sniffing as a further parameter of exploratory behaviour is decreased, too, except for CUS rats which displayed even significantly more periods and total amount of sniffing than controls. Excessive sniffing after large lesions of this region was already reported by Parker and Feldman [33]. Sniffing is not only activated by olfactory stimuli, but also when input from any of several senses arouses the animal to explore [1, 42].

The modulating olfactory information comes through the lateral olfactory tract to the limbic system and directly from the anterior olfactory nucleus to the anterior mesencephalon [26]. Lesions of the anterior olfactory nucleus lead to similar changes of the sniffing parameters [11].

We suggest that the CUS has some inhibitory function in the regulation of sniffing depending on the situational context. Disturbances of these functions may be involved in strong changes of alimentary behaviour which we observed in agreement with other authors [3, 12, 33] after lesions in the CUS. On the other hand, the interruption of a great part of the pallidotegmental [31] and other inputs from the periaquaeductal gray [35], the medial hypothalamus [35], the subthalamus [34] and the area preoptica [27] to the ventral and medial parts of the MRF may also contribute to the syndrome of disturbed alimentary and drinking behaviour.

Mesencephalic lesions influence grooming, too (Fig. 4). The grooming duration increased and the grooming latency is extremely shortened. This may have two reasons. We consider that the missed activating effect of novelty in the open field situation after MRF lesions may contribute to grooming not being so long suppressed or secondly a descending inhibitory influence of the anterior MRF is taken off.

Also the investigation in the open field situation brings us to the conclusion that the old too much generalized concept about the MRF is not correct.

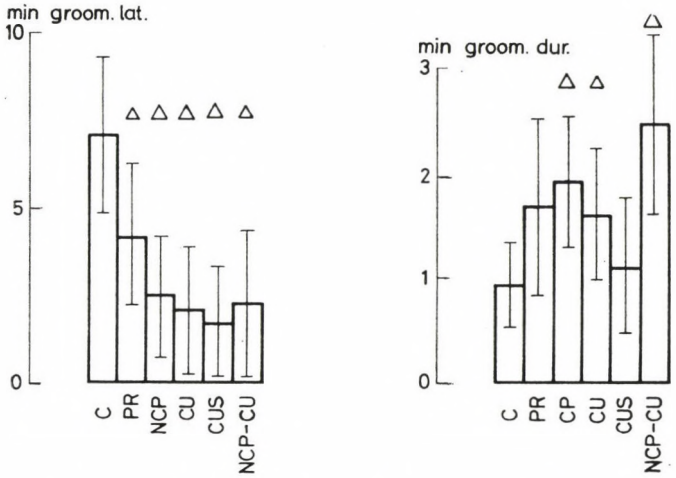

Fig. 4. Grooming duration and grooming latency of rats during 10 min exposure to the open field. Group mean values ± s. d.

4. We further investigated goal-directed behaviour induced by avoidance conditioning procedures after various lesions of the MRF. In these experiments the following tests were used:

a) acquisition of the conditioned avoidance response (CAR) in a Y-maze with the right branch closed (runway left, 10 trials per session).

b) Direction change of this test (runway right, 10 trials p. s.).

c) Continuation with both branches open but only one exit unlocked in a 2 : 2 alternation schedule. In this test the open exit was illuminated, the closed one was dark (dark-light discrimination test 20 trials per session).

The start-box and the two goal-boxes of the Y-maze were separated from the branches by one-way doors connected with electric stopwatches. Both were set on by the conditioned stimulus (CS), which was a five second buzzer sound. The first watch was stopped by pushing the start-box door and indicating the reaction time; the second one was stopped by pushing the goal-box door. The time difference was the running duration, from which the running speed (1 m distance) was simple to calculate. The unconditioned stimulus (UCS) consisted of a 90 V square-wave impulse series with 30 imp/s and 1.5 ms impulse duration. It was switched on to the grid floor 7 s after the onset of the CS.

NCP rats indicated by filled circles in Fig. 5 were better in the acquisition of CAR in the simple runway test compared to controls indicated by open circles. In this case it was a good advantage to start early, to perform less orientation and to run straight on. Thus, the direction change in the fifth sessions was not noticed by the lesioned rats, whereas control rats hesitated and performed exploratory behaviour before passing the goal. In the first

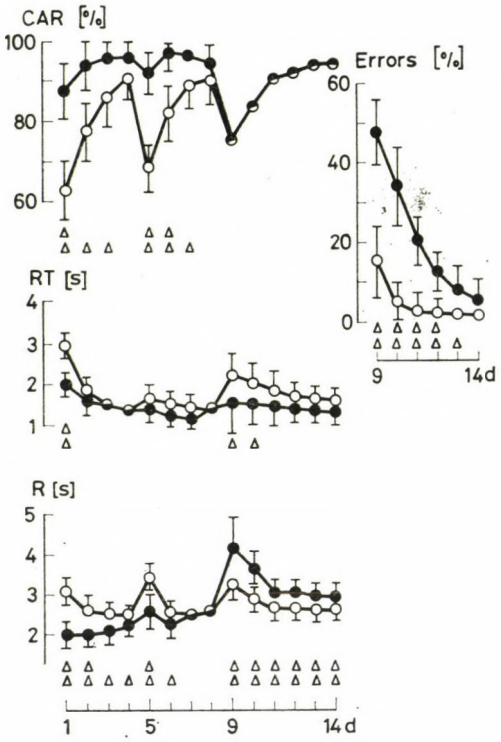

Fig. 5. Mean values and standard deviations of conditioned avoidance responses (CAR) in % of trials, of reaction times (RTs) in s, of running durations (Rs) in s and of errors during brightness discrimination in % of trials, after lesion of NCP (•, n = 8) compared with sham-operated controls (o, n = 8). day (d) 1—4: runway left, d 5—8: runway right, d 9—14: alternation of the goal. In Fig. 5—9 indicates one triangle (or cross) $p < 0.05$, two triangles (crosses) $p < 0.005$

session with goal alternation after every second trial the NCP rats entered one of the branches by chance — that's why they had error rates about 50%. In such a case differring from the controls they immediately and quickly turned to the other branch which was illuminated and open. Thus, in spite of their error rate their avoidance rate was equal to that of the controls. CU_1 rats had not shown such high error rates but were lower in their CAR rate because they moved slower than controls. The greatest deficit was found after CUS lesions (Fig. 6) because this group had high error rates and was the slowest. The PR group had the highest error rate.

We investigated whether rats could perform the CAR well if they had learnt it perfectly before the lesion. The results in most groups with preoperative experience were really better. However, the error rate in dark-light discrimination retention task 3 weeks after the lesion respectively during relearning was still rather high. Figure 8 demonstrates these results from a CU_2

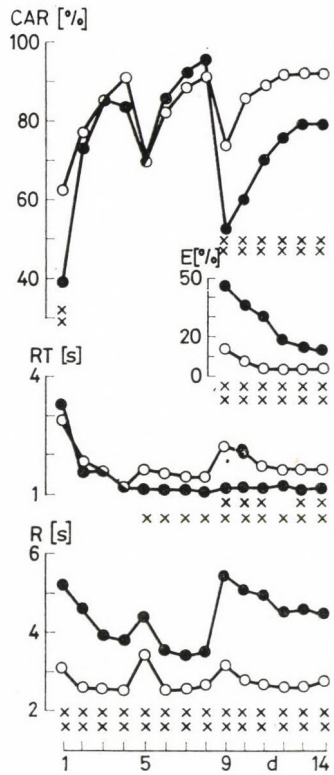

Fig. 6. Mean values and s. d. of CAR, RT, R and of errors (E) after lesion of CUS (•, n = 8) compared with controls (o, n = 8).
d 1—4: runway left; d 5—8: runway right; d 9—14: alternation of goal

group: error rates above 40%, slowed running, difficulties to correct the errors or to reactivate the memory are typical, whereas the start (reaction times, RT) was more rapid. In this investigation CUS rats had the most troubles, too.

We may conclude that animals with MRF lesions are able to see, to move, to react stimuli, to habituate to situations or even to learn, but evidently they have troubles to select informations which may serve as a cue and to couple them with the correct or even most effective behaviour. In the case of lesions in the anterior part of the mesencephalon these troubles are mostly connected with visually guided behaviour.

d) More difficulties were expected in the acquisition of a jump (pole-climbing) test in which the rats could avoid footshocks from the grid floor (50 V alternating current) by jumping or climbing on a wooden rod hanging in the middle of the apparatus. After the onset of the CS (a 1.5 kHz tone) they had 3.5 s time until the UCS was switched on. Both stimulations were switched off by the animal's weight on the rod. RTs and hanging durations were auto-

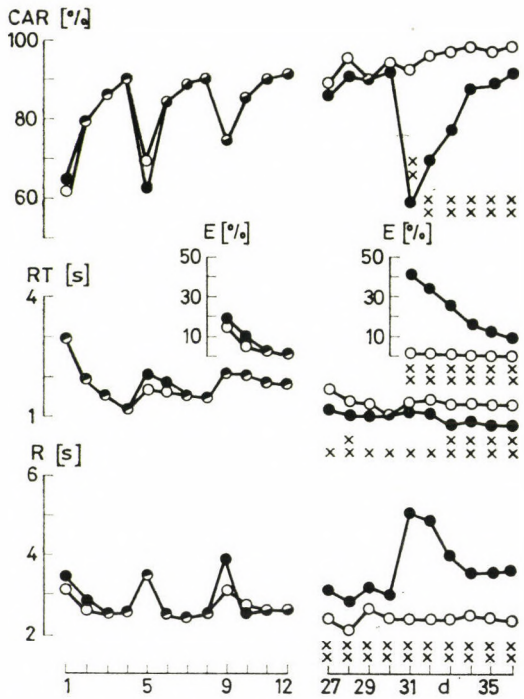

Fig. 7. Mean values of CAR, RT, R and E during preoperative and postoperative testing in the Y-maze of CU_2 group (●, n = 8) compared with controls (o, n = 8). d 1—4: runway left; d 5—8: runway right; d 9—12: alternation of goal; d 13: lesioning, d 27—28: runway left, d 29—30: runway right; d 31—36: alternation

matically measured by stopwatches connected to the rod. Neither CU_1 rats nor CU_2 rats nor CUS rats were able to learn this task and to successfully avoid punishment without previous preoperative training. None of them found the escape possibility. PR rats detected this way in the first and NCP rats in the third session with 10 trials each. PR rats remained slow while performing throughout 8 sessions with a maximal CAR rate below 50%. NCP rats continued to perform a great number of variant jumps beside the goal as if they couldn't see it (Fig. 8). They were also unable to learn the context between CS, UCS and the correct goal-directed movement. Even after 80 trials they poorly avoided punishment, and their RTs were still too long. The increasing number of intertrial responses and the slow decrease of variant jumps (ITR and VJ in fig. 8) reveal that they were not inhibited as in learned helplessness (compare [10, 17, 25]. Their parameters indeed point to increased motivational activity.

The situation was quite different when the NCP rats had preoperatively learnt the task. Then they correctly responded in time and their CAR rate was normal. The CU and CUS groups, however, found the escape possibility, but always waited for reinforcement and could not relearn the jumping test (Fig. 9).

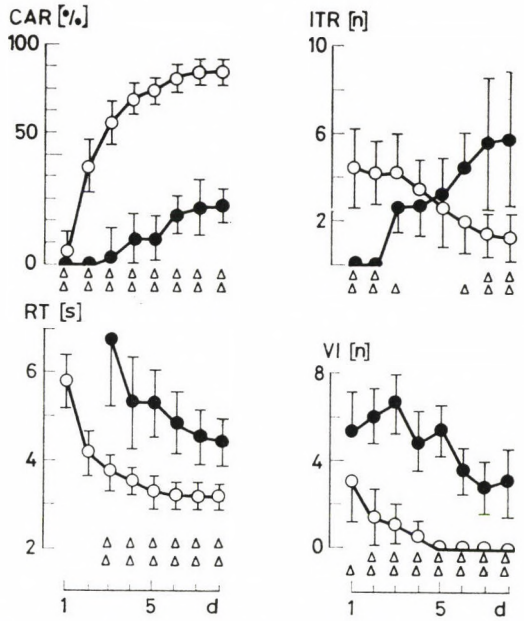

Fig. 8. Mean values and s. d. of CAR, RT, R, intertrial responses (ITR) and variant jumps (VJ) per session in the postoperative jump test training of NCP rats (•, n = 6) compared with sham-operated controls (o, n = 8)

In all these experiments it was surprising that CU rats never displayed any response of the arrest reaction type during which movements are blocked in any posture of the body. The strong excitation of the CU by immediate local effect of injected kainic acid [14] produced frozen postures of very long duration in cats concommitted by EEG desynchronization. We had found a similar tonic behavioural response in classical aversive conditioning of rats who had no possibility to escape or to avoid punishments [17]. There is evidence that the striatum plays a dominant role in this response type [15] which is possibly influenced by an ascending excitatory pathway from the CU to medial thalamic nuclei, in cats to the centrum-medianum-parafascicularis complex [40] which itself has a strong projection to the striatum.

Reports on the effects of MRF lesions upon learning and retention in rats or cats are as contradictory as results published on the problem whether MRF stimulation facilitates memory consolidation or disrupts retention. We think that the reasons for divergent conclusions come from methodical differences in the investigations. We have discussed several problems elsewhere [15, 19, 24]. Our results clearly demonstrate that MRF lesions produce special behavioural syndromes and impairment in different types of goal-directed behaviour. There is no longer evidence to believe in a common reticular structure with unspecific functions or any socalled widespread projections. With respect to

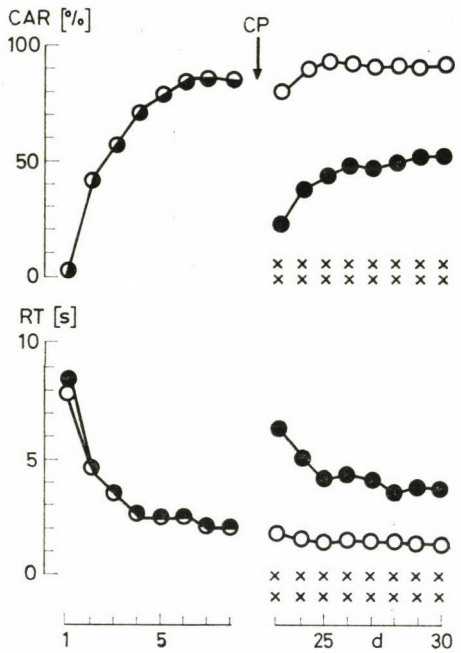

Fig. 9. Mean values of CAR and RT of CUS group (●, n = 8) compared with sham-operated controls (o, n = 8) in the preoperative and postoperative jump test training. d 9: operation; d 23—30: postoperative sessions

many unknown details about networks and functional systems in which brain stem RF and especially MRF or better to say in which the many reticular nuclei and divisions of the brainstem are involved, we should avoid further simplifications like "unspecific", "arousing system", "activating system" etc. From the anatomical point of view there are more local, segmental or intersegmental interactions which are strongly controlled by descending cortical and limbic structures, than ascending functions.

There is further evidence that MRF participates in information selection, tuning and coupling of these informations with goal-directed movements of any type. On the other hand, MRF lesions do not interrupt sensory pathways to special sensory fields or any motor system directly, but they severely disturb the proper use of some information for a cue in goal-directed behaviour. This concerns more often visual cues when lesions are in the anterior part of the mesencephalon.

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CHANGES OF ACID SOLUBILITY AND FLUORIDE CONTENT OF THE ENAMEL SURFACE IN CHILDREN CONSUMING FLUORIDATED MILK

Zsuzsanna TÓTH, P. ZIMMERMANN, Z. GINTNER, Jolán BÁNÓCZY

Department of Conservative Dentistry, Semmelweis University Medical School, Budapest, Hungary

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Changes of the acid solubility and fluoride content of the surface layer of the enamel of the first incisor of seventy-nine 9 to 10-year-old children living in a closed community were measured by the method of enamel biopsy at the 6th and 12th months of the consumption of fluoridated milk. Half-year after the beginning of consumption of fluoridated milk the acid solubility decreased significantly, while one-year-long consumption led to significant increase of the fluoride content of the enamel surface.

Keywords: milk fluoridation, enamel biopsy, phosphorus dissolution, F^- content.

The aim and desired result of the consumption of fluoridated milk is to decrease the extent of the decay of both the deciduous and the permanent teeth. The first conclusive data originated from Japan where 2 to 2.5 mg NaF was added to the milk or soup provided in the school, and after 4 years 36.3% reduction in the increment of caries could be observed [5]. In the United States of America 35% reduction of caries increment was seen 3.5 years after the beginning of the supplementation of the milk consumed by 6 to 9-year-old children with F^- in a concentration of 3.5 mg/285 ml [12]. Analysis of a numerous Swiss sample revealed that in the case of those children who from the age of 9 to 44 months onwards drank milk containing F^- in a concentration of 1 mg/l the reduction of caries increment was between 25.5 and 73.5% as far as the deciduous and 62.7% as far as the permanent teeth were concerned [21]. In Scottish children who consumed every day for 5 years, from the age of 4.5 to 5.5 years onwards, 200 ml milk containing 1.5 mg F^- significantly (by 31.2%) lower DMF-T values were seen [15, 16]. Three-year-long and five-year-long Hungarian follow-up investigations which began at the age of 2 to 3 years showed 60 to 70% reduction of caries increment in the permanent teeth [1]. After 3 years the caries reduction of the first permanent molars in the 5 to

Correspondence should be addressed to
Zsuzsanna TÓTH
Department of Conservative Dentistry, Semmelweis
University Medical School
H-1088 Budapest, Mikszáth Kálmán tér 5. Hungary

6-year-old children was 74%.Moreover, even as early as 2 years after the children began to drink fluoridated milk significant difference could be observed when the dmf value and the decay of the first permanent molars were compared to the controls [23]. Other authors found 77% reduction in the caries increment of the permanent teeth after two-year-long consumption of chocolate-flavoured milk containing F^- in a concentration of 1.0 mg/236 ml [8].

As far as the effect of fluoridated milk on the enamel was concerned, animal experiments revealed considerable increase of the F^- content of samples obtained by enamel biopsy [11]. Fluoride incorporation into the enamel could be demonstrated *in vitro* when extracted human teeth were steeped in fluoridated milk [7]. After the consumption of fluoridated milk increased amount of fluoride was measured in the ash of enamel and dentine of human deciduous teeth [9].

Following two years of milk fluoridation *in vivo* enamel biopsy investigations revealed considerable decrease of the acid solubility of the enamel [19]. Milk fluoridation for 5 years led to significant increase of the F^- content of the enamel surface [18].

We were unable to find, however, any data in the literature on the time period necessary for the increase of the fluoride content of the enamel to be demonstrated after continuous consumption of fluoridated milk. Therefore the present investigations were aimed to study, on the one hand, whether the acid solubility and fluoride content of the enamel surface exhibit any change half-year or one year after the beginning of the consumption of fluoridated milk and, on the other, how these changes develop in the dependence of time.

Materials and methods

The investigations were carried out on seventy-nine 9 to 10-year-old children living in the Children's City of Fót. Between 1978 and 1984 the children living there, were already given fluoridated milk; the circumstances of the investigation and the favourable effects (reduction in the incidence rate of caries) have been reported elsewhere [2]. Due to technical reasons (renovation of the kitchen) milk fluoridation was interrupted and began anew only one year later. One milligram of fluoride in the form of NaF salt was freshly mixed into 200 ml milk and given to the children once a day, at breakfast. The F^- content of the drinking water in the children's home is lower than 0.1 mg/l.

The children were investigated by the method of enamel biopsy (4,3) before the reintroduction of fluoridated milk administration, half-year after and one year after. After the isolation, cleaning and drying with alcohol of the (1) tooth an area of 3 mm diameter was isolated in the middle of the buccal surface by a self-adhesive synthetic (polymer) tape. Then 4 μl of 0.5% $HClO_4$ solution was dropped to a Whattmann filter paper disc of 3 mm diameter and the disc was placed on the isolated tooth surface for 60 seconds. After the removal of the first disc the remaining acid was blotted by two other discs and all the three discs were stored in a polyethylene tube containing 200 μl bidistilled water. The amount of phosphorus dissolved from the enamel surface was considered to represent acid solubility and was measured photometrically [22]. Determination of fluoride content was carried out by means of an ionselective electrode (Radelkis OP-7443). The results were evaluated by the one-tailed t-test. Only complete follow-up data, i. e. those containing initial, half-year and one-year results, were taken into account at the final evaluation. This selection accounts for the lower number of the sample in the case of phosphorus determination.

Results

Before the introduction of fluoridated milk administration the acid solubility was 2.95 $\pm$ 0.14 μg dissolved phosphorus per 7.1 mm^2 surface area. At the 6th month of fluoridated milk feeding the corresponding data was 2.47 $\pm$ 0.13 μg/7.1 mm^2, whereas at the 12th month 1.92 $\pm$ 0.07 μg/7.1 mm^2. The amount of dissolved phosphorus became significantly lower ($p < 0.05$) already by the end of the first half-year and further significant decrease

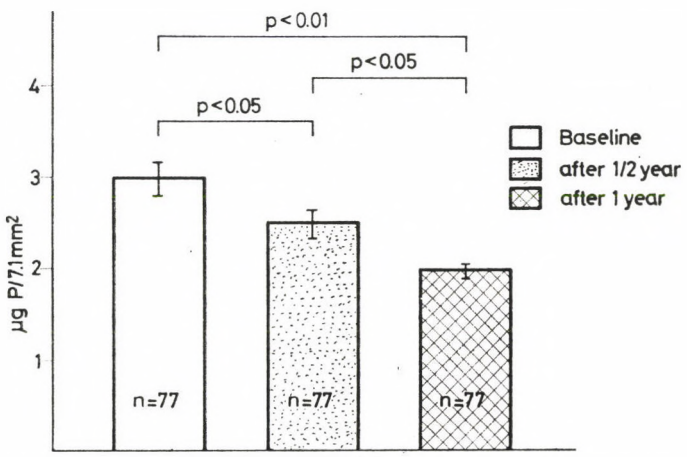

Fig. 1. Amount of phosphorus dissolved from the enamel surface of the first incisor of children consuming fluoridated milk

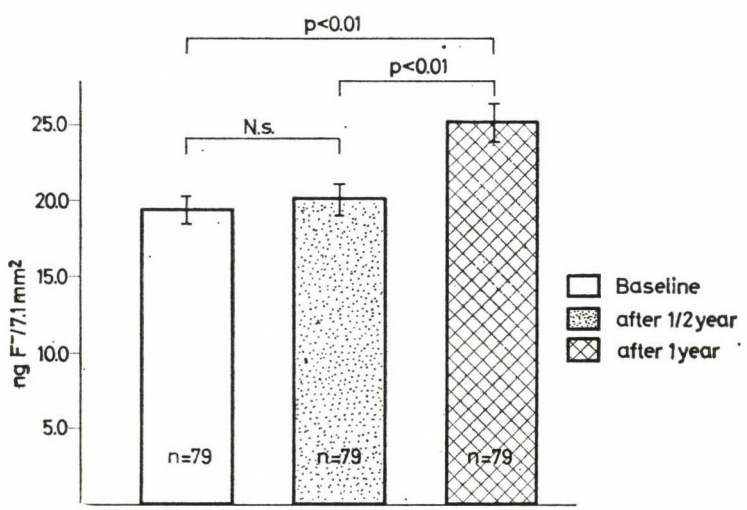

Fig. 2. Fluoride content of the enamel surface of the first incisor of children consuming fluoridated milk

($p < 0.05$) could be observed by the end of the one-year follow-up period (Fig. 1).

The fluoride content of the samples obtained by enamel biopsy were as follows: initial: 19.37 ± 0.85 ng/7.1 mm^2, half-year: 20.01 ± 1.00 ng/7.1 mm^2 and one year: 25.08 ± 1.21 ng/7.1 mm^2. The increase of fluoride content became statistically significant ($p < 0.01$) by the end of the one-year follow-up period (Fig. 2).

Discussion

The investigations were carried out in a closed community, under standard conditions, excluding thereby the organisation difficulties summarized in the literature [10]. With the help of the teachers the fluoride enrichment and distribution of the milk were carried out without any difficulty. Urinary F^- excretion of the children was checked regularly. Enamel biopsy proved to be a quick method of sample collection and caused no unpleasantness to the children.

Contradictory data can be found in the literature on the adequacy of enamel biopsy for the measurement of F^- incorporation into the enamel surface. On the one hand, in rats considerable increase of the F^- content of enamel biopsy samples was seen following the fluoridation of milk and water [11]. Also the results of human investigations indicated that the F^- concentration of the enamel was higher in those who continuously consumed fluoridated water than in those who were never, or just accidentally, exposed to fluoride [17]. On the other hand, following 30 months of administration of fluoride tablets no difference could be observed when the F^- content of the enamel surface was compared to the controls [13]. On the contrary, other authors reported on the effect of three-year-long administration of tablets containing NaF to increase the F^- content of the enamel of the deciduous teeth significantly [20]. It is worthy of attention that marked increase of the F^- content of the adult enamel could be demonstrated by enamel biopsy as early as four weeks after the introduction of NaF tablet therapy [6].

At the 6th month following the beginning of fluoridated milk consumption significant decrease of phosphorus dissolution compared to the initial value could be observed. By the end of the year further considerable decrease of solubility was seen. The demonstration of these changes proves not only the sensitivity and adequacy of the methods used but supports the opinion that as early as six months after the initiation of fluoride intake in the above-mentioned amounts significant favourable changes can be seen in the enamel as well. For the evaluation of the clinical significance of the statistically significant differences further long-term follow-up investigations on the caries incidence rate of these children are needed.

Fluoride content of the enamel increased significantly after one year. In the present study the (1) tooth was investigated which was already erupted in all the 9 to 10-year-old children studied. Due to the self-controlled nature of the study it is indifferent from the point of view of the changes observed that the children had already intermittently consumed fluoridated milk from the ages of 4 to 5 years onwards. Certainly, previous exposure to fluoride might have exerted systemic effect on the tooth germs, in the present investigation, however, the condition of the same, already erupted tooth of the same child was compared half-year and one-year later to the initial condition. Therefore the changes observed may be considered to represent the effect of local influences — though the possibility of the local effect of fluoridated milk is doubted [14] —: partly direct and partly indirect (salivary excretion) influence. It can be assumed with reason that both the direct and the indirect (salivary excretion) effects would be more pronounced if the same quantity of fluoridated milk could be divided into more than one daily portion.

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THE EFFECT OF RINGER SOLUTION INDUCED EXTRACELLULAR VOLUME EXPANSION ON KIDNEY FUNCTION

G. KÖVÉR, HILDA TOST, Anna DARVASI

Department of Physiology, Semmelweis University Medical School, Budapest, Hungary

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The present study quantitated the effects of extracellular volume expansion on sodium and water excretion in 118 anesthetized dogs.

The animals received a priming injection of 10 ml kg^{-1} Ringer solution i. v. which was followed by a constant Ringer solution infusion at a rate of 0.25 ml · min^{-1} · · kg^{-1} until the end of the experiment.

Fifteen minutes after the start of the constant infusion the renal parameters were examined in 11 subsequent 15 min periods (the total time was 3 hours).

Volume expansion produced no significant change in arterial blood pressure, glomerular filtration rate (GFR), plasma sodium and potassium concentration or, haematocrit, but did reduce the C_{PAH} from 284 ml · min^{-1} to 218 ml · min^{-1} (the data were calculated for 100 gram wet kidney weight).

There were constant significant increases in the urinary excretion rate from 0.84 ml · min^{-1} to 4.06 ml · min^{-1} and the 39% of the infused water was excreted during the experiment.

Volume expansion also caused a significant increase in sodium excretion during the three first periods from 120 μmol · min^{-1} to 329 μmol · min^{-1} followed by a small but significant decrease. The sodium excretion at the end of the experiment was 221 μmol · min^{-1} and the 23% of the infused sodium was excreted in the course of the experiment.

The increase of the water excretion during the volume expansion was associated with fall of the urine osmolality and the urine became hypoosmotic as compared to the plasma.

We have provided evidence that vasopressin was not involved in the control of water excretion in our experiments.

It is concluded that neither filtered sodium nor decreased aldosterone secretion can account for the increase in sodium excretion that occurs after Ringer solution loading in the dog.

It has been proposed that a decrease in plasma protein concentration may decrease passive sodium reabsorption due to oncotic forces in the proximal tubule. The Ringer solution diuresis elicits a rise in medullary blood flow, thereby causing a washout of medullary sodium. This might dissipate the osmotic force for the back-diffusion of water from the collecting duct.

Our studies indicate that the response of the diluting segments of the distal nephron to increased delivery of sodium depends upon the presence or absence of volume expansion. However the increase of the distal tubular loading activates the tubuloglomerular feedback which increases the proximal tubular reabsorption.

Based on these assumptions our studies provide further evidence that the tubuloglomerular feedback regulates the blood pressure in the peritubular capillaries in the cortex around the proximal tubules.

Keywords: volume expansion, water excretion, sodium excretion, urinary osmotic activity, tubuloglomerular feedback.

Correspondence should be addressed to
György KÖVÉR
Department of Physiology, Semmelweis University Medical School
1088 Budapest, Puskin u. 9., Hungary

The maintenance of fluid and electrolyte balance by the kidney is achieved by integrated adjustments in glomerular filtration and tubular reabsorption.

When dogs are fed or infused with sodium chloride, both sodium excretion and glomerular filtration rate (GFR) increase. Since the quantity of filtered sodium is usually more than that of the excreted one it is commonly assumed that the sodium diuresis can be explained by the increase in filtered load [22, 42, 43].

The error of measurements of GFR is probably about 5% [9]. Since sodium excretion even at the peak of a saline diuresis is usually a small fraction of filtered sodium, it is difficult to rule out the possibility that small errors in measurements are sufficient to account for occasional instances of apparent dissociation between filtered and excreted sodium.

Although factors possibly influencing tubular reabsorbtive activity, such as the adrenal and neurohypophyseal hormones, are not yet amenable to a quantitative study, the general correlation between filtration rate and sodium excretion in the dog has led many investigators to believe that any change in glomerular activity constitute one of the most important mechanisms in the maintenance of salt and water balance in this animal.

An extensive literature is available on the excretion of sodium, chloride and water in dogs receiving saline infusion, but in great part the information is merely descriptive concerning the fact of diuresis and is not correlated with the measurable physiological mechanisms in the excretion of sodium and water. The comparison of the data has many difficulties, because the administration of isotonic saline or Ringer solution to dogs in quantities equal to one-half or more of the volume of extracellular fluid leads to unphysiological responses.

It has occasionally been suggested, however, that augmented sodium excretion is due to decreased tubular reabsorption of sodium rather than to increased filtration.

Several studies on the renal mechanisms of sodium excretion have focused attention on the proximal tubule as the site of an important regulatory function. It has become increasingly apparent, however, that the transport system in the more distal parts of the nephron are of critical importance in determining the final rate of sodium excretion [8, 13, 23, 31].

The factors which affect sodium reabsorption in the distal nephron are poorly understood. The magnitude of saline diuresis during saline loading is determined to a large extent by the fraction of sodium escaping proximal reabsorption which is reabsorbed in the distal nephron. Evidence has been presented suggesting that during volume expansion sodium reabsorption in the diluting segment of the nephron in the dog may be inhibited and that under some circumstances sodium reabsorption in this segment tends toward a limit [40].

Tubuloglomerular feedback (TGF) is an intrarenal homeostatic mechanism whereby increased sodium chloride uptake at the macula densa causes vasoconstriction in the afferent arteriole of the same nephron [35, 36, 45]. Intrarenal arterial infusion of hypertonic saline decreases renal blood flow (RBF) and GFR in the canine kidney by activation of the TGF [17, 32]. Micropuncture observations during intrarenal arterial infusion of hypertonic saline confirm that the reduction in renal blood flow and glomerular filtration rate are due to activation of tubuloglomerular feedback [37].

Intravenous isotonic sodium salt-infusions are given for both experimental and therapeutic purposes. Only few data exist detailing the effect on glomerular filtration of acute infusions of various sodium salts, but it is suggested that each may have characteristic effects. Studies using either intraluminal infusion of solute in micropuncture experiments, or intrarenal arterial hypertonic saline infusions in the intact kidney, have demonstrated that the potential for a renal response is dependent on the salt status of the experimental animal. TGF activity is enhanced by salt depletion, but alternated by sodium chloride loading [41].

Several investigators are consistent with the view that the excretion of sodium may be regulated in part by yet unidentified factors that alter net tubular reabsorption [3, 12, 25, 30].

The present study was undertaken to evaluate the effects of volume expansion on sodium and water excretion in anesthetized dogs, when the Ringer solution loading was 1—3% of the body weight (this volume expansion is in the physiological range). The Ringer solution infused did not change the plasma sodium, potassium and calcium concentrations. No significant differences related to changes in plasma osmolality were noted.

The purpose of the present study was, to determine whether specific changes in urinary osmolality associated with volume expansion could be prevented by exogen vasopressin or not.

We have used this model to test wether or not the potential for TGF activity is maintained in a situation in which the kidney is actively conserving sodium chloride despite an expanded extracellular fluid volume.

Materials and methods

The experiments were performed on mongrel dogs of either sex weighing between 10 and 30 kg. For 24 hours prior to the experiment the animals were kept on water only. Anesthesia was induced by intravenous administration of pentobarbital sodium (Nembutal, Abott 30 mg/kg body wt.) and maintained with periods of additional administration. After the anesthesia each animal received an infusion of Ringer solution equal to 1% of the body weight, containing para-aminohippuric acid (PAH) and inuline, ensuring a plasma concentration of 2 mg and 30 mg per 100 ml respectively, followed by an intravenous infusion of 0.25 ml/kg of Ringer solution containing 154 mmol/l NaCl, 4 mmol/l KCl, 1.78 mmol/l $NaHCO_3$, 2.16 mmol/l $CaCl_2$ per min for the rest of the experiment.

Cannulation of the femoral arteries and veins of both sides was performed in all animals for arterial blood collections and infusions. Mean arterial blood pressure was measured by a Statham strain gauge transducer connected to a polyethylene catheter in the left femoral artery and recorded on a RADELKIS OH 814/1 recorder.

From a lower midline incision the bladder was exposed and the ureters were catheterized supravesically using fine polyethylene cannulas.

About 15 minutes after starting the intravenous infusion urine was collected from both kidneys in 15 min periods. Blood was taken at the midpoint of each clearance period. After centrifugation the supernatant plasma was collected and the erythrocytes were reinfused in Ringer solution. In the middle of the periods arterial blood pressure was registered.

In the present paper we demonstrate the results of 3 experimental series. In group No. 1 the effects of the Ringer solution infusion were examined in eleven subsequent 15 min periods. The total experiment lasted for three hours.

In group No. 2 after the 5th period 10 mU/kg vasopressin was infused intravenously with the constant Ringer solution infusion during 10 minutes. Following the vasopressin infusion the renal function was examined in two 10 min periods (periods 6 and 7). After the period 7 the animals received a constant vasopressin infusion, when 50 mU/kg vasopressin was infused during sixty minutes (it was given to the Ringer solution administered in constant infusion intravenously).

In ten dogs the lysine-8-vasopressin (SERVA) and in ten dogs the arginine-8-vasopressin (SIGMA) was used.

In group No. 3 the effect of a large volume expansion on kidney function has been studied. Following the 7th period 25 ml per kg body weight of Ringer solution of body temperature was infused for 60 minutes (periods 8, 9, 10, 11), when volume expansion was assumed to amount to 5% of body weight.

PAH concentration in urine and plasma was determined by the method of Smith et al. [39], that of inuline by the method of Little [27]. Urinary and plasma sodium and potassium concentration was measured by flame photometry. Total osmolarity of urine and plasma was measured by the method of freezingpoint depression in a Fiske osmometer. Hematocrit was determined by means of Hawskley microhematocrit centrifuge, plasma protein concentration by the biuret method [19].

The clearance of PAH (C_{PAH}) and the clearance of inuline ($C_{inuline}$) were determined by the usual formulas. The data for C_{PAH}, $C_{inuline}$, urine flow (water excretion), sodium excretion ($U_{Na} \times V$), potassium excretion ($U_K \times V$), and free water clearance (C_{H_2O}) were referred to 100 g kidney tissue weight.

Significance levels of the data was determined by paired and unpaired analysis using Student's "t" test. A p value of less than 0.05 was considered significant.

Results

The first series of studies concerns the effects of Ringer solution infusion in anesthetized dogs. The results of the group are summarized in Figures 1, 2, 3. Experiments were performed on 118 dogs whose body weight was 16.5 ± 0.57 kg ($\bar{x} \pm$ S.E.M.), the kidney weight 77.10 ± 2.65 g, and the kidney weight calculated for 1 kg body weight was 4.67 g.

As the Fig. 1 shows the mean arterial blood pressure during the first period was 126 ± 2 mmHg and at the end of the experiment 123 ± 2 mmHg. While the arterial blood pressure did not change, the C_{PAH} decreased from 284 ± 8 ml/min to 218 ± 7 ml/min ($p < 0.001$), and the glomerular filtration measured by clearance of the inuline was 94 ± 3 ml/min during the first period and 87 ± 3 ml/min during the last one (period 11).

As shown in Fig. 2 the urine flow rose progressively from 0.84 ± 0.07 ml/min to 4.09 ± 0.25 ml/min and the water retention increased too, which

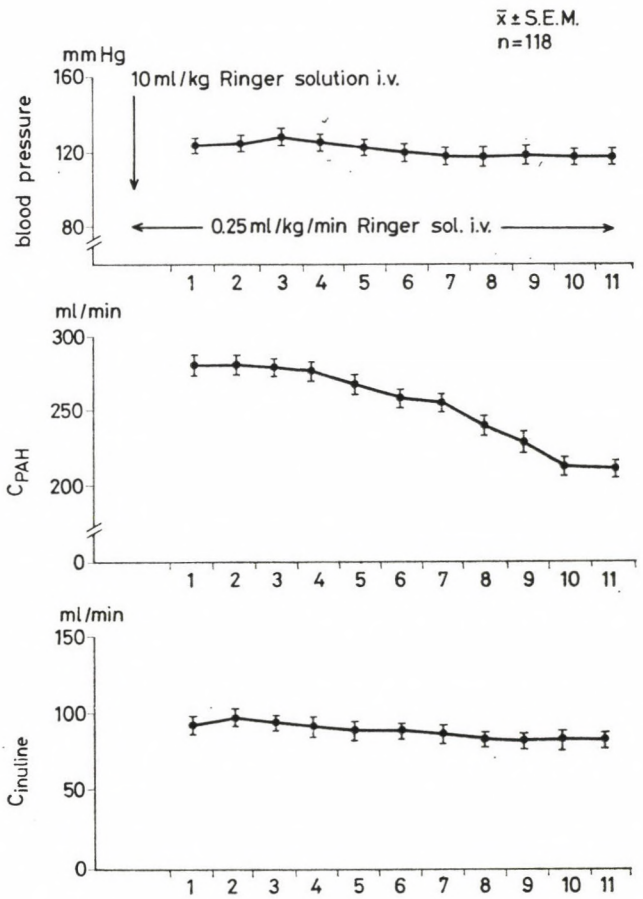

Fig. 1. The blood pressure, PAH clearance (C_{PAH}) and glomerular filtration rate ($C_{inuline}$) during Ringer solution infusion. Points are means ± S. E. M.

was 15 ml/kg of body weight during the first period and 31 ml/kg during the period 11. The 39% of the infused water was excreted by the kidneys during the experiment.

During the periods 1, 2, 3, 4 the sodium excretion increased significantly from 110 ± 12 μEq/min to 329 ± 24 μEq/min and following this period despite progressive increments in sodium retention it tended to stabilize.

The sodium retention was 2000 μEq/kg during the period 1 and 6000 μEq/kg during the last period, the 23% of the infused sodium was excreted by the kidneys during the experiment.

The potassium excretion increased slightly during the periods 1, 2, 3, 4 from 47 ± 4 μEq/min to 73 ± 4 μEq/min and after that, did not change while there was a loss of potassium by the kidney and at the end of the experiment the decline of the potassium content was 300 μEq/kg of body weight.

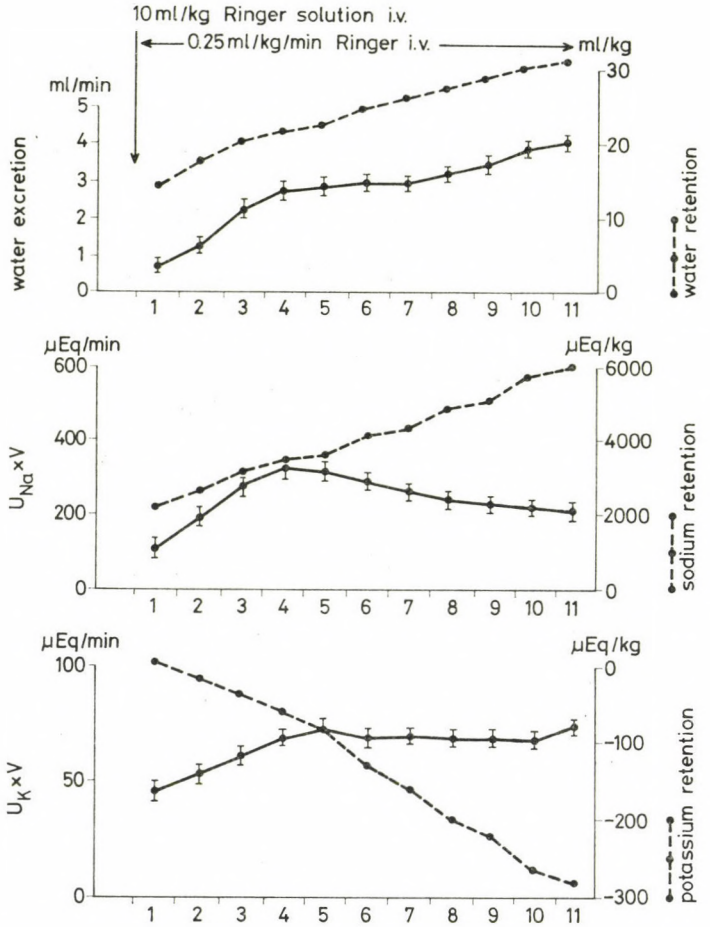

Fig. 2. The urine flow (water excretion), sodium excretion ($U_{Na} \times V$) and potassium excretion ($U_K \times V$) during Ringer solution infusion are solid lines, points are means ± S. E. M. The water retention (ml/kg), the sodium retention (μEq/kg) and potassium retention (μEq/kg) during the experiments, dropped lines. Points are means

As we can see in Fig. 3 the urine osmolality decreased during the course of the experiment and in spite of the administered isosmotic Ringer solution infusion the urine became hypoosmotic as compared to the plasma. At the beginning of the experiment the urine osmolality was 725 ± 17 mosm/l and during the last period 251 ± 14 mosm/l. The plasma total osmolality did not change during the experiment its value was about 310 ± 2 mosm/l.

A striking increase in free water clearance (C_{H_2O}) was noted from −0.86 ± 0.05 ml/min to +1.38 ± 0.17 ml/min.

The plasma sodium concentration did not change during the experiment and its value was 150 ± 1 mEq/l. The hematocrit remained constant during the

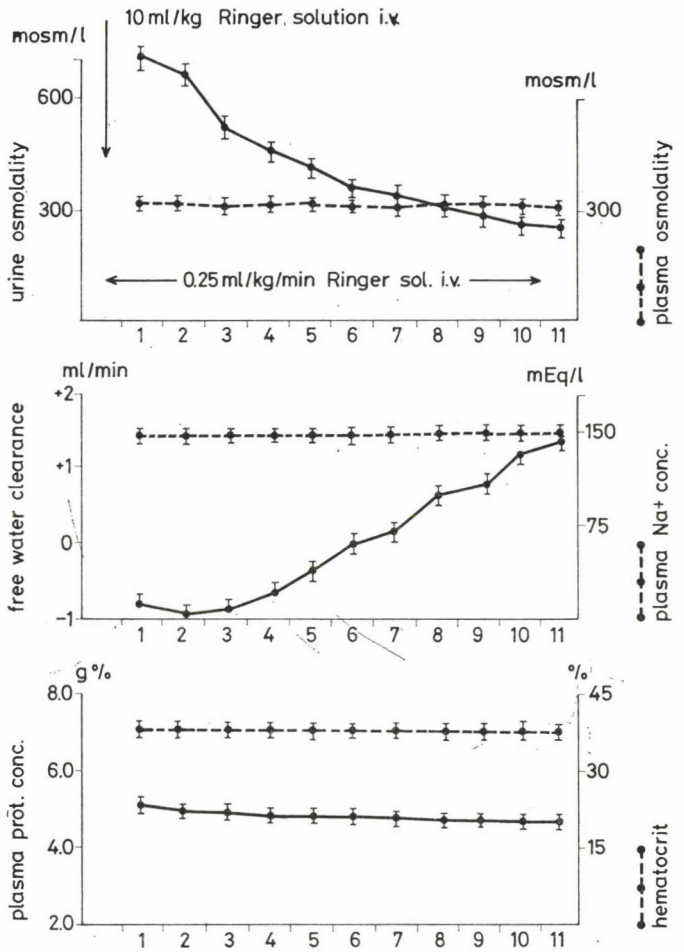

Fig. 3. The urine osmolality, the free water clearance and plasma protein concentration (plasma prot. conc.) during Ringer solution infusion, solid lines. Points are means ± S. E. M. The plasma osmolality, plasma sodium concentration (plasma Na^+ conc.) and haematocrit during the experiment, dropped lines. Points are means ± S. E. M.

course of the experiment, and the plasma protein concentration fell slightly from 5.03 ± 0.07 g/dl to 4.69 ± 0.11 g/dl.

The level of the plasma potassium concentration was 3.88 ± 0.08 mEq/l during the first period and 3.74 ± 0.06 mEq/l during the last one.

In 20 animals vasopressin was administered during the Ringer solution induced diuresis. Following the period 5 10 mU/kg of vasopressin was infused during 10 minutes and after this the kidney parameters were examined during the period 6 and period 7. After the period 7 50 mU/kg of vasopressin was given intravenously in a constant infusion during 60 minutes.

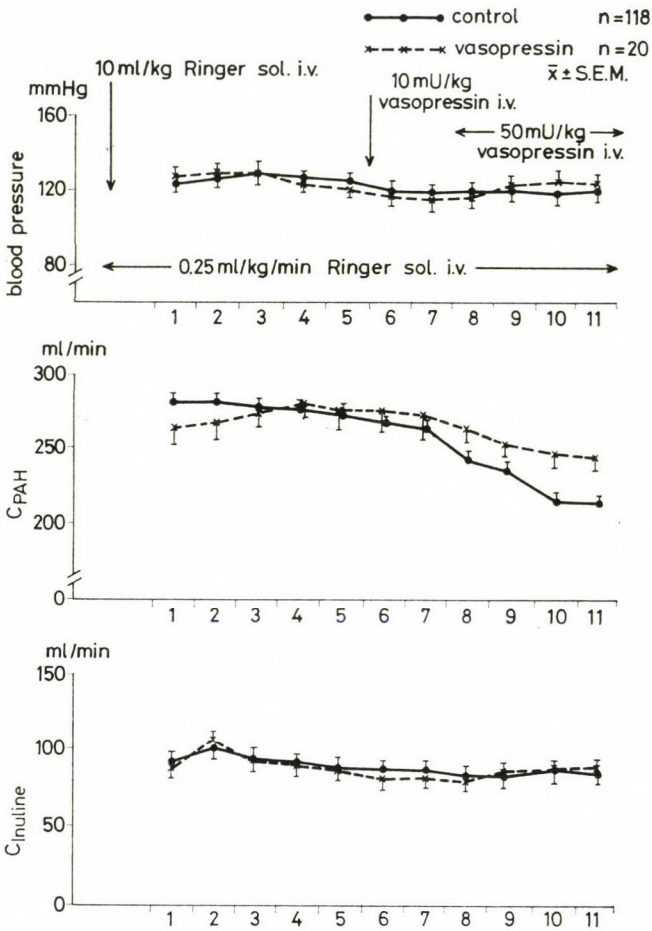

Fig. 4. Effects of vasopressin on blood pressure, PAH clearance (C_{PAH}) and glomerular filtration rate ($C_{inuline}$). Values are means ± S. E. M. for successive 15 min periods

As the Fig. 4. shows the arterial blood pressure, the C_{PAH}, and glomerular filtration rate remained relatively stable throughout each study.

When a vasopressin infusion was superimposed on the Ringer solution diuresis, the GFR remained unchanged, and urine excretion rose strikingly (Fig. 5). The urine flow during the period 11 was 4.09 ± 0.25 ml/min in the Ringer loaded animals, when a vasopressin infusion was superimposed on the Ringer solution the urine excretion was significantly higher 5.54 ± 0.72 ml/min ($p < 0.01$).

The administration of vasopressin provoked a modest rise in sodium excretion and potassium output.

While in the Ringer loaded dogs the sodium excretion during the last period was 221 ± 17 μEq/min, in the vasopressin infused animals 340 ± 48

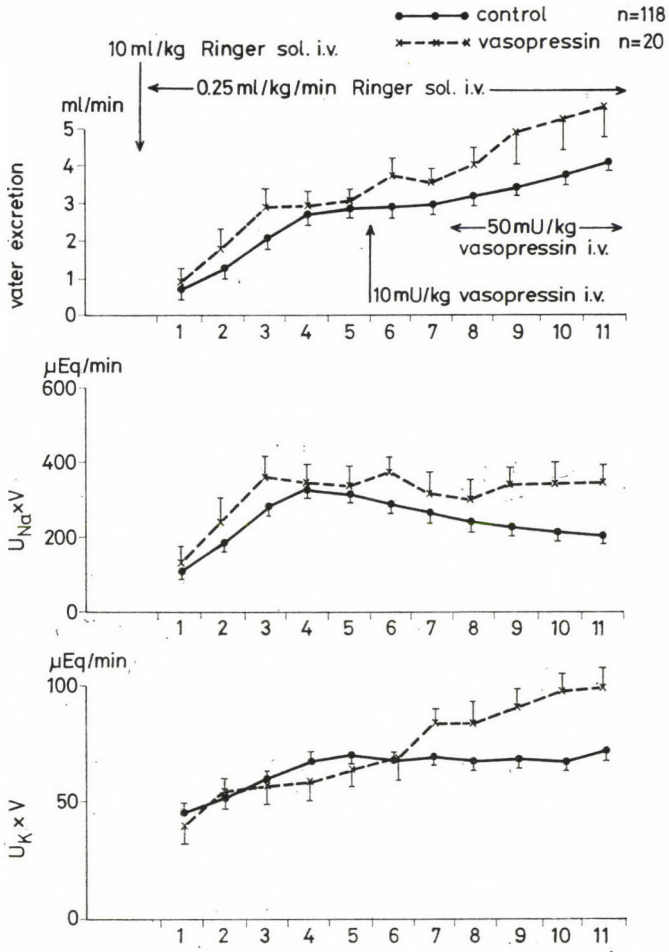

Fig. 5. Effects of vasopressin on urine flow (water excretion), sodium excretion ($U_{Na} \times V$) and potassium excretion ($U_K \times V$). Values are means $\pm$ S. E. M. for successive 15 min periods

μEq/min ($p < 0.01$), and the potassium excretion was 75 ± 4 μEq/min and 101 ± 10 μEq/min, respectively.

Figure 6 shows that the pattern of the urine osmolality, C_{H_2O}, plasma protein concentration, and hematocrit changes are similar in the two series during the experiment.

An additional study was performed on 22 anesthetized dogs, when the Ringer solution load was increased following the period 7. The animals received 25 ml/kg Ringer solution intravenously during 60 minutes (period 8, 9, 10, 11), so the increase in extracellular volume at the end of the experiment was about 5% of the body weight. Results obtained from this study are summarized in Figures 7, 8, 9.

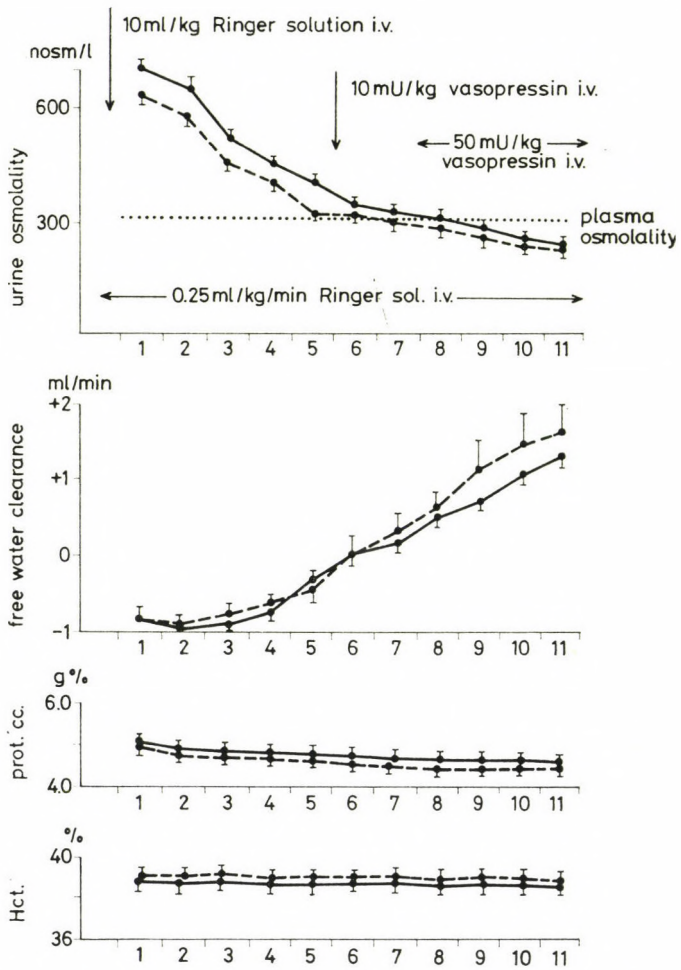

Fig. 6. Effects of vasopressin on urine osmolality, free water clearance, plasma protein concentration (prot. cc.) and haematocrit (Hct). Values are means ± S. E. M. for successive 15 min periods

As we can see on Fig. 7 the increment of the Ringer solution loading did not change either the arterial blood pressure, or C_{PAH}, or glomerular filtration rate.

Figure 8 shows that the overhydration provoked a three-fold increase in the urine excretion, while the sodium excretion was doubled, and the potassium output increased slightly.

On the Figure 9 we can observe that in the course of Ringer solution overhydration C_{H_2O} rose sharply and the urine osmolality became hypoosmotic as compared to the plasma osmolality. During the Ringer solution overhydration the plasma protein concentration decreased by about 0.45 g/dl and the hematocrit by 3%.

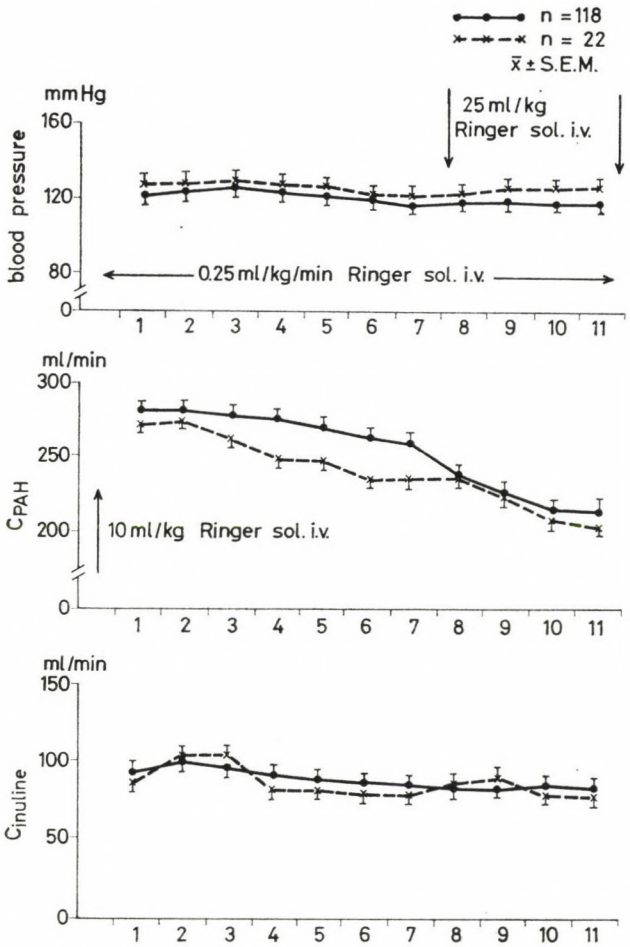

Fig. 7. Effects of the "Ringer solution over loading" on the blood pressure, PAH clearance (C_{PAH}) and the glomerular filtration rate ($C_{inuline}$) during Ringer solution infusion. Values are means ± S. E. M.
The solid lines represent the data of the control experiments, the dropped lines demonstrate the parameters during "double loading" experiments

Discussion

The present studies were undertaken to quantify the magnitude and time course of changes in glomerular filtration rate, the urine and sodium excretion during isosmotic extracellular volume expansion induced by intravenous Ringer solution infusion. Clearance experiments were performed in 118 anesthetized dogs.

These experiments clearly show that some factor other than increased filtered sodium accounts for part of the sodium and water excretion increase

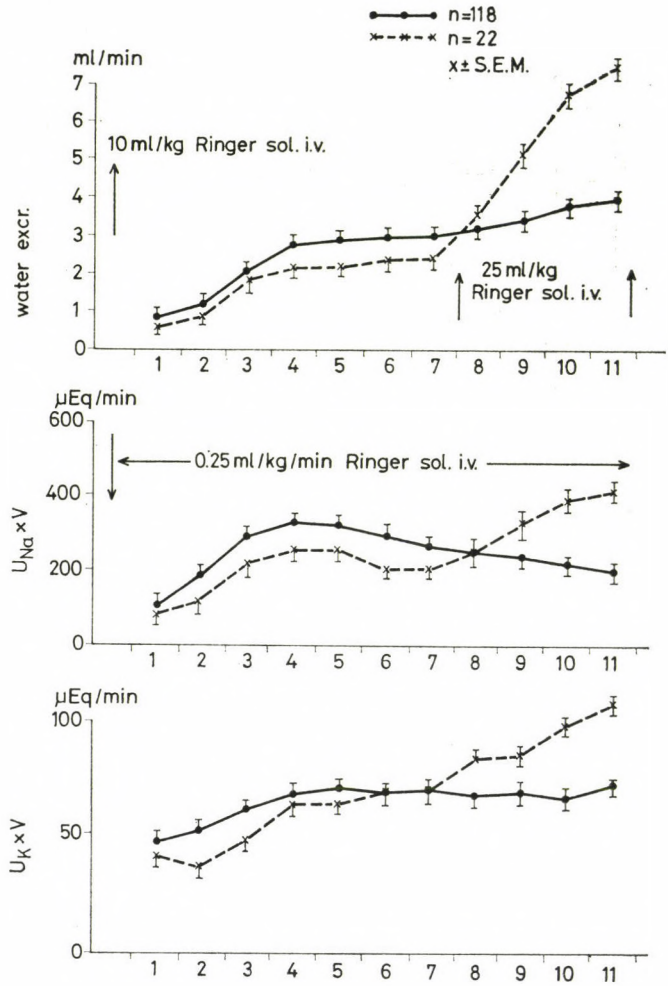

Fig. 8. Effects of the "Ringer solution over loading" on the urine flow (water excr.), sodium excretion ($U_{Na} \times V$), and potassium excretion ($U_K \times V$). Other details as in Figure 7

that follows Ringer solution infusion in the dog. In all experiments in the present series the glomerular filtration rate was unchanged. Plasma sodium concentration was relatively constant in most experiments.

De Wardener and co-workers [12] have suggested that the increase in sodium excretion after saline infusion is essentially independent of the changes in glomerular filtration rate. In their experiments GFR increased or decreased with approximately equal frequency when saline was infused, excretion always increased.

It is well established that saline loading has an extensive inhibition of renal tubular sodium reabsorption. On the basis of micropuncture and clearance

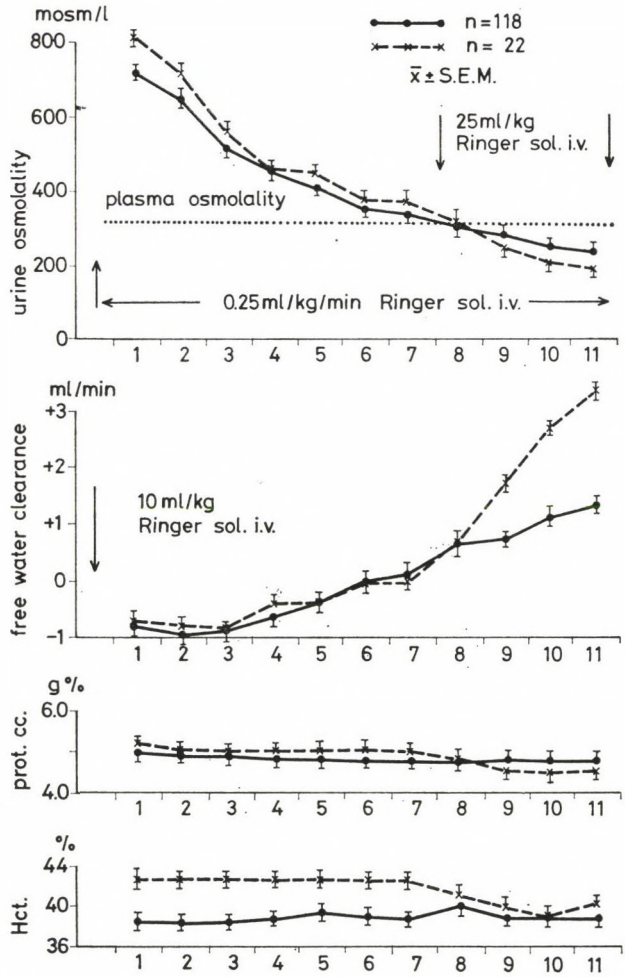

Fig. 9. Effects of the "Ringer solution over loading" on the urine osmolality, free water clearance, plasma protein concentration (prot. cc.), and haematocrit (Hct). Other details as in Figure 7

studies in dog, it was concluded that the inhibition of sodium transport develops primarily within the proximal tubule [23, 40]. Despite considerable reductions in proximal reabsorption suggested by these studies, sodium excretion remained at relatively low levels. The major increment in sodium excretion apparently develops only after saline loading alters the capacity for sodium transport in the distal tubule.

The magnitude of saline diuresis during saline loading is therefore determined to a large extent by the fraction of the sodium escaping proximal reabsorption and is reabsorbed in the distal nephron.

The factors which affect sodium reabsorption in the distal nephron are poorly understood.

In the present study before collection of the first period the dogs had received in excess 10 ml/kg of Ringer solution, an amount sufficient to activate the "tubular effect". At least 200 μEq per minute of the elevated rate of sodium excretion was presumably due to the action of the tubular factor. In the first series of the experiments this amount rather decreased than increased (Fig. 2).

The results of the "double loading" (third series) experiments suggest that the magnitude of this change in tubular reabsorption is related to the volume of Ringer solution infused.

After the second Ringer solution load, an additional tubular effect was demonstrated; at least 150—200 μEq per minute of the increase above control excretion was not related to increased filtered sodium (Figs 7 and 8).

The initial infusion of Ringer solution had not elicited the maximal "tubular effect", the magnitude of this effect increased when rising quantity of Ringer solution was infused.

The data summarized in Fig. 2 do not suggest that the magnitude of the "tubular effect" increases with progressive Ringer loading. Our results demonstrated that the greater part of the infused sodium is retained in the organism.

The results of the experiments in which Ringer solution was infused to keep the plasma chloride, potassium and bicarbonate concentrations relatively constant were not different from those in which saline was given. Changes in the concentrations or proportions of the principal filtrable anions thus do not account for the results.

Three possible explanations for the decrease in tubular reabsorption of sodium have been tested. It has been proposed that a decrease in plasma protein concentration may decrease passive sodium reabsorption due to osmotic forces in the proximal tubule [5, 28]. In a previous article we have suggested that the decrease of tubular sodium reabsorption is attributed partly to the decreased plasma protein concentration [21].

Brenner et al. [4], Lewy and Windhanger [26] observed that the oncotic and hydrostatic pressure existing in the peritubular capillaries might influence the proximal tubular sodium reabsorption.

But in some experiments when dilution of plasma proteins was prevented by adding albumin to the saline infusion, the decrease in tubular reabsorption of sodium was entirely comparable to that noted when saline alone was infused [25]. Changes in protein oncotic forces, therefore do not account for the "tubular effect" on sodium excretion.

De Wardener and associates [12] have proposed that a humoral agent other than aldosterone is the "tubular factor" involved.

Atrial natriuretic peptide (ANP) has been postulated to have a role in the regulation of fluid and sodium homeostasis [11, 33, 34]. It was demonstrated

that the intrarenal infusion of ANP at a dosage that evokes a potent natriuretic effect, without changes in renal blood flow (RBF) or glomerular filtration rate, is capable of significantly decreasing the release of renin. The renin-angiotensin system, on the other hand is known to exert a marked antinatriuretic effect by acting directly on the kidney. Further results suggest that the modulatory activity of angiotensin II. on the natriuretic effect of ANP could be negligible under normal conditions [7].

However, other observations are inconsistent with the concept that atrial peptides participate in blood volume regulation and these results suggest that in the dog, endogenously released ANP is not responsible for the diuretic and natriuretic response caused by atrial distension [18]. Chronic bilateral atrial appendectomy in the dog does not alter the renal response to volume expansion or attenuate the increase in ANP [1]. In addition these results show that vagal pathways are not required for the release of ANP and that vagotomy fails to uncover any effect of atrial appendectomy on renal function. The question concerning the role of ANP in the renal response to volume expansion remains unanswered.

In the dog the net tubular reabsorption of sodium may diminish during saline loading, independently of salt retaining hormones and even in spite of a decline in glomerular filtration rate.

It has been suggested that increased renal blood flow during saline loading may be one factor that contributes to the decreased tubular reabsorption of sodium since in the saline loaded dog when renal blood flow is decreased experimentally the net tubular reabsorption increases [15].

The natriuretic effect of increased plasma flow is greater in the animals receiving small saline loads than in the hydropenic group, although control rates of blood flow were similar in both groups [16]. This latter observation is consistent with the view that relatively small saline loads may activate natriuretic factors other than blood flow, and thereby may condition the kidney for a greater (natriuretic) response to increased blood flow.

In our experiments the sodium excretion increases in the first part of the observation when the C_{PAH} is unchanged (we suppose that the C_{PAH} measures the effective renal plasma flow), but in the second part the C_{PAH} decreases and there is a fall in the sodium output too. But in the third series in our experiments during the "doubling load" when the C_{PAH} declines significantly, the sodium excretion increases without any change in the glomerular filtration rate (Figs 7 and 8).

Tubuloglomerular feedback (TGF) is an intrarenal homeostatic mechanism whereby increased sodium chloride uptake at the macula densa causes vasoconstriction of the afferent arteriole of the same nephron. TGF has been shown to be stimulated by plasma volume or extracellular volume (ECF) depletion and inhibited in states of ECF volume expansion. Thus TGF probably

helps the kidney in maintaining ECF volume homeostasis. Evidence from in vivo microperfusion studies support distal nephron tubular chloride concentration and/or its transport as being at least one signal [6, 44], while other studies support an important role for distal tubule fluid osmolality. Other investigators have suggested that both of these parameters may work in concert to stimulate TGF [29, 41].

Our results show that during Ringer solution infusion the effective renal plasma flow, and certainly the renal blood flow too, decreases progressively. We may suppose that the increase of distal tubular load activates the TGF, thus decreasing the RBF.

But it is well recognized that renin secretion is inhibited by acute expansion of plasma volume. Intravenous infusion of sodium chloride inhibits renin release by a renal tubular mechanism [10].

It has been suggested that the increased metabolic work associated with high solute reabsorption in the proximal tubule and in the region of the macula densa enhances the conversion of ATP to adenosin, which is a potent vasoconstrictor in the kidney [31].

Controversy exists concerning the effector limb (s) of the TGF mechanism, but many investigators feel that part of this mechanism may involve local alteration in synthesis and/or release of intrarenal hormones. If this is the case, then baseline hormonal status may influence the ultimate outcome of the TGF response initiated by the signals (or others) described above.

On the basis of the present observations it may be suggested that the decreased net tubular reabsorption of sodium and water which accompanies extracellular volume expansion in the dog occurs through a mechanism that also decreases the ability to concentrate the urine. In our experiments the urine became hypoosmotic as compared to the plasma.

The ability of mammalian kidneys to produce urine that is more concentrated than plasma is usually attributable to the action of vasopressin (ADH) which increases the permeability of the distal nephron to water, thereby allowing osmotic equilibration of collecting duct fluid with the surrounding hyperosmotic medullary interstitium.

Under the conditions of the present experiments the decrease in urinary osmolality cannot be attributed to a decreased availability of vasopressin. In the second series of the experiments exogenous vasopressin was infused and the changes of the kidney function were similar to those observed in the experiments without ADH loading.

An additional factor that may influence urinary osmolality is the rate of medullary blood flow since an inverse relationship must exist between medullary blood flow and the degree of medullary hypertonicity [2, 20]. This factor could be of importance in producing the decreased urinary osmolality observed in the present studies, and it is entirely possible that changes in medullary

blood flow may indirectly influence sodium reabsorption and sodium excretion. If medullary blood flow rises, the degree of medullary hypertonicity will decline, and secondarily the volume of water lost from the descending limb would be diminished. Therefore, the rate of flow through the ascending limb would be increased, and the concentration of sodium in the fluid reaching this latter segment would be decreased. It is entirely possible that the net transport of sodium by the ascending limb would be diminished under such conditions. Lassiter, Mylle and Gottschalk [24] have reported that during saline loading fractional reabsorption by the proximal tubule is unchanged, whereas the tubular fluid to plasma inulin ratio in the early part of the distal convolution of cortical nephrons may be decreased. This observation is consistent with the view that volume reabsorption within Henle's loop is diminished during saline loading. Such changes could be even more marked in juxtamedullary nephrons with long loops of Henle.

The possibility cannot be excluded that Ringer solution loading through some unidentified pathway directly affects the transport system of the Henle's loop ascending limb.

Rector and associates [30] have reported that during water diuresis urinary dilution may increase with saline loading and reduced glomerular filtration rate and on the basis of the observation these authors have suggested that the decreased tubular reabsorption occurs at some site proximal to the ascending limb of Henle's loop.

It may appear unlikely that an increased flow through Henle's loop could have occurred in the present studies at a time when glomerular filtration rate was unchanged, unless a simultaneous decrease in proximal reabsorption occurred.

On the basis of the present data it is assumed that Ringer solution infusion in dog provokes a progressive inhibition of proximal tubular sodium reabsorption which additively with the medullary flow increase and the sodium reabsorption decrease in the ascending limb of Henle's loop produces a considerable sodium and water loading increase in the distal part of the nephron. Subsequently an alteration develops in distal tubular fluid sodium and chloride concentrations and the concentrations of these ions or the transport of the sodium or chloride activates the tubuloglomerular feedback and the renal blood flow decreases.

It is well established that Ringer solution loading decreases renal blood flow without modifying the glomerular filtration rate. Thus the present studies demonstrate that in this experiments the tubuloglomerular feedback regulates the blood pressure of peritubular capillaries.

The present studies may help to reconcile the conflicting conclusions regarding the tubular sites of altered sodium transport during Ringer solution loading in dog. Ringer solution infusion (in the physiological range) apparently

provokes a progressive inhibition of proximal sodium reabsorption. Initially the sodium and water excretion increases similarly and the free water clearance doesn't change. Thereafter a progressive alteration in tubular sodium reabsorption appears to develop as reflected by the sharp rise in C_{H_2O} with only modest increases in sodium excretion. It is during this phase that the TGF moderates the proximal rejectate and the water excretion increases while the sodium excretion remained constant or decreased and the urine osmolality became hypoosmotic as compared to the plasma.

The present experiments may permit an analysis of several factors that affect tubular function during Ringer solution loading in dog.

In any event, the major increment in salt excretion provoked by saline or Ringer solution loading apparently develops only after saline loading alters the regulatory capacity of the different nephron parts.

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PRESENCE OF PHOSPHO-TYROSINE IN ALKALINE HYDROLYSATE OF PIG SKELETAL MUSCLE MYOSIN

S. Fazekas, I. Óváry*, Vilma Székessy-Hermann

Second Institute of Biochemistry and *Psychiatric Clinic, Semmelweis University Medical School, Budapest, Hungary

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A small part of the P-containing fraction of myosin hydrolysate which could be eluted with basic solvent (triethylamine) buffer was accumulated on chromatographic column. On the basis of the elution profile, P- and tyrosine content determination, moreover the thin layer chromatographic separation and specific Tyr reaction, this fraction is suggested to be phosphotyrosine.

The concentration of P-Tyr is about 2 mol in pig muscle myosin calculated for 500 kDa protein. As the higher P-containing myosins also have an approx. 2 mol P-Tyr, it is thought that this concentration had been present in myosins already before alkaline hydrolysis.

Keywords: phosphotyrosine, pig muscle myosin.

The total P content of the hydrolysates of myosins cannot be recovered with a chromatographic method elaborated previously [4, 5] for the P containing fractions of myosins. A smaller part of P content (11—14%) remained on the ion-exchange resin (Dowex 1 × 8 column), however, in the course of the column regeneration a perfect elution is reached with 1 M HCl and 1 M NaOH solutions. Earlier this fraction was thought as a byproduct of alkaline hydrolysis.

Different amounts of endogeneous P containing fraction are found in the alkaline hydrolysates of myosins prepared from different sources [6—9]. During the studies of elution profiles it was observed that this P fraction from the column can be eluted with appropriate concentrations of basic organic compound buffers such as pyridine, triethylamine.

Studies of elution profiles and the observations of P-binding stability, the effect of alkaline hydrolysis on the fraction called our attention to the P-Tyr. Therefore, for analysis we produced the chromatographic preparations of the fraction to compare to the commercial P-Tyr and synthetic P-Tyr preparations. The presence of P-Tyr in alkaline hydrolysate was confirmed by paper- and thin-layer chromatographic separations as well as specific tyrosine and phosphate reactions.

Correspondence should be addressed to
Sándor Fazekas
Second Institute of Biochemistry, Semmelweis University Medical School
H-1444 Budapest, P. O. B. 262, Hungary

Materials and methods

The chemicals used were:
O-phospho-L-Tyrosine-t (P-Tyr) Sigma, Germany; Phosphoric acid, Phosphoric acid anhydrid, P_2O_5, HNO_3, H_2SO_4, Ammonium molybdate, 2-mercaptoethanol, Merck, Darmstadt; Perchloric acid, Aldrich, Germany; ATP, DEAE-cellulose, Dextran blue, Serva, Switzerland; Sepharose 4B (CL 4B), Pharmacia; Triethylamine, Dowex 1 × 8, Fluka, Switzerland; Tris [= Tris-(hydroxymethyl)-amino-methan], KCl, $KHCO_3$, Acetic acid, Chloroform, Methanol, Reanal, Budapest; "Fixion Fertigfolien" TLC chromatoplates with cation-exchange Dowex resin layer, Chinoin, Budapest; DC-Alufolien Polyamid 11 F 254 sheet, Merck, Darmstadt.

The synthesis of P-Tyr was performed by the Plimer method [19] modified by Mitchell and Lunan [17]. The P-Tyr from hydrolysate was separated with ion-exchange chromatographic technique of Mitchell and Lunan [17]. The synthetic and commercial P-Tyr preparations were also chromatographied successively on a Dowex 1×8 column (0.9×6 cm) to identify their elution volumes and profiles. For the separation of the P-Tyr containing fractions of the myosin the same Dowex 1×8 column was applied.

The myosin was prepared from domesticated pig skeletal muscle (m. long. dorsi) of 8 months' old pigs. The elaborated method is detailed elsewhere [4, 8]. For the production of P-containing hydrolysates gel filtered and lipid-free myosin preparations were applied [11].

Protein concentration was determined by biuret micro method [13] and uv absorbance measurements checked by dry weight gravimetric measurements of lipid and salt-free preparations.

The P-binding of P-Tyr has a significant alkali-stability [12, 16]. When the lipid-free myosin was hydrolysed in teflon ampoules (3 M KOH, 105 °C, 10 h) in such circumstances the P binding of P-Tyr was moderately hydrolysed. The bulk of the P containing fraction of the hydrolysate was removed from the column by chromatographic separation technique [4, 5], except the P-Tyr fraction which remained on the column (Fig. 2).

The P-content of gel filtered and lipid-free myosin preparation and also of each elution fraction of alkaline hydrolysate was determined by the Fiske and Subbarow method [10] in the presence of 0.1 M $HClO_4$ and the final reduction was reached with 1% ascorbic acid [15].

When the separation of the hydrolysate was finished with 2 M KCl the column was washed with 0.005 M $KHCO_3$ exhaustively to remove all Cl ions. Then a second portion of diluted myosin hydrolysate was percolated through the column and separated again as before. The separation process, with a third portion of hydrolysate was repeated again. Thus altogether 148—150 mg myosin hydrolysate was chromatographied on the column. From this quantity of myosin a sufficient amount of P-Tyr could be accumulated to be identified by chromatographic and TLC separations and specific reactions [1, 20, 24]. Finally, after hydrolysing the P-Tyr, the free Tyr was run on cationic ion-exchange-coated TLC sheet by a method of Sajgó and Dévényi [21]. The spots were observed by uv light and identified with specific reaction and R_f values.

Results

The gel filtration profile of ultracentrifuged (uc-) myosin is shown in Figure 1. The KCl concentration in uc-myosin was increased to 3 M (final conc.) before gel filtration in order to facilitate the dissociation of associated proteins, lipids and RNA traces. The first peaks of protein (tubes 14—19) were collected as myosin.

The protein phosphatase activity of the preparations was checked with Ehler's method [3]. No phosphatase activity was perceived in gel filtered myosin, it disappeared in the course of the first DEAE-cellulose treatment. Only a small RNA-P-content was found in the preparations (0.1—0.3 mol P/mol myosin), when it was determined by Schneider's pentose analysis method [22] and the results were corrected at the calculation of real P-content. Always

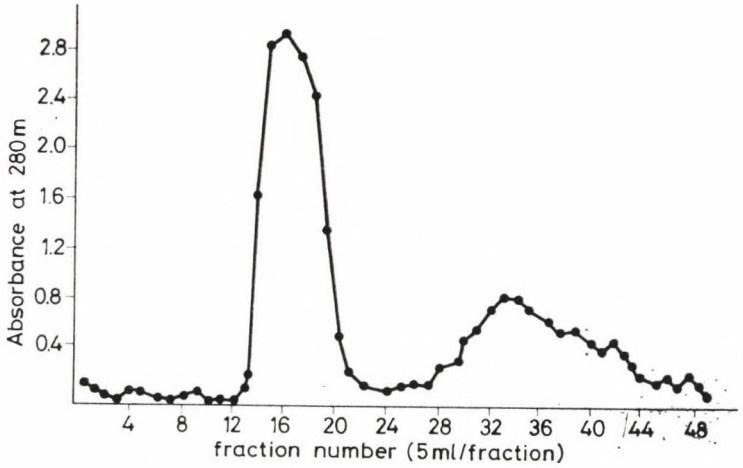

Fig. 1. Gel filtration and elution profile of pig skeletal muscle myosin on Sepharose 4 B column (1.9×82 cm). The column was equilibrated and eluted with 0.5 M KCl containing 8 mM $KHCO_3$. About 130 mg uc-myosin was stored in 3 M KCl for a night and applied onto the column in 8 mL volume. Flow rate, 18—19 mL/h. The protein content was detected by uv absorbance at 280 nm. The content of tubes 14—19 was pooled as myosin, about 90 mg protein in 30 mL volume. Additional batches of uc-myosin were applied onto the same column to obtain higher amounts of gel filtered myosin

the lipid- and phospholipid-free myosin samples were hydrolysated in teflon ampoules. The total P-content of the hydrolysate was determined before the separation of the P-containing fractions, then the KOH concentration of the hydrolysate was diluted to 0.005 mol and percolated through a Dowex 1×8 column and separated as shown in Figure 2.

The separation on the column was repeated two times successively in order to accumulate such a quantity of P-Tyr which was sufficient for analysis and identification (see method).

The separations and elution volume of commercial P-Tyr and the P-Tyr fraction of the hydrolysate accumulated from the three different portions of the myosin can be eluted using a 0.5 mol triethylamine-acetate buffer shown on Figure 3. In the elution volume of our synthetic P-Tyr an insignificant deviation was found only. It is interesting to note that the separation of the P-containing fractions, like the basic amino acid phosphates and the P-Tyr fractions, could be performed on the same Dowex 1×8 column where the synthetic and the commercial P-Tyr samples and samples originating from myosin were gained and investigated.

The tubes of the P-Tyr fraction were collected, divided into two equal parts, and were lyophilized. A part of the lyophilized remnant was applied on a TLC-sheet and separated with the method of Rubin and Ears [20] (Fig. 4), while another part of it was developed on a Polyamid "Alufolien" layer with a mixture of isobutanol and 0.5 M NH_4OH. The spots of the latter sample were

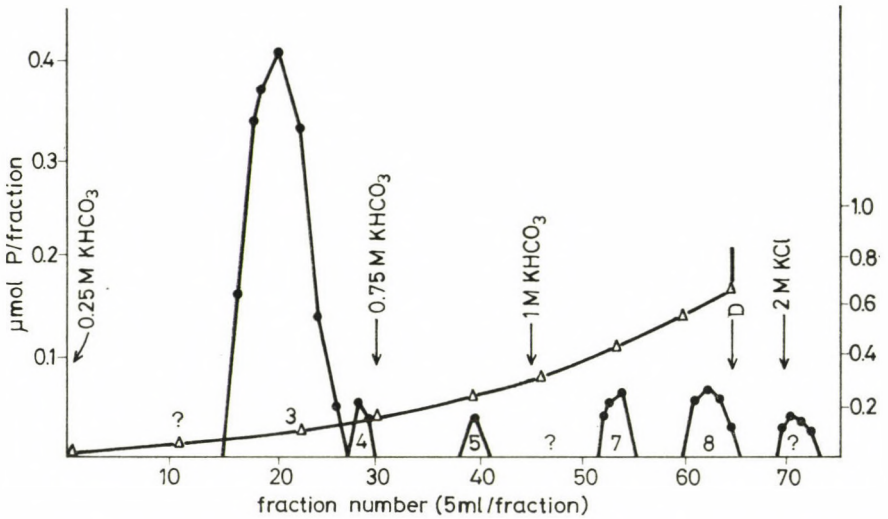

Fig. 2. Elution profile of amino acid phosphates of myosin hydrolysate obtained from 48 mg protein before P-Tyr chromatography. Hydrolysis was performed in a teflon ampoule (3 M KOH, 105 °C, 10h). The hydrolysate was diluted to 0.005 M KOH concentration and percolated through Dowex 1×8 column (0.9×6 cm). Separation was performed with a linear step gradient chromatographic technique using a mixing chamber with a capacity of 160 cm^3 containing 0.02 M $KHCO_3$ and 120 mL of 0.25 M $KHCO_3$ in the reservoir equipped with an electromagnetic stirrer. The $KHCO_3$ concentration was increased and changed as indicated by the arrows. 1 M $KHCO_3$ was applied onto the column directly (D) without mixing and 2 M KCl for regeneration of the column. The peaks are represented by P content of tubes in apparent μmol P per total fraction. Peaks in turn: 2.. P-amidate, 3.. P-Arg, 4.. Pi, 5.. P-Lys, 7.. N^{π}-P-His, 8.. N^{τ}-P-His, 6, 9.. unidentified. Generally from the most of myosin sources 9 different P-containing chromatographic fractions can be produced [8]. The P-fractions which were small to be detected or still unidentified are marked by ?

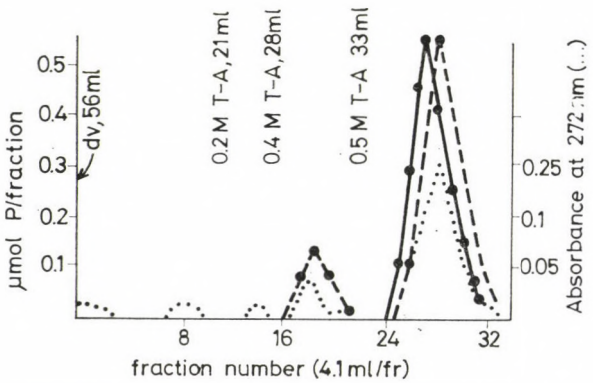

Fig. 3. Elution profile of P-Tyr Sigma Co (. —. —.) and P-Tyr from the hydrolysate of 148 mg myosin (— —) on Dowex 1×8 column (0.9×6 cm). Elution was performed with tryethanolamine — acetate (T—A) buffer, pH 5.0, by step gradient method of [8]. The concentrations are increased and changed as indicated by arrows. Uv absorbance was measured at 272 nm in 0.2 M NaOH solution

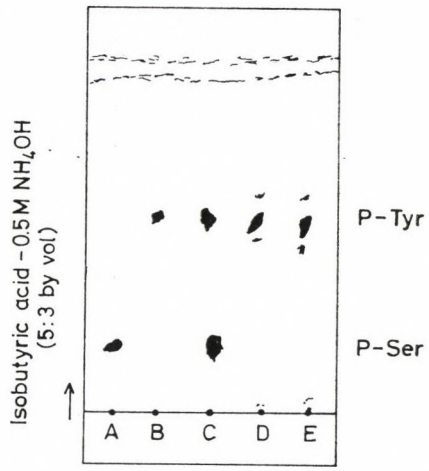

Fig. 4. Thin-layer chromatography of P-Tyr fraction of pig myosin in presence of controls developed with the method of [20]. The spots checked by uv light were stained with ninhydrin spray. A.. synth. P-Ser, B.. synth. P-Tyr, C.. mixture of P-Ser and P-Tyr, D.. P-Tyr from hydrolysate

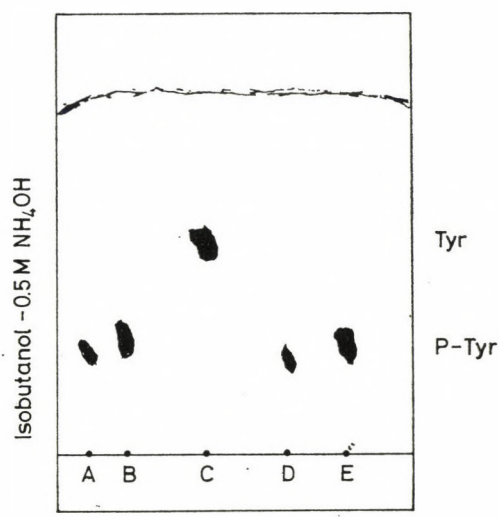

Fig. 5. Thin-layer chromatography of P-Tyr samples on DC-Alufolien Polyamid 11 F 254 sheet, developed with isobutanol — 0.5 M NH_4OH mixture. P-Tyr: A.. synthetized by us, B.. Sigma preparation, D, E.. myosin derived samples, C.. Tyr (control), respectively. The R_f values for P-Tyr and Tyr is 0.23 and 0.62, respectively

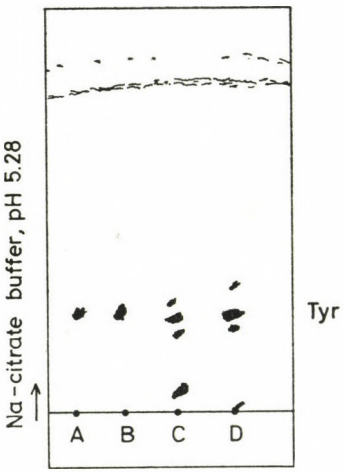

Fig. 6. Thin-layer chromatography of Tyr fractions obtained with the 6 N HCl hydrolysis from P-Tyr samples developed on "Fixion" X 8 ion exchange resin coated plates with the method of [21]. Tyrosine originating from P-Tyr compounds: A.. synthetic, B.. Sigma preparation, C, D.. myosin hydrolysate, E.. control Tyr (Sigma). Other control amino acids seen on the plate are as follows: Lys, Phe, Tyr and Leu

visualized by uv light. During the process we found that the P-Tyr had a 0.23 R_f value (Fig. 5).

In the presence of high salt concentration when the chromatogram was developed in 1 M NaCl-containing buffer with the Bardócz method [1], the spots of P-Tyr could not really be evaluated as they were near the front.

The second portion of liofilized residue was hydrolysed in 6 N HCl and the P-Tyr was identified on a TLC-chromatoplate developed in the presence of control amino acid samples using the Sajgó and Dévényi method [21] (Fig. 6).

Discussion

The phosphate ester bond in P-Tyr is far more resisting alkaline than acidic hydrolysis. The bond of the P-Tyr tolerated the alkaline hydrolysis fairly well in 5 N KOH (NaOH) for 30 min, at 155 °C [12, 16]. In our conditions (3 M KOH, 105 °C, 10h) the P-Tyr ester bonds were moderately hydrolysed in teflon ampoules.

Summing the results 4.2 μmol P was found in the hydrolysate of 150 mg lipid-free myosin applied on a chromatographic column. Thus, the total P-content of the myosin was 14 mol P/mol myosin calculated for 500 kDa protein as approx. molecular mass.

After the usual separation of P-containing fractions 11—13.1% (0.50—0.55 μmol P) of the total P-content remained on the Dowex 1×8 column.

It could be eluted with an organic buffer only, just like the P-Tyr. Consequently the amount of P-Tyr in the pig skeletal myosin was 2 mol. As to earlier studies it was concluded that the P-Tyr concentration did not increase above 2 mol, thus the phosphorylation test could not be accomplished. Still the question arises whether the P-Tyr may exist in the myosin before the hydrolysis or it was produced in the course of the hydrolysis from the N-P types of P-amino acids by transesterification as it had been proved earlier using model reactions [23].

Similarly to the present amount of P-Tyr originating from pig skeletal muscle myosin, from the different myosin sources such as skeletal and smooth muscle, even from cell myosin nearly 2 mol P-Tyr could be gained.

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EFFECTS OF THE LIPOLYSIS INHIBITING AGENT β-PYRIDYLCARBINOL (RONICOL®) IN ACUTE MYOCARDIAL ISCHAEMIA

T. FAZEKAS, L. SZEKERES*

First Department of Medicine, and Institute of Pharmacology*,
Szent-Györgyi Albert University Medical School, Szeged, Hungary

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The antilipolytic, nicotinic acid analogue β-pyridylcarbinol (Ronicol®) has prel viously been reported to decrease the free fatty acid (FFA) concentration of the arteria-blood, and to moderate the FFA-uptake and O_2-consumption of the myocardium; on this basis, the drug may be expected to exert a cardioprotective action.

The cardiac effects of Ronicol were therefore studied on a self-control, 'single-vessel' coronary artery ligature dog model. The left anterior descending coronary artery (LAD) was prepared in the in situ heart of anaesthetized, thoracotomized animals. Following the control ligation, a stabilization period and Ronicol infusion (1 mg/kg iv. during 10 minutes), the LAD was repeatedly ligated. The duration of the individual occlusions was 10 minutes. Ronicol significantly decreased the arterial FFA concentration and the epicardial ST segment elevation; its antilipolytic and anti-ischaemic effects were protracted and were still observed 120 minutes after pretreatment. The drug did not decrease the inhomogeneity of ventricular depolarization in the isch-aemic myocardium and in the dose applied it had no influence on the heart rate, arterial blood pressure, left ventricular end-diastolic pressure and left ventricular contractility (LV dP/dt_{max}).

In the canine myocardial infarction model employed it was observed that the duration of the anti-ischaemic effect of Ronicol (1 mg/kg iv.) is about 120 minutes. It has the advantage that it does not possess the unwanted cardiovascular side-effects displayed by nicotinic acid observed by us too in this model earlier (Cardiol. Hung. 13, 33—41, 1984).

Keywords: experimental myocardial ischaemia, inhibition of lipolysis, Ronicol, antiischaemic effect.

It is now widely known that FFAs and fatty acid esters accumulating in the ischaemic myocardium play an important pathogenetic role in the development of postocclusion myocardial injury, and in the genesis of the often life-threatening disturbances of impulse formation and conduction arising as a complication of acute myocardial infarction [AMI; 4, 6, 8, 18, 25, 26, 27]. Fatty acid excess impairs both the electrical and mechanical function of the heart: it enhances myocardial O_2 consumption [17, 26], and concomittantly decreases its contractile force [4, 11] and aggravates myocardial ischaemia [14].

Correspondence should be addressed to
Tamás FAZEKAS,
First Department of Medicine, Szent-Györgyi University Medical School,
H-6701 Szeged, Korányi fasor 10, P. O. Box 469, Hungary

The *inhibitors of lipolysis* block TG hydrolysis both in the fat tissues and within the myocardium, thereby diminishing the arterial FFA supply and the local myocardial FFA release [10, 15, 24]. Therefore, on the basis of the above considerations, antilipolytic drugs are expected to exert an anti-ischaemic effect [6, 8, 25].

One of the antilipolytics most widely used for a long time is nicotinic acid, which decreases the FFA and fatty acid ester contents of the blood and the ischaemic myocardium [10, 27], at the same time it increases the uptake and utilization of glucose [18]. Despite these beneficial metabolic effects, nicotinic acid is not a suitable antagonist of the FFA excess accompanying AMI, for it also has unfavourable side-effects (hypotension, increase of cardiac output, coronary 'steal' phenomenon, arrhythmias; 7, 21). On the contrary, clinical and experimental experience with *long-acting nicotinic acid analogues* with no considerable cardiovascular side-effects is highly promising [16, 21, 23, 25]. This group of drugs includes one studied in the present work: β-pyridylcarbinol (Ronicol®; F. Hoffmann-La Roche).

The primary aim of our experiments was to establish whether this drug may possess an appreciable anti-ischaemic effect in the self-control 'single-vessel' ligature dog model applied [5, 7] and, if so, what is the duration of this action. We were also interested in learning how Ronicol may influence the electrical and mechanical function of the ischaemic canine heart [9].

Methods

The experiments were performed on mongrel dogs weighing 8—18 kg fasted for 14—16 hours, anaesthetized with Na-pentobarbital (30 mg/kg iv), thoracotomized and ventilated artificially. Unipolar and multipolar (composite) electrodes were sutured onto the anterior surface of the left ventricle, i. e. to the area to be made ischaemic after ligation. Unipolar electrodes were used to measure the epicardial ST segment elevation (reflecting the severity of the ischaemic myocardial injury), and the composite electrode to determine the activation time of the ischaemic myocardial area [4, 5]. The left ventricular end-diastolic pressure (LVEDP) and contractility (LV dP/dt_{max}) were recorded, as was the limb standard II ECG. The arterial blood pressure was measured, and the heart rate was calculated from the RR-interval.

Ischaemia of the anterior wall of the left ventricle was induced by ligation of the left anterior descending coronary artery (LAD). In our experience, the haemodynamic, electropathological and metabolic changes accompanying the consecutive 10-minute coronary ligations are comparable after a 1—2 minute precontrol occlusion [4]. After a control ligation and an equilibration period, β-pyridylcarbinol (Ronicol; F. Hoffmann-La Roche, Basel, Switzerland) was administered in a dose of 0.1 mg/kg/min, in the form of an intravenous infusion over 10 minutes [2, 11]. After Ronicol infusion, the LAD was repeatedly ligated for 10 minutes and the data measured at the times of the successive ligations were compared. Before the Ronicol infusion and after the release of the occlusions, arterial blood was taken for FFA determination. The one- and two-tailed *t*-tests were used for statistical evaluation of the results. The methods applied were described in detail earlier [4, 5, 7].

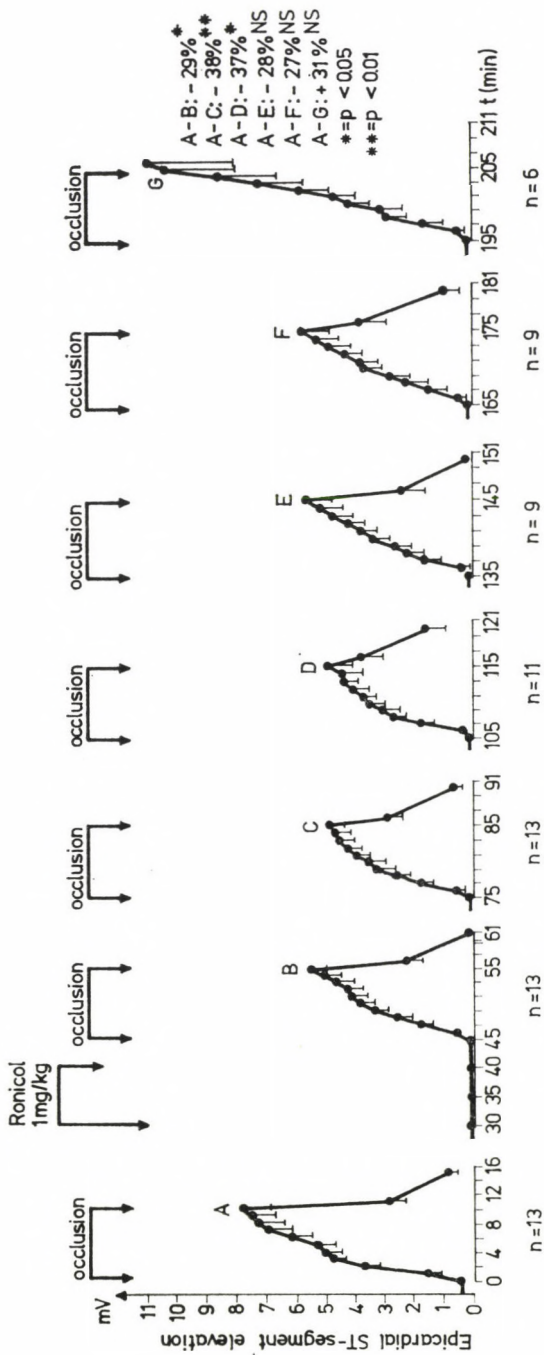

Fig. 1. Effect of Ronicol (1 mg/kg iv.) on epicardial ST segment elevation recorded from the ischaemic myocardial area in anaesthetized dog. Ronicol pretreatment significantly reduces ST segment elevation; duration of action ca. 120 minutes

Results

Ronicol pretreatment significantly reduced the epicardial ST segment elevation; the anti-ischaemic effect of the drug was long-acting and could be observed even 2 hours after injection (Fig. 1). As to the control ligation, the arterial FFA level increased from the preocclusion value (from 305 $\pm$ 47 to 409 $\pm$ 71 μmol/l); Ronicol significantly decreased the FFA blood concentration (367 $\pm$ 54 μmol/l; $p < 0.05$) and the antilipolytic effect was manifest even 2 hours after administration (318 $\pm$ 64 μmol/l; $p < 0.05$). The drug did not decrease the activation time of the ischaemic myocardial region, and in the dose applied it did not influence the heart rate, the arterial blood pressure, the LVEDP and the LV dP/dt_{max}.

Discussion

Like its parent compound nicotinic acid [7], β-pyridylcarbinol (Ronicol) blocks the lipolysis stimulated by sympathomimetics (isoproterenol, dopamine, noradrenaline), without appreciably decreasing the cardiac manifestations (positive chronotropic and inotropic effects) of the β-stimulus [15, 17, 19]. The inhibition of FFA accumulation results in a lower myocardial O_2 consumption and an improvement of the O_2 supply/O_2 demand ratio [19, 26]. Through this beneficial myocardial metabolic effect, Ronicol moderates the ST segment elevation following coronary artery occlusion in dog experiments [14], and also the myocardial infarct size, estimated by epicardial ST segment mapping and by serial CPK determination [12]. The anti-ischaemic effect of Ronicol is linked to intact myocardial catecholamine stores, as demonstrated by the fact that the cardioprotective action of the drug is not exerted in dogs pretreated with reserpine [12]. After Ronicol pretreatment, the left ventricular failure (LV stroke volume and contractility decreases) associated with coronary occlusion does not occur [11].

In patients suffering from coronary heart disease, Ronicol therapy prevents the arterial FFA blood level increase induced by isoproterenol infusion, and moderates the O_2 demand of the myocardium [22]. Ronicol treatment introduced in the early hospital phase of AMI and maintained for 15 hours reduced the spatial QRS and ST vector changes and the rate of their development; it did not increase the heart rate, and decreased the arterial blood pressure only slightly, and not significantly [13].

Another nicotinic acid analogue, 5-fluoronicotinic acid, displays a considerable antiarrhythmic effect in dogs and in patients with AMI: it diminishes the frequency of the life-threatening ventricular arrhythmias (paired extrasystoles, ventricular tachycardia) without cardiovascular side-effects (heart

rate increase, arterial blood pressure and cardiac index decrease [21, 23]). It is also effective in stable effort angina pectoris, significantly decreasing the ST segment depression in response to physical exertion [16]. We too have observed the anti-ischaemic and antilipolytic effects of a nicotinic acid salt, K-Mg-nicotinate, in an angina pectoris model in dogs: the long-acting anti-ischaemic effect of the drug was not complicated by the undesirable haemodynamic side-effects of nicotinic acid (unpublished).

This can also be said of Ronicol, which when given iv. in a dose of 1 mg/kg during 10 minutes exerts an antiischaemic and antilipolytic effect lasting for about 120 minutes. In dogs, this dose does not increase the heart rate or the LVEDP, nor does it reduce the arterial blood pressure and LV dP/dt_{max}. This indicates that the haemodynamic effects of Ronicol are more favourable than those of nicotinic acid [4, 21, 23]. In the mammalian liver, nicotinic acid is formed from Ronicol through enzymatic oxidation, but this process requires time; this probably explains the long-lasting effect of the drug and the rare occurrence of side-effects [1].

Concerning the mechanism of the anti-ischaemic action of Ronicol, it is probable that besides the antilipolytic effect other factors too may play a role in the cardioprotective action. Nicotinic acid [20] and Ronicol [3] have been shown to release prostacyclin (PGI_2) from the endothelium, and PGI_2 also possesses cardioprotective effect [25]. It cannot be excluded that, besides the antilipolytic and prostacyclin-like effects, the fibrinolytic activity of Ronicol plays a part in the emergence of its anti-ischaemic action [1].

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ROLE OF THE SUBSTANTIA NIGRA IN THE BEHAVIOURAL-CARDIOVASCULAR INTEGRATION IN THE CAT

L. ÁNGYÁN

Institute of Physiology, University Medical School, Pécs, Hungary

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Electrical stimulation of the substantia nigra elicited complex patterns of response composed of somatomotor and circulatory changes. Increase in blood pressure associated with acceleration in heart rate was consistently produced by substantia nigra stimulation both in conscious and in anaesthetized cats. The respiration was either accelerated or arrested by the stimulation. Also self-stimulation of the substantia nigra elicited pressor responses. Electrical stimulation of the nucleus of the tractus solitarius paired with self-stimulation of the substantia nigra, produced regular changes in the lever-pressing rate for self-stimulation. It is suggested that the substantia nigra is probably involved in the neural mechanisms coupling the circulatory changes with the somatomotor responses.

Keywords: blood pressure, heart rate, nucleus tractus solitarii, respiration, self-stimulation, substantia nigra.

The substantia nigra (SN), a major structure in the extrapyramidal motor system, is a matter of considerable interest, primarily because it has been related to human movement disorders. Animal experiments have revealed multiple function of the SN, namely, it participates in the control of movements [14, 19, 29], it is involved in mediating somatomotor conditioning [18], consummatory behaviour [5, 11, 20], defense reaction [1, 12], and it has the ability to support electrical self-stimulation (SS) [3, 4, 5, 11, 23]. It is assumed that most if not all behaviours are associated with appropriate circulatory changes, thus the behaviours elicited by SN stimulation should have circulatory concomitants, too. However, the exact brain mechanisms of coupling circulatory changes with widely different behaviours are not known yet. The question raised in this study is whether the SN may have a role in the behavioural-cardiovascular integration. Previous studies concentrated on the somatomotor responses to SN stimulation and less attention was paid to the circulatory concomitants of these responses. Therefore, our intention is, first,

Correspondence should be addressed to
Lajos ÁNGYÁN
Institute of Physiology, University Medical School,
H-7643 Pécs, Szigeti út 12, Hungary

to investigate the circulatory changes produced by SN stimulation, and, second, to study the question whether the behavioural responses to SN stimulation are modified by the activation of the cardiovascular afferents. This question is based on previous studies [9, 10, 21] showing that in addition to the medulla, supramedullary structures also receive cardiovascular inputs. The nucleus of the tractus solitarius (NTS) is known as an important relay station for cardiovascular afferents, so the self-stimulation of the SN was tested under simultaneous stimulation of the NTS. In spite of the well-known limitations of electrical stimulation studies, e.g. cell bodies or axons are activated, describing the particular patterns of response to SN stimulation may provide a useful approximation of the neural circuitry involved in behavioural-cardiovascular integration.

Methods

Animals and surgery

Results were obtained in 6 male and 8 female adult cats weighing 2.5—3.5 kg at the time of surgery. Under pentobarbital sodium (40 mg/kg iv.) anaesthesia bipolar electrodes were implanted into the substantia nigra and in the nucleus of the tractus solitarius by the conventional stereotaxic techniques. The coordinates were based on the Snider and Niemer's brain atlas [27]. The electrodes were constructed of two insulated nikrothal wires (0.3 mm in diameter, bared 0.5 mm at the tip) having tip separation of 1 mm. The tip resistance was 0.1—0.2 megohm. A thermocouple was inserted into the nasal orifice with which the respiration was monitored as a change in temperature of tidal air [8]. The electrodes and the thermocouple were connected to a miniature multipin connector which was fixed with dental acrylic to the cat's skull. Each cat was allowed 7 to 10 days recovery before being tested.

Behavioural procedures. Overt behavioural responses

At each electrode site the threshold intensity for overt behavioural responses was determined at 100 cps and 0.3 ms pulse duration. This intensity varied between 0.1 mA and 1.5 mA at the different electrode localizations. During stimulation sessions the stimulus intensity was systematically increased till strong forced movements were elicited by the stimulation. The interstimulation interval was 20 s or longer. The duration of stimulation was between 1.0 s and 10 s. The somatomotor and autonomic responses (dilation of the pupils, salivation etc.) to SN stimulation were visually observed.

Self-stimulation

The cats were trained to press a pedal to obtain SN stimulation of medium intensity. Each pressing produced a 0.3 s train of pulses at 100 cps. The lever-pressing rate was counted on an electromechanical counter.

Self-stimulation paired with NTS stimulation

Both the SN and the NTS stimulations were triggered by the cat pressing on the pedal. Two types of stimulation procedures were used (Fig. 1). The first type consisted of successive pulse trains, i. e., each pulse train delivered in one locus was immediately followed by a pulse train of same duration in the other locus. The second type of stimulation consisted of overlapping pulse trains, but each pulse at one locus was followed by a pulse at the other locus with a delay from 0.5 ms to 9.0 ms. The pulse train duration was 300 ms in both cases. The sequence of SN and NTS stimulations was systematically changed from one experimental session to the next. The cat was allowed to self-stimulate during 5 min under each combination of stimulation. A daily experimental session consisted of ten 5 min trials. The lever-pressing rate measured in each 5 min trial was expressed as percent of the control value measured at the beginning of each

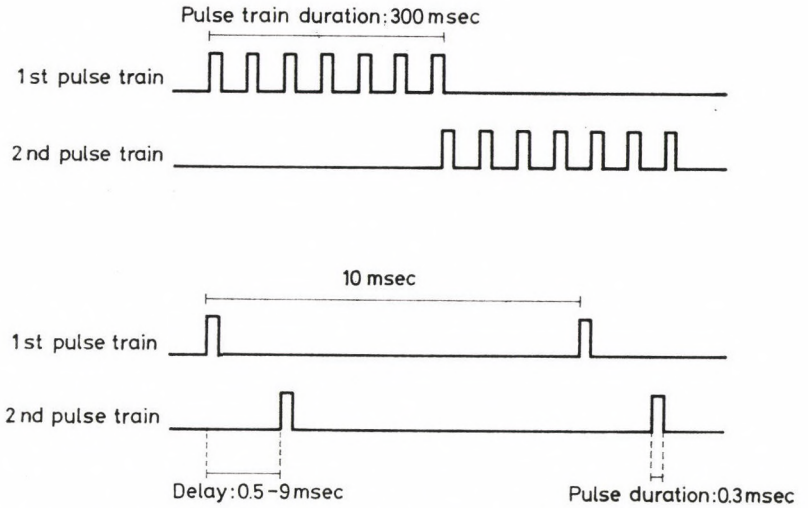

Fig. 1. A scheme for the two types of SN stimulation paired with NTS stimulation: 1. successive pulse trains (A), the first pulse train was immediately followed by the second one; 2. overlapping pulse trains (B), the two pulse trains were simultaneously delivered but each pulse of the first pulse train was followed by each pulse of the second pulse train with a delay of 0.5—9.0 ms

experimental session without NTS stimulation. All animals were tested in at least three sessions for both sequences of SN and NTS stimulations. The Student's t-test was used for statistical comparisons.

Recordings

A second operation was made to tie a catheter into one of the common carotid arteries. Recording sessions were conducted after 1—2 days recovery from this second operation. Arterial blood pressure, monitored by a Statham transducer connected to the catheter in the carotid artery, heart rate computed by a beat-to-beat analyser, and respiration monitored by the thermocouple, were recorded on a Hellige polygraph. The effects of self-stimulation, and those of sustained stimulation of the SN were studied on separate experimental sessions. The effects of sustained stimulations were recorded under pentobarbital sodium or chloralose (40 mg/kg iv.) anaesthesia, too. After anaesthesia, three cats were paralyzed with Flaxédil (gallamine triiodoéthylate, SPECIA) and ventilated with a respirator for the duration of the recording session.

Histology

Upon completion of the experiment, the animals were sacrificed, their brain was perfused, removed, cut and blocks were embedded in paraffin. The position of electrode tips were determined by microscopic examination of frontal sections stained with luxol fast blue and cresyl violet.

Results

Electrode localizations

Histological verification of electrode placement showed that all electrodes were properly placed in the substantia nigra (Fig. 2). All of the NTS electrodes were localized in the region rostral to the obex.

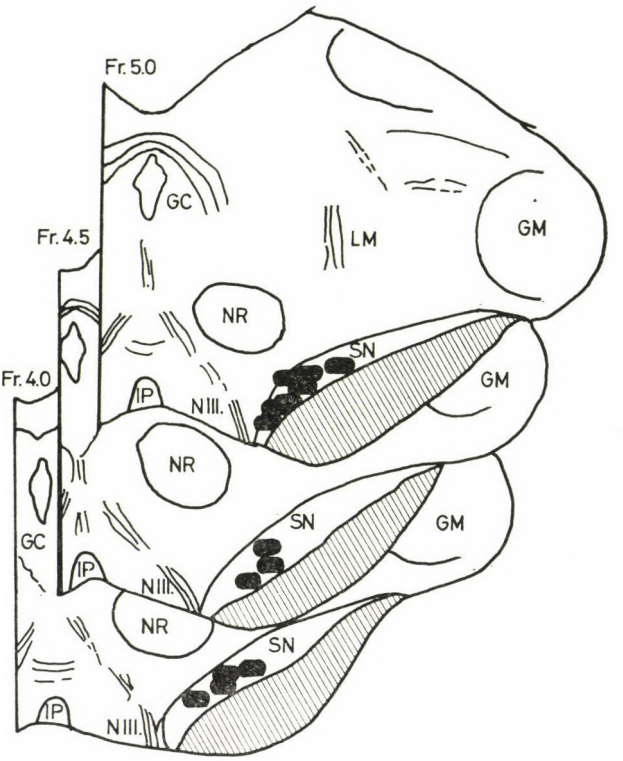

Fig. 2. Diagram showing the localization of the electrode tips in the substantia nigra. Abbreviations: GC: substantia grisea centralis, GM: corpus geniculatum mediale, IP: nucleus iuterpeduncularis, LM: lemniscus medialis, NIII: nervus oculomotorius, NR: nucleus ruber, SN: substantia nigra

Effects of substantia nigra stimulation in unrestrained cats

Stimulation with threshold intensity produced orienting behaviour. Head turning, oral-face movements, contraversive circling appeared by increasing the intensity. Stimulation at high level of intensity elicited tonic leaning of the body to the ipsilateral side, and stretching of the foreleg contralateral to the side of stimulation. In all cases dilation of the pupils, many times salivation, occasionally piloerection and urination might be observed.

The arterial blood pressure, heart rate and respiration effects are summarized in the Table 1. The SN stimulation produced consistently rise of blood pressure and acceleration in heart rate. The higher stimulus intensity was applied the higher increase in blood pressure could be obtained. The somatomotor and the circulatory effects of SN stimulation varied in parallel, i.e., more intensive somatomotor activity was associated with circulatory changes of higher magnitude. The blood pressure response was a monophasic increase during stimulation associated with acceleration in heart rate (Fig. 3A). After

Table I

Changes in cardiorespiratory parameters elicited by substantia nigra stimulation in freely moving cats

	Prestimulation control	%	no	p
Heart rate	171 ± 9	+29 ± 4	12	0.001
Systolic blood pressure	131 ± 4	+40 ± 4	12	0.001
Diastolic blood pressure	108 ± 5	+45 ± 5	12	0.001
Respiration rate	33 ± 3	+221 ± 33	8	0.001

%, change in percent of prestimulation control. Prestimulation control values given in beats/min for heart rate, mm Hg for blood pressure, and breathes/min for respiration rate. All values are means ±SE.

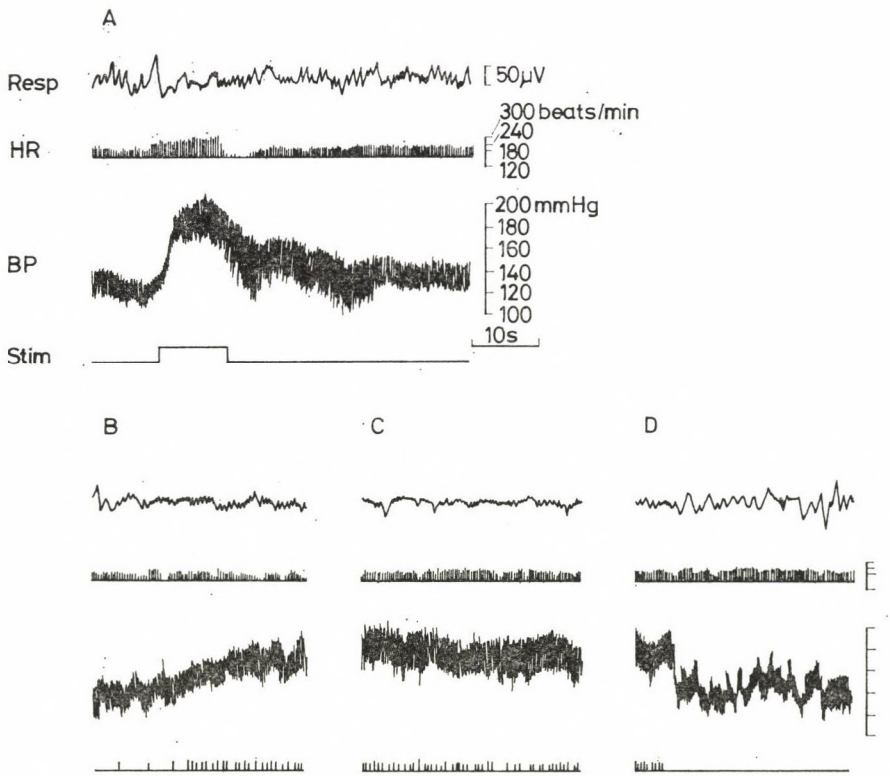

Fig. 3. Respiration (Resp), heart rate (HR) and blood pressure (BP) effects of sustained (A), as well as self-stimulation of the substantia nigra. The cardiorespiratory effects rapidly increased at the beginning of SS (B), they were maintained at a high level during continuous SS (C), and returned to the prestimulation level when the stimulation was cut off (D)

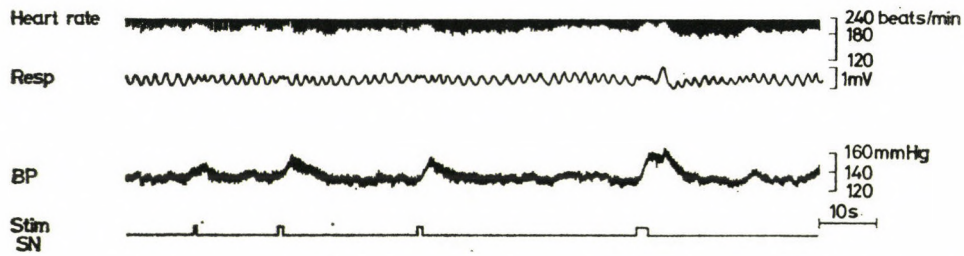

Fig. 4. Heart rate, respiration (Resp) and blood pressure (BP) responses to SN stimulations of different pulse train duration

the cessation of stimulation both blood pressure and heart rate returned to the prestimulation level, but the descending phase was much longer than the ascending phase. The latency of blood pressure and heart rate responses was in the order of several hundreds ms. The pulse pressure decreased in the ascending phase, and increased in the descending phase of blood pressure response. These changes in pulse pressure were produced by the somewhat higher magnitude changes in the diastolic than in the systolic pressure. It is important to note that also rebound-like after effects in the heart rate and occasionally in the blood pressure occurred, i.e., the descending phase fell below the prestimulation level for a short time. As a consequence of repeated stimulating at medium or high level of intensity the prestimulation level of blood pressure and heart rate increased up to about 130% of the resting level measured at the beginning of the experimental session. This elevated high level was maintained for several minutes.

The respiration effects of SN stimulation were variable. In 9 out of 13 animals the stimulation produced decrease in amplitude and increase in frequency of breathing. In the other 4 cats, during stimulation at suprathreshold intensities, expiratory apnea was obtained. This apnea ceased immediately when the stimulation was cut off.

In 4 cats also the effects of different pulse train duration [1, 2, 3, 5 and 10 s] on the blood pressure and heart rate responses were studied. The general form of the response was similar regardless of pulse train duration, but the response reached higher magnitude when duration was lengthened (Fig. 4). The shortest train duration required for a response of full size was from 3 to 5 s.

Effects of SN stimulation under anaesthesia

As an attempt to eliminate the possibility that the cardiovascular responses to SN stimulation were secondary to the somatomotor responses, the effects of SN stimulation on blood pressure, heart rate and respiration were studied either under pentobarbital sodium (3 cats), or chloralose (5 cats)

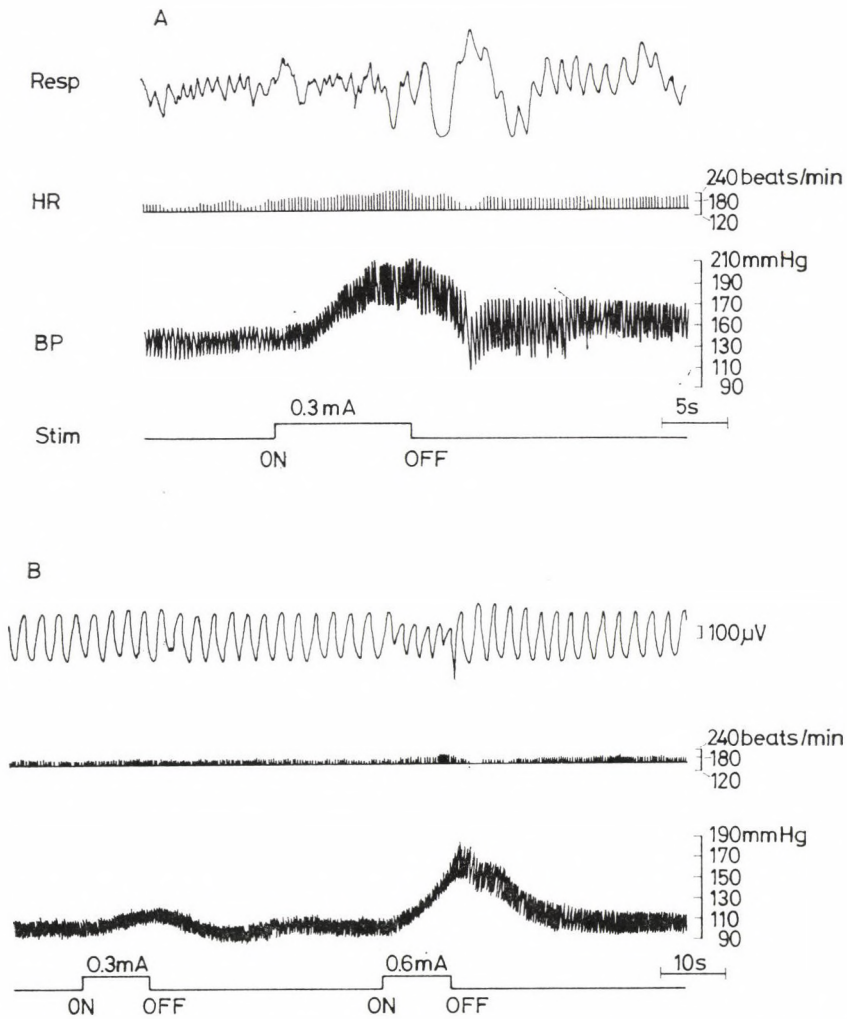

Fig. 5. Respiration (Resp), heart rate (HR) and blood pressure (BP) responses to SN stimulation at the same locus in conscious cat (A), and under pentobarbital sodium anaesthesia (B)

anaesthesia. The results are summarized in the Table II. The general form of the response was similar to that obtained in conscious cat, but some differences might be observed. The threshold intensity for cardiovascular responses was higher in the anaesthetized cats than in the conscious ones (Fig. 5). Under pentobarbital sodium anaesthesia only a slight acceleration in heart rate appeared. The blood pressure response was as high as in conscious cat, but smaller changes in pulse pressure occurred. Under chloralose both the blood pressure and the heart rate responses were smaller than those obtained in conscious cats (Table II). However, besides the direct effects of stimulation

Table II

Cardiorespiratory effects of substantia nigra stimulation under anaesthesia

	Pentobarbital sodium				Chloralose			
	Prestimulation control	%	no.	P	Prestimulation control	%	no.	P
Heart rate	208 ± 24	+ 4 ± 0.5	3	0.05	168 ± 10	+13 ± 4	5	0.05
Systolic blood pressure	108 ± 8	+38 ± 7	3	0.05	130 ± 9	+15 ± 2	5	0.01
Diastolic blood pressure	90 ± 5	+39 ± 8	3	0.05	108 ± 5	+17 ± 2	5	0.01
Respiration rate	15 ± 2	+50 ± 35	2	NS	14 ± 1	+27 ± 5	2	NS

%, change in percent of prestimulation control.
Prestimulation control values are given in beats/min for heart rate, mm Hg for blood pressure, and breathes/min for respiration rate. All values are means ±SE.

the rebound-like after effects might be produced by increasing stimulation intensity (Fig. 6). The respiration effect was apnea in 3 cats, while small amplitude high frequency breathing appeared in the two others.

Seeing that some muscular contractions were occasionally elicited by SN stimulation in anaesthetized animals, the SN stimulations were repeated in three cats paralyzed with Flaxédil. Also under these experimental conditions pressor responses might be produced by SN stimulation.

Effects of substantia nigra self-stimulation

The substantia nigra supports self-stimulation at a high lever-pressing rate in cats, too. The mean ± SE values obtained in this study were 370 ± 25 presses per 5 min. The powerful somatomotor activity of the self-stimulating cat often resembled to eating behaviour, namely, licking, chewing movements

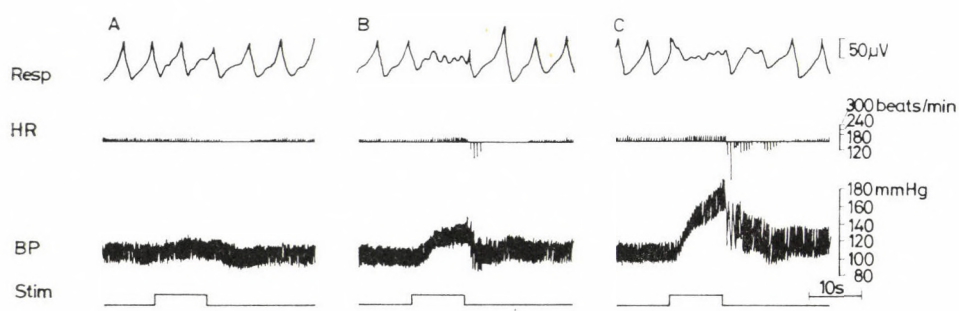

Fig. 6. Respiration (Resp), heart rate (HR) and blood pressure (BP) effects of increasing stimulus intensity in a cat anaesthetized with chloralose. The stimulus intensities were: 0.5 mA (A), 1.0 mA (B), and 1.5 mA (C)

accompanied by salivation might be observed. The SS elicited pressor responses. The mean ± SE values in % of the prestimulation control are as follows: 120 ± 5 for the heart rate, 149 ± 5 for the systolic blood pressure, 152 ± 6 for the diastolic blood pressure, and 151 ± 17 for the respiration rate. At the beginning of SS the arterial blood pressure increased rapidly up to about 180 mm Hg, but in certain cases it might exceed even 200 mm Hg. After the initial period of increasing blood pressure during SS a fairly constant value was reached and maintained (Fig. 3B, C, D). The level of the elevated blood pressure depended upon the intensity of stimulation, while the constancy of blood pressure was related to the lever-pressing rate for SS. The blood pressure decreased during the interstimulation interval, therefore, high amplitude waves appeared in the blood pressure curve whenever a short train of lever-pressings was followed by an interstimulation interval of about the same duration. Such behaviour often occurred at the beginning of the experimental session. The increase in blood pressure was associated with acceleration in heart rate and respiration.

Effects of NTS stimulation on the substantia nigra self-stimulation

In accordance with our previous studies [4, 7], the substantia nigra self-stimulation was modified by NTS stimulation. However, the changes in SS depended on the sequence of NTS and SN stimulations (Fig. 7). Namely, in cases when the first stimulation was applied to the SN and the second to

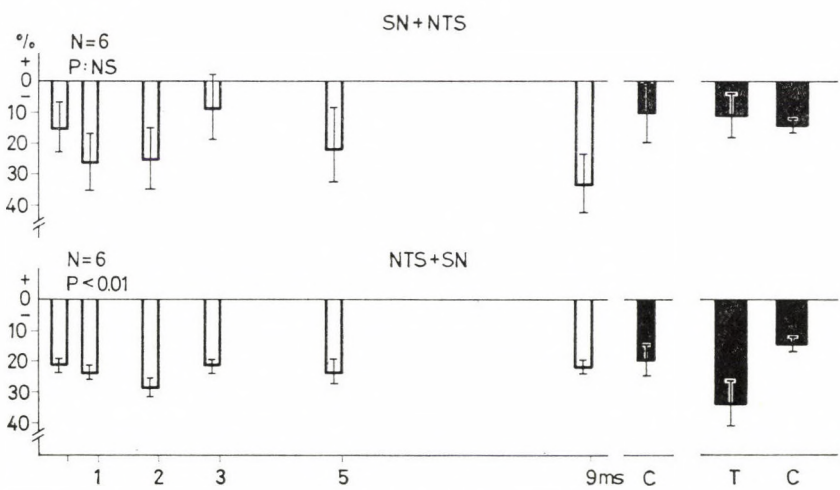

Fig. 7. Changes in lever-pressing rate for SN self-stimulation in percent of control self-stimulation measured at the beginning of each experimental session. The empty columns show the effects of self-stimulation with overlapping pulse trains at different pulse delay. C_1 and C_2 are poststimulation controls, i. e. self-stimulation without NTS stimulation. T columns show the effects of self-stimulation with successive pulse trains

the NTS, the lever-pressing rate for SS decreased. However, this effect was not significant statistically for the group of six cats. Interestingly, stimulations with overlapping pulse trains produced accelerations in SS in one out of 6 cats.

In the case, when the NTS stimulation preceded the SN stimulation, the suppression of SS was statistically significant, and the decrease in lever-pressing rate was between 20 and 40 percent of the prestimulation control level. Interestingly, stimulations with successive pulse trains were more suppressive on SS than stimulations with overlapping pulse trains. In the latter case, however, similar effects were produced by the stimulations regardless of the pulse delay (Fig. 7).

It is important to note that also the poststimulation controls, i.e., the lever-pressing rate measured after the paired stimulations without NTS stimulation, were below the prestimulation control level. After stimulations with successive pulse trains the decrease in poststimulation control was the same in size regardless of the order of SN and NTS stimulations. After stimulations with overlapping pulse trains no statistically significant change was obtained in the poststimulation control when the first stimulus pulse was applied to the SN, while the highest effect on poststimulation control occurred when the order of stimulation pulses was inverted (Fig. 7).

Discussion

The results of the present study show that the patterns of response elicited by SN stimulation contain consistently circulatory components. The circulatory effects of SN stimulation are pressor responses. The first question to be discussed is whether both somatic and circulatory effects are initiated and controlled by the same neural mechanisms activated by SN stimulation, or the circulatory changes are secondary to the somatomotor effects of SN stimulation. The long latencies of the circulatory effects support the latter possibility. However, SN stimulation elicited circulatory changes in anaesthetized and paralyzed cats, too. Thus, the muscular activity seems to have no determinative role in the circulatory changes elicited by SN stimulation.

The next question concerns the anatomical basis for the circulatory responses to SN stimulation. There is now extensive literature dealing with the neuroanatomy of the SN [2, 9, 11, 17, 22, 23, 24, 26]. Peculiar bundle configurations generated by dendrites of SN were observed [24]. Scheibel and Scheibel [25] suggested that the dendrite bundle is a site for central programs coding output patterns. Recent studies have demonstrated that in addition to the principal projections from the SN to the striatum and thalamus, the SN projects also to brain stem areas which give rise to descending spinal projections. These nigral projections may play an important role in the SN stimula-

tion-evoked somatomotor, circulatory and respiration effects. However, a dichotomy of the somatomotor and the autonomic functions of the SN was revealed by Kao and Powell [18]. They have reported that lesions of SN retarded Pavlovian somatomotor learning but did not affect autonomic conditioning. The conditioned heart rate response was bradycardia. Considering that SN stimulation elicited invariably acceleration in heart rate, it can be understood that lesions of SN failed to affect the decelerative heart rate response.

The increase in heart rate, the rise in blood pressure with simultaneous decrease in pulse pressure indicate that probably adrenergic sympathetic activation produces these effects. This suggestion is in accordance with the results of Gravante et al. [15] showing inhibitory effects of SN stimulation on the electrical and mechanical activities of the duodeno-jejunal loop. It seems also probable that the baroreflex mechanism is suppressed by the SN stimulation, which is shown by the marked bradycardia and increased pulse pressure after cessation of stimulation. Such inhibition of baroreceptor reflexes on hypothalamic stimulation was demonstrated by Hilton [16]. The elevated control level of blood pressure after repeated stimulating of the SN suggests that also long-lasting change in the baroreflex mechanism might be produced by SN stimulation.

It is a difficult question that the cells in SN, or the fibers of passage mediate the effects of SN stimulation. A definite answer to this question is still lacking, but the following data indicate the crucial role of the cells in responses to SN stimulation. Injections of 6-hydroxydopamine into the SN produced profound learning deficits in rats [13]. Thompson et al. [28] reported that neurotoxic lesions to SN can produce a nonspecific learning impairment, but the combined destruction of cells and fibers of passage within the SN produces a more profound learning deficit. By recording unit activity from the SN of monkey it was revealed that a substantial number of neurons are concerned with the conditioned arm movement [19]. The present study gives further evidences showing the importance of the cells in SN in mediating SN stimulation-elicited responses: 1. The self-stimulation of the SN was more affected by NTS stimulations with successive pulse trains than with overlapping ones; 2. The pulse delay did not make much difference in the effects of overlapping stimulations; 3. Significant differences were found between the prestimulation and the poststimulation controls for SS. All of these effects may be explained by the modulatory effects of NTS stimulation on the cells in SN, and not on the fibers passing through the SN region. However, it is possible to argue that changes in SS may be due to pain or aversion elicited by NTS stimulation. Our earlier experiments showed that NTS stimulation produced no aversion in shuttle-box [7]. The acceleration in lever-pressing rate obtained in one cat, as well as, the dependence of the changes in SS upon the

sequence of SN and NTS stimulations, also support the assumption of modulatory influences ascending from the NTS to the SN.

In conclusion, the data obtained in this study suggest a possible role for the SN in integrating behavioural-cardiovascular patterns of response. The following evidences are supporting this assumption: 1. The electrical stimulation of the SN elicited regularly both somatomotor and circulatory effects. 2. The intensity of the somatomotor effects varied parallel with the magnitude of circulatory changes both during sustained stimulation and self-stimulation. 3. Changes in the cardiovascular input, produced by NTS stimulation, exerted modulatory influences on the substantia nigra self-stimulation.

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OXYTOCIN REGULATES Ca^{2+} LEVEL IN MYOMETRIUM BY INFLUENCING PHOSPHOINOSITIDE METABOLISM*

Ágota VÉR, N. MÜLLNER, L. SZOLLÁR,+ J. SOMOGYI

Institute of Biochemistry I. and Institute of Pathophysiology+, Semmelweis University, Medical School, Budapest, Hungary

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The effect of oxytocin on phosphoinositide metabolism as well as on membrane protein phosphorylation in myometrial tissue was studied. Oxytocin enhanced the ^{32}P incorporation into phospholipids in myometrial tissue. The effect of oxytocin on phosphoinositide metabolism was also detected in plasma membrane of 20 days pregnant rats. Phosphorylated membrane lipids have been analysed and phosphatidylinositol 4, 5-bisphosphate proved to be the main reaction product. Oxytocin enhanced the ^{32}P incorporation into phospholipids measured in the first 30 sec then the labeling decreased more rapidly then in case of the control. The effect of oxytocin proved to be concentration dependent. The protein phosphorylation was also influenced by oxytocin. However the amount of alkylphosphate formed depended on the presence or absence of Ca^{2+}, Ca^{2+}-calmodulin and cyclic AMP, oxytocin influenced the protein phosphorylation in the presence of Ca^{2+}-calmodulin only.

Keywords: myometrium, oxytocin, phosphoinositide metabolism, protein phosphorylation.

It is widely accepted that oxytocin plays an important role in the stimulation of uterine activity during delivery. Considerable evidences support that oxytocin action is mediated via oxytocin receptor localised at the surface of the plasma membrane [22] by triggering an increase in sarcoplasmic free Ca^{2+} concentration [3, 23].

The elevation of intracellular Ca^{2+} level induced by several hormones has been linked to the stimulation of phosphoinositide metabolism [4, 9]. The correlation between phosphoinositide breakdown and increased level of intracellular calcium led to the idea that the actual level of phosphatidylinositides in the plasma membrane could alter calcium channels or some other Ca^{2+}-transporting systems [5, 15, 25]. Alternatively, products of phosphatidylinositide metabolism may serve as calcium mobilising agents [4].

The experiments performed in this report were designed to examine the effect of oxytocin on phosphoinositide turnover and Ca^{2+}-transport processes of close to term pregnant uterus smooth muscle.

Correspondence should be addressed to
Ágota VÉR
Institute of Biochemistry I., Semmelweis University Medical School
H-1088 Budapest, Puskin u. 9., Hungary

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Methods

Plasma membrane enriched fraction was prepared from 20 days pregnant rats as described by [19]. Sarcoplasmic reticulum fraction was prepared according to [8]. Plasma membrane phosphorylation was performed as [28] and after hydroxylamine treatment, ^{32}P incorporation was measured [26]. Acrylamide gel electroforesis was carried out according to Laemmli [12].

Autoradiograms were prepared from dried gels using Kodak X-ray film and analysed by densitometer. Lipids were extracted as in [26]. The polar lipids were identified by thin layer choromatography in silica gel [2]. Ca^{2+}-transport processes were measured as described previously [27]. Protein content of the samples was determined by Lowry et al. [13]. Tissue incubation with ^{32}P was carried out on the basis of Seyfred and Wells [21].

Results and discussion

The effect of oxytocin on ^{32}P incorporation into phospholipids is shown in Table I. In this study myometrial tissues were incubated with ^{32}P for 5 min before addition of oxytocin. Oxytocin produced a rapid stimulation of ^{32}P incorporation into phospholipids during the first 5 min. The hormon sensitive

Table I

Effect of oxytocin treatment on ^{32}P labeled phospholipids in myometrial tissue

	cpm/g tissue
addition	
no	3450
oxytocin	4120

Myometrial tissue derived from 20 days pregnant rats was incubated with ^{32}P for 5 min and then with and without 10^{-7} M oxytocin for additional 5 min. ^{32}P labeled phospholipids were extracted and determined as described in Methods.

pool of phosphoinositides localises in plasma membrane and appears to constitute a small fraction of total cellular phosphoinositides, therefore we investigated the effect of oxytocin on phosphoinositide metabolism on isolated plasma membrane. Oxytocin enhanced the ^{32}P incorporation from labeled ^{32}P ATP into phospholipids measured in the first 30 sec in plasma membrane preparation (Table II) then the labeling decreased more rapidly in the presence of oxytocin than in case of the control both in Ca^{2+} free and Ca^{2+} containing medium [28]. The effect of oxytocin proved to be concentration dependent [28]. Phosphorylated membrane lipids have been analysed and phosphatidylinositol 4,5-bisphosphate proved to be the main reaction product, but phosphatidylinositol 4-phosphate and phosphatidic acid could be also detected, and no incorporation into the other phospholipids was observed (Fig. 1).

The demonstration that oxytocin induced enhancement of phosphoinositide metabolism is partly independent from the presence of Ca^{2+} strongly

Table II

^{32}P incorporation into myometrial plasma membrane prepared from pregnant rats

	protein	lipid
	pmol ^{32}P/mg protein	
Addition		
EGTA	41	30
cyclic AMP	51	32
PKI, EGTA	32	32
Ca^{2+}	54	47
Ca^{2+}-calmodulin	60	49
EGTA, oxytocin	39	49
Ca^{2+}-calmodulin oxytocin	48	78

The membrane phosphorylation was carried out as described earlier (28) using ^{32}P ATP. The concentration of additives EGTA 1 mM, cyclic AMP 0.1 μM, Ca^{2+} 10 μM, calmodulin 0.1 μM, oxytocin 0.1 μM.

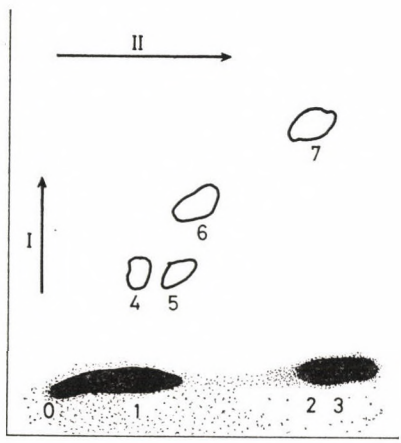

Fig. 1. Autoradiogram of phospholipids. The lipids were extracted [24] and the polar lipids were identified by two dimensional thin layer chromatography [2].
1. phosphatidylinositol 4, 5-bisphosphate; 2. phosphatidylinositol 4-phosphate; 3. phosphatidic acid; 4. sphingomyelin; 5. phosphatidylcholin; 6. phosphatidylserine, phosphatidylinositol; 7. phosphatidylethanolamine

imply that phosphoinositide breakdown is not the consequence of an increase of intracellular Ca^{2+}. The hydrolysis of phosphatidylinositol 4,5-bisphosphate results in a subsequent formation of diacylglicerol and inositol 1,4,5-trisphosphate (IP_3). Whereas diacylglycerol is an activator of protein kinase C (25), IP_3 releases Ca^{2+} from intracellular stores [24]. Oxytocin induced IP_3 production has already been reported [14], and the demonstration of IP_3 induced Ca^{2+} release from myometrial sarcoplasmic reticulum was the pivotal question. Figure 2 shows that IP_3 causes a dose dependent release of Ca^{2+} from sarcoplasmic reticulum previously filled with ^{45}Ca. IP_3 can either be

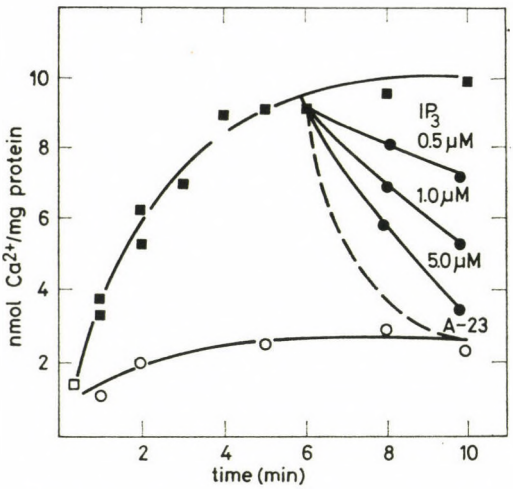

Fig. 2. IP_3 induced release of Ca^{2+} from sarcoplasmic reticulum. Sarcoplasmic reticulum vesicles were allowed to take up ^{45}Ca in the presence of ATP [27]. The additives were administered at 6 min of incubation period, the calcium contents of vesicles were determined as described in 27

stepwise dephosphorylated or converted to inositol 1,3,4,5-tetrabisphosphate (IP_4). Although the physiological role of IP_4 is rather unclear it has been suggested that it opens up channels for Ca^{2+} in plasma membrane to cause further rise in free Ca^{2+} concentration [11].

We have investigated the effect of oxytocin on membrane protein phosphorylation too. The amount of alkylphosphate formed in plasma membrane depended on the presence or absence of Ca^{2+}, Ca^{2+}-calmodulin and cyclic AMP (Table II). Oxytocin influenced only the protein phosphorylation in the presence of Ca^{2+}-calmodulin, i.e. the phosphorylation of about 20 000 Dalton mol. w. protein was completely abolished by 100 nM oxytocin [27]. Recently it has been suggested that IP_3 and IP_4 might activate protein phosphatases [18].

The relevance of these phenomena to known stimulatory effects of oxytocin on uterine smooth muscle activity is open to further consideration. The enhancement of PIP_2 hydrolysis in plasma membrane might result in a change in lipid components of the membrane. In this respect it is interesting that changes in PIP_2 level have also been implicated in the regulation of plasma membrane Ca^{2+}-pump. Thus deficient Ca^{2+}-pumping ATPase is associated with decreased PIP_2 levels in erytrocytes [17]. It has been recently reported that oxytocin inhibits sarcolemmal Ca^{2+} ATPase from human gestation myometrium [1, 20], however, the data obtained in myometrial Ca^{2+}-ATPase are rather contradictory [16]. Beside the sarcolemmal Ca^{2+}-ATPase the sodium dependent Ca^{2+} exchange also plays an important role in the regulation of

intracellular free calcium [6]. Our preliminary data show that oxytocin specifically inhibits Na^+-Ca^{2+} exchange. These data suggest that oxytocin receptor activation in myometrial tissue is coupled to an enhanced phosphoinositide metabolism, and resulting in IP_3 that may be a link between oxytocin receptor binding and intracellular calcium rise. Further analysis of the oxytocin mediated processes and their interrelationships is in progress.

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ZINC AS A POSSIBLE MEDIATOR OF SIGNAL TRANSDUCTION IN T LYMPHOCYTES*

P. CSERMELY, J. SOMOGYI

Institute of Biochemistry I., Semmelweis University Medical School, Budapest, Hungary

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Our recent findings indicate that phorbol esters, the specific activators of protein kinase C induce the translocation of heavy metals (mostly: zinc) from the nucleus and mitochondria to the cytosol and microsomes of T lymphocytes. Phorbol ester treatment impairs the action of Ca-ionophores, this effect is mediated by intracellular heavy metal ions (most probably: by zinc). Zinc activates cytosolic protein kinase C, increases its affinity towards phorbol esters and contributes to its binding to plasma membranes. These results suggest that zinc may play a role in the "cross-talk" of second messengers and hence in signal transduction in T lymphocytes.

Keywords: zinc, heavy metals, T lymphocytes, phorbol, protein kinase C, Ca-ionophores, Ca^{2+}-dependent processes.

The pleiotropic effects of zinc on the activation of T lymphocytes were known decades ago. Zinc deprivation results in many symptoms of immune-deficiency, zinc in itself is mitogenic for T lymphocytes, while at higher concentrations it effectively blocks the action of other mitogens such as antigens or lectins [13]. However, we have very few information about the biochemical mechanisms of these processes.

On the other hand there are some recent findings which suggests that zinc may play a role in signal transduction. Zinc can substitute calcium in a number of calcium binding sites [14]. In this way changes in the intracellular zinc concentration may induce conformational and functional changes of numerous calcium binding proteins. On the other hand several DNA-binding proteins such as the cortisol, oestradiol and thyroid hormone receptors [17, 18] contain a zinc binding site, a so-called "zinc-finger" [2, 3, 11]. Parker et al. [16] defining the primary structure of protein kinase C demonstrated the presence of these "zinc-fingers" in the regulatory domain of the enzyme. However, the function of this zinc-binding site of protein kinase C is not yet known.

Correspondence should be addressed to
Péter CSERMELY
Semmelweis University School of Medicine, Institute of Biochemistry I.
H-1088 Budapest, Puskin u. 9., Hungary

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Based on these results we planned some experiments to get an insight into the possible role of zinc in the regulation of Ca^{2+}- and protein kinase C-dependent signal transduction in T lymphocytes.

Methods

Subcellular fractions of T lymphocytes were prepared by differential centrifugation after the cells have been ruptured by nitrogen cavitation [12]. The amount of various heavy metals was analysed by X-ray fluorescence [5] and plasma emission spectroscopy [4]. The intracellular calcium concentration was determined by the fluorescent indicators quin2 and fura-2 [6]. Zinc was depleted from T lymphocytes and from their subcellular fractions by the intracellular heavy metal chelator tetrakis-(2-pyridyl-methyl)-ethylenediamine (TPEN, [1]). The activity of protein kinase C was determined by measuring the transfer of ^{32}P to the basic H1 histone (Type III-S, Sigma) as described earlier [9]. Binding of ^{3}H-phorbol-dibutyrate was measured by polyethylene-glycol precipitation [9].

Results and discussion

Studying the possible role of zinc in signal transduction of T lymphocytes we have selected an approach which may indicate that the concentration of heavy metals (zinc) changes during the activation of T lymphocytes. The detection of such concentration changes is a prerequisite for the postulation that zinc indeed may participate in signal transduction. Since we wanted to study the role of zinc in the "cross-talk" of known signal transduction systems we used phorbol esters, the selective activators of protein kinase C for lymphocyte stimulation.

Isolating the subcellular fractions of control and 12-O-tetradecanoyl-phorbol-13-acetate (TPA) treated rabbit thymocytes and analysing their heavy metal content by X-ray fluorescence [5] and plasma emission spectroscopy [4] we have found that phorbol ester treatment (20 nM TPA for 90 minutes) induces the translocation of approximately 270 nmol Zn from the nucleus and mitochondria to the cytosol and microsomes of 5×10^9 cells. Smaller amounts of other heavy metals (Cu and Fe) are also translocated in a similar way. Our results suggest that the activation of protein kinase C induces a substantial increase in zinc concentration of cytosolic and microsomal fractions of T lymphocytes. Investigation of changes in *free* zinc concentration during lymphocyte stimulation is in progress in our laboratory.

As a next step we investigated the interrelationship between zinc and Ca^{2+}-induced signal transduction. It is well-known that phorbol esters attenuate the calcium signals induced by various hormones and agonists. In this systems protein kinase C has a number of possible targets to prevent the agonist-induced increase in intracellular Ca^{2+} concentration. In this way the

action of heavy metals (zinc) may be concealed. Therefore we have selected a more simple, "model" system for our studies. In our model system we induced an increase in intracellular Ca^{2+} concentration by means of Ca-ionophores such as A23187 or ionomycin and the effect of phorbol ester treatment on these calcium-signals was studied.

We have found that phorbol ester treatment impairs the action of Ca-ionophores. This effect is concentration- and time-dependent being maximal at TPA concentrations higher than 10 nM (this is the range where protein kinase C is fully activated) and after an incubation time of 90 minutes. Our studies indicate that the effect of phorbol esters can not be explained by the activation of Ca^{2+}-extrusion systems, "freezing" of Ca-ionophores to the lymphocyte plasma membrane or by other means of ionophore immobilization. On the other hand the intracellular heavy metal chelator TPEN restores the Ca-ionophore-induced Ca^{2+} translocation to the control level in TPA, or diacyl glycerol treated lymphocytes. This suggests that intracellular heavy metals (most probably the increased amount of zinc in the cytosol and microsomes — see above —) may be responsible for the attenuation of Ca-ionophore-induced Ca-signals in T lymphocytes [7].

As a third step we investigated whether the protein kinase C-induced excess of zinc in the cytosol and microsomes had any regulatory effect on protein kinase C itself. This question is interesting all the more because Parker et al. [16] suggested the existence of putative zinc binding site(s) on protein kinase C.

We have found that zinc activates cytosolic protein kinase C at nanomolar concentrations while it does not affect the membrane-bound form of the enzyme [8, 9]. Our results are in agreement with that of Murakami et al [15] who also demonstrated a zinc-induced activation of protein kinase C. Zinc increases the phorbol ester binding affinity of cytosolic protein kinase C also at nanomolar concentrations while it has a much smaller effect on the membrane-bound form of the enzyme [10].

The presence of the intracellular heavy metal chelator TPEN prevents the phorbol ester- and antigen-induced translocation of protein kinase C from the cytosol to the microsomes of T lymphocytes [9]. This suggests that heavy metals (most probably: zinc) may contribute to the membrane binding of protein kinase C. Taken our data together we suggest that cytosolic protein kinase C is a mixture of enzymes which are differently saturated with zinc. Zinc saturation may be a prerequisite of membrane binding of the enzyme. This may explain the relative insensitivity of membrane-bound protein kinase C to the addition of zinc. However, to strengthen this hypothesis further experiments are needed.

Figure 1 summarizes our present view on the possible role of zinc in signal transduction of T lymphocytes. The activation of protein kinase C

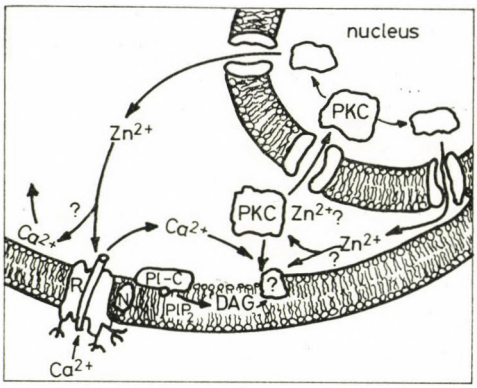

Fig. 1. The possible role of zinc in signal transduction of T lymphocytes
Abbreviations: DAG, diacyl glycerol; N, GTP-binding protein; PIP_2, phosphatidyl inositol bis-phosphate; PKC, protein kinase C; PI-C, phosphatidyl inositol specific phospholipase C; R, T cell receptor

(PKC) releases zinc from the nucleus. The mechanism of this action is unknown. In Figure 1 we illustrated one possibility which supposes that after prolonged activation of the enzyme protein kinase C translocates to the nucleus, phosphorylates some of the zinc-containing nuclear proteins inducing a decrease in their zinc-binding affinity. As a consequence these proteins release zinc which appears in the cytosol and microsomes. However, we would like to stress that this mechanism is merely speculative (at the moment).

The excess of zinc in the cytosol may disturb the calcium-signalling mechanisms and causes the activation and membrane binding of protein kinase C. This "secondary" activation of protein kinase C may represent a new mechanism of the autoregulation of the enzyme since it occurs at a later phase of lymphocyte activation (after 1.5 hours) when the initial activators of the enzyme (e.g.: diacyl glycerols) have been already metabolized.

Our results present pieces of evidence that zinc should be considered as an active constituent of the sophisticated signalling mechanism of T lymphocytes. These observations might help to clarify the heretofore mainly unknown mechanisms of both the action of zinc and tumor promoter phorbol esters in these cells.

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THE EFFECT OF ETHANOL ON RAT VAS DEFERENS

F. A. WALI*, A. HAYTER

Anaesthetics Unit, The London Hospital Medical College, Whitechapel, London El, U. K.

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The effect of ethanol (3.9—390 mM) on resting tension of vas deferens, isometric contractions produced by field stimulation of the vas deferens and on the contractions produced by neurotransmitters, acetylcholine (ACh 0.55—55 mM), noradrenaline (NA 0.058—580 μM) and 5-hydroxytryptamine(5-HT 0.25—250 μM) was studied in rat vas deferens to see if ethanol modified neurotransmission and muscle contraction in this preparation.

The results showed that ethanol had no significant effect on resting tension of the muscle, but reduced the contractions produced by field stimulation and by neurotransmitters. It was concluded that ethanol modifies neurotransmission in the rat vas deferens preparation, by reducing the contractions produced by field stimulation and by depolarizing agents.

Keywords: ethanol, vas deferens, skeletal muscle, smooth muscle, neurotransmission and muscle contraction.

It has been reported that ethanol has peripheral effects, i.e. it affects skeletal and smooth muscles [1, 2], causing intoxication [3] and potentiation of the effects of other drugs [4, 5].

In the present investigation, we were interested in the effects of ethanol on neurotransmission and muscle contraction in the isolated vas deferens preparation. The effects of field stimulation and neurotransmitters on the rat vas deferens were studied in the absence and presence of ethanol.

Methods

Preparation: Adult male Sprague-Dawley rats, weighing 300—400 g, were killed by a blow to the head and bled. The abdomen was opened and the vas deferens was removed [6]. The preparation was set up in an organ bath containing Krebs-Henseleit solution (20 ml) maintained at 38 $\pm$ 2 °C and bubbled with 5% CO_2 in O_2. The pH of the Krebs solution was 7.2.

Stimulation and recording

The vas deferens was stimulated at 1—100 Hz, with 10—20 V (maximal) and 0.1—0.5 ms pulse duration. The preparation was set up under 0.5 g tension, and the contractile responses produced by electrical stimulation or by drug action were recorded isometrically, using a

Correspondence should be adressed to
F. A. WALI
The London Hospital Medical College, Anaesthetics Unit,
University of London
Whitechapel, El 1BB, London, England

force-displacement transducer (Dl 50 g) and a Washington pen recorder (model 400 MD 2C, Palmer Bioscience, U. K.). Stimuli were delivered from a stimulator (Sartorius) to the preparation via a pair of stimulating electrodes (silver/silver chloirode).

Procedure

After the preparation had been set up, it was left there for 15 min to settle in physiological Krebs solution. Control recordings, without any drug, were made and these were found to be stable for a long period of time (2—3 h). The effects of ethanol (3,9—390 mM), noradrenaline (0.058—580 μM), acetylcholine (0.55—55 mM) and 5-hydroxytryptamine (0.25—250 μM) on rat vas deferens were studied. Concentration-effect curves were constructed and the mean EC_{50} values (i.e. concentrations to produce 50% maximum contraction) were calculated. In constructing the concentration-effect curves, single concentrations were used (0.2 ml or 0.4 ml) and these were given at random. Contact time for ethanol varied with the concentration used (4—8 min). In general, the drug was left there in the bath until a maximum effect was obtained. The neurotransmitters, ACh, NA and 5-HT were left in the bath for 20—60 s and until a maximum effect was produced. No attempt was made to characterize the nature of transmitters released by nerve stimulation nor to record the excitatory junction potentials, since details of such investigation are well-known and are out of the scope of the present investigation.

Drugs and solutions

All drugs and solutions were made up in de-ionized distilled water. The Krebs solution used had the same composition (mM) as that previously used [7]. The drugs used were: ethanol (pure 90%, i.e 900 $mg.ml^{-1}$), acetylcholine chloride, noradrenaline, 5-hydroxytryptamine creatinine sulphate, atropine sulphate, tetrodotoxin, propranolol HCl, methysergide bimaleate and guanethidine.

Statistical analysis

Means$\pm$S.E. were calculated. Student's (t) test was employed for the significance between sets of paired and unpaired observations. A probability value of $p < 0.05$ was considered significant.

Results

1. *Effect of ethanol on the contractions produced by field stimulation of the rat vas deferens*

In the rat, the vas deferens was devoid of spontaneous activity. Ethanol (0.39—39 mM) had no apparent effect on the resting tension of the vas. Repetitive field stimulation of the vas, at 1—100 Hz with 10—20 V (maximal) and 0.1—0.5 ms pulse duration, produced frequency-dependent contractions. These contractions were reduced in the presence of ethanol (39 mM). Fig. 1 shows contractile responses of the rat vas deferens in response to field stimulation at 50 Hz for 30 s, in the absence and presence of ethanol. Repetitive nerve stimulation at 50 Hz, produced a biphasic response; a fast (phasic) and a slow (tonic) contraction. (Fig. 1a, b, c). In this experiment, we measured the slow tonic contractions. Ethanol was administered 4 min before record (1b) was taken. The slow tonic contraction (in Fig. 1a) was 1.6 g tension and this was reduced

to 1.1 g in Fig. 1b, i.e. in ethanol. After washing out (w)ethanol, the response returned towards the control height. The phasic contraction (2.3 g control) was only slightly reduced (2.0 g) in ethanol. Figure 2 shows frequency-response curves for the tonic contractions produced by field stimulation at 1—100 Hz. The mean $Freq._{50}$ (i.e. the frequency which produced 50% maximum contraction) values of the control, in ethanol and during recovery period were: 21 ± 1.5 Hz, 43 ± 4.1 Hz and 27 ± 2.2 Hz, respectively (means$\pm$S.E., $n = 8$, $P < 0.001$, P: N.S.) (for curves a, b, c). To make sure that these (phasic and tonic) contrac-

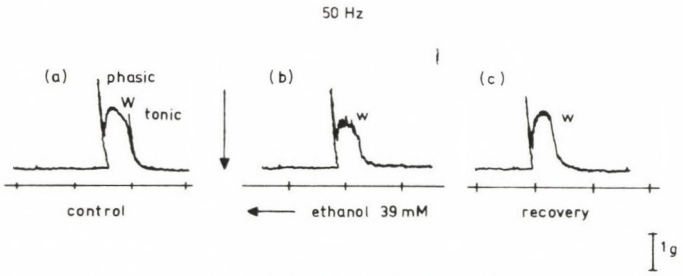

Fig. 1. Contractile responses produced by field stimulation of the rat vas deferens and the effect of ethanol. Field stimulation of the vas deferens produced phasic and tonic types of contractions which were reduced in the presence of ethanol (39 mM). Stimulus parameter: 50 Hz for 30s with 20 V and 0.2 ms pulse duration. At (w), the recorder was stopped and the preparation was washed out. Ethanol was administered 4 min before record (b) was taken

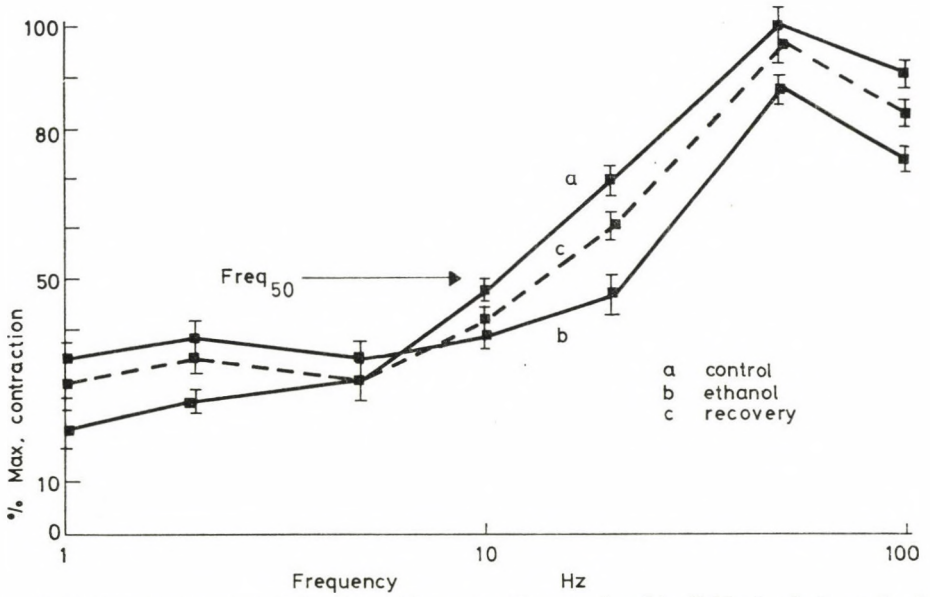

Fig. 2. Frequency-response relationship of the contractions produced by field stimulation at 1—100 Hz in the rat vas deferens. Ethanol (39 mM) shifted the control curve (a) to the right (b). In (c) recovery of the responses occurred. See text for more details. Each point is a mean$\pm$S.E. of eight experiments

tions were of a nervous origin, i.e due to neurotransmitter release from nerve terminals, tetrodotoxin (1 μM) was added to abolish these responses. In the presence of guanethidine (10 μM), propranolol (1μM), and methysergide (1 μM), field stimulation at 50 Hz, produced only small tonic contractions (0.5 g) which were greatly reduced by atropine (1 μM). These experiments indicated that the major neurotransmitter released is noradrenaline, not acetylcholine.

2. *Effect of ethanol on contractions produced in vas deferens by neurotransmitters, acetylcholine, noradrenaline and 5-hydroxytryptamine*

The contractile responses produced by neurotransmitters ACh (28 mM), NA (29 μM) and 5-HT (63 μM) are shown in Fig. 3 (a—f) in the absence and presence of ethanol (39 mM). Ethanol was introduced 5 min before the records in ethanol were taken. It can be seen that ethanol reduced the NA-induced

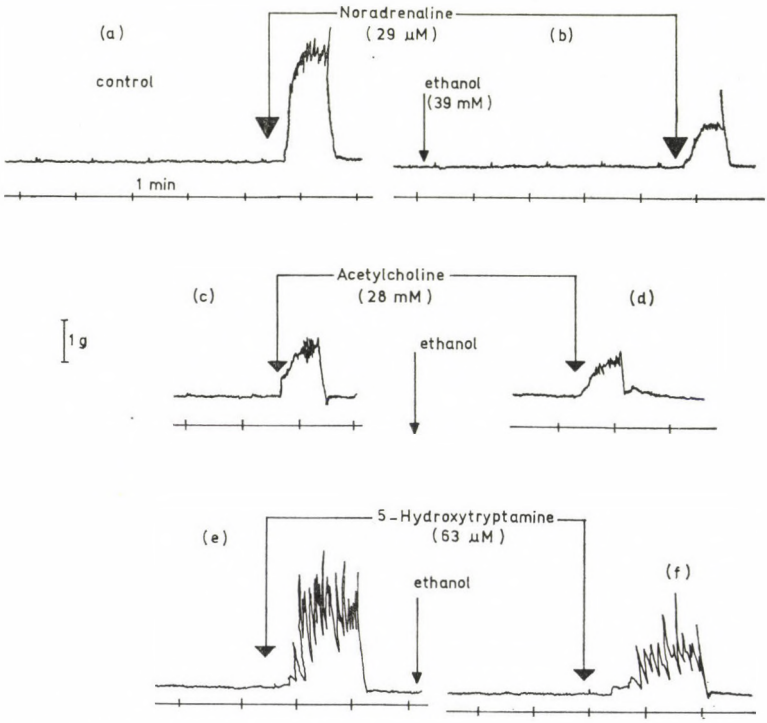

Fig. 3. Contractile responses produced by noradrenaline, acetylcholine and 5-hydroxytryptamine and the effect of ethanol in the rat vas deferens. (a) control noradrenaline response (2.8 g tension), (b) ethanol (39 mM) had no effect on resting tension but markedly reduced the noradrenaline-induced contraction (by 61% of control value). Ethanol was added 5 min before record (b) was taken. (c) ACh-induced contraction (control), (d) ACh response in the presence of ethanol (39 mM) (reduced by 31%). (e) 5-HT-induced contraction (control), (f) 5-HT response in the presence of ethanol (39 mM) (reduced by 58%). For details see text

contraction (control 2.8 g tension) by 61% (see a, b). Ethanol reduced the ACh response (1.3 g) by 31% and the 5-HT response (2.4 g) by 58% (see c—d and e—f). Pooled results are shown in Table 1.

Figure 4 shows concentration-response relations of NA-induced contractions and the effect of ethanol (39 mM). Ethanol shifted the control NA curve

Table I

*Contractions produced by neurotransmitters, noradrenaline, acetylcholine and 5-hydroxytryptamine and effect of thanol on rat vas deferens preparation (means ± S.E., n = 8, *P < 0.001)*

	(g) Maximum Contraction	EC_{50} Control (C)	EC_{50} Ethanol(E)	EC_{50} Ratio(E/C)
Noradrenaline (0.058—580 μM)	4.9±1.1	32±0.9	95±3.1*	3:1
Acetylcholine (0.55—55 mM)	1.6±0.9	14±0.4	46±1.2*	3.3:1
5-hydroxytryptamine (0.25—250 μM)	2.6±0.5	65±3.1	164±10.4*	2.5:1

* Ethanol (39 mM) (E) significantly increased the mean EC_{50} values of control (C) concentrations, indicating a marked depression of neurotransmitter responses

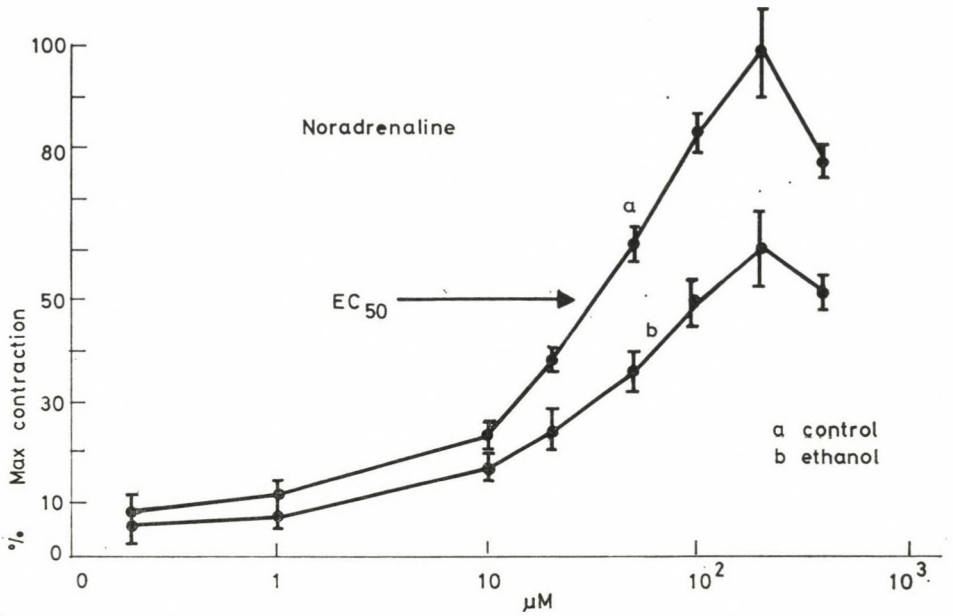

Fig. 4. Concentration-response curves of noradrenalin-induced contractions in control and ethanol solutions (bathing rat vas deferens preparations). Ethanol (39 mM) shifted the control (a) noradrenaline curve to the right and downwards (b). For more details see text. Each point is a mean± S.E. of eight experiments

to the right and downwards (a to b). The mean EC_{50} values of NA-induced contractions in control and ethanol solutions were (32 ± 0.9 μM and 95 ± 3.1 μM, respectively (means $\pm$ S.E. $n = 8$, $P < 0.001$).

Discussion

It has been reported that ethanol, unlike ethyl ether, or other general anaesthetics, stimulates (depolarizes) the skeletal muscle of the frog sartorius [8, 9]. In the gut smooth muscle, ethanol inhibited the spontaneous contractions and reduced the contractions produced by depolarizing agents [1, 3, 4]. However, in high concentrations (390 mM), ethanol produced marked contractions in the gut muscle [10].

We have chosen the vas deferens preparation since this preparation is mainly adrenergic mechanism [11]. We aimed at two specific points: (a) to see if ethanol modified neurotransmission, and (b) if ethanol influenced the vas deferens and its contractility, with respect to neurotransmitters, ACh, NA and 5-HT [12, 13]. It is certain that ACh is involved in neurotransmission in the vas deferens, this is in accordance with the theory of Burn & Rand [14] and with the electrophysiologic studies of Richardson [15], who suggested that the longitudinal coat of the guinea-pig vas deferens may be innervated by cholinergic fibres. That 5-HT may be involved in vas deferens neurotransmission is not yet known. However, noradrenaline is the main neurotransmitter involved in neurotransmission in the rat vas deferens, and we have demonstrated this using various adrenergic and other blocking agents in the present investigation.

The present results showed that ethanol had a dual action, depending on the contraction used, and on the rate of field stimulation. Ethanol (39 mM) enhanced the contractions produced by low rates of stimulation (1—5 Hz), whereas it depressed the contractions produced at 10 Hz or more. A typical response of field stimulation consisted of two phases: fast (phasic) and slow(tonic) contractions. These responses probably reflect the mechanism of action of noradrenaline, released by nerve stimulation to produce excitatory junction potential (phasic), followed by a contraction of low amplitude and prolonged duration(tonic). The contraction produced by NA is due to a release of Ca^{2+}, from intracellular stores [16—18]. Thus Na can affect both receptor and voltage-dependent calcium channels.

The results also suggested that ethanol may have pre- and postsynaptic effects at the rat vas deferens preparation, since ethanol reduced the contractions produced by field stimulation and by neurotransmitters added to the preparation. Among other actions of ethanol, is the blockade of nerve or muscle conduction, i.e acting like the local anaesthetic lignocaine. Since the vas deferens is a quiescent preparation, i.e it produces no action potential, it is possible

that the contraction produced in the muscle, due to electrical or chemical stimulation, is calcium based. More experiments are needed to explain the effect of ethanol on the contractions produced in the vas deferens in relation to the role of calcium.

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THE DETERMINATION OF URINARY FLUORIDE/CREATININE RATIO (Q) IN MONITORING FLUORIDE INTAKE

Pálma KERTÉSZ, Jolán BÁNÓCZY, B. RITLOP, Andrea BRÓDY, Mária PÉTER

Dept of Conservative Dentistry and Second Dept. of Internal Medicine, Semmelweis University, Medical School, Budapest, Hungary

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Linear correlation ($r = 0.6811$, $p < 0.001$) was found between the 24 hour urinary excreted fluoride quantity and fluoride/creatinine ratio (Q) of the morning urine sample of 21 school children living in a children's home in Hungary. The mean value of fluoride/creatinine ratio (Q) in groups of children from eight different parts of Hungary, also showed linear correlation ($r = 0.9720$, $p < 0.001$) with the measured fluoride concentration of drinking water. The urinary fluoride/creatinine ratio (Q) seems sufficiently informative for controlling fluoride intake in the course of caries preventive field studies.

Keywords: Urinary fluoride/creatinine ratio (Q), monitoring of fluoride intake

The role of fluoride is well known in caries prevention and it is widely used in collective and individual prevention. In human caries preventive studies where fluoride is systemically administered the control of the total fluoride intake is a task of fundamental importance [2, 3, 4, 5, 7, 9, 10, 11, 12, 13, 14, 15, 18]. The determination of the 24 hour urinary fluoride is considered to be one of the most acceptable indices of fluoride intake in the steady state of fluoride balance [8, 1]. It yields reliable results only when fluoride supplementation is monitored continuously. However this close monitoring, for technical reasons, is not feasible within the conditions of field studies involving hundreds of participants. To monitor the fluoride intake in order to avoid the difficulties of the 24 hour urine collection, the above cited authors, except Hefti et al. [7], measured the fluoride concentration of one or two urinary spot samples per day, taking into consideration that it reflected the fluoride metabolism with a lower degree of precision. Wespi and Bürgi [18] reported that the fluoride/creatinine ratio (Q) of a single urinary spot sample could reflect the fluoride ingestion at group level, but they did not discuss the possible advantages of its usage. The aim of our present study was to investigate whether the fluoride/creatinine

Correspondence should be adressed to
Pálma KERTÉSZ
Department of Conservative Dentistry, Semmelweis University Medical School
1088 Budapest, Mikszáth Kálmán tér 5., Hungary

ratio of urinary spot samples was just as informative of fluoride intake as the amount of the 24 hour urinary fluoride or not, and whether it was usable within the frame of field studies.

Materials and methods

The material consisted of 326 healthy subjects between 8 and 13 years of age, living in 11 children's homes in Hungary. These children live under almost identical conditions and have similar eating habits due to the centralised standardisation of homes. Alimentary fluoride intake was negligible. There was no sizeable fluoride exposure through the drinking water and the air (Table I., II and personal communication of Dept. of Air Hygiene of National Institute of Public Health). Home No 7. (Table I.) where the highest fluoride concentration of drinking water was measured, represented about 30% of the optimal fluoride level recommended for caries prevention. In three separate children's homes (Table II.) fluoride supplementation was introduced via fluoridated milk. The protocol for preparation of milk called for 0.75 mg fluoride in 0.2 ml milk in form of NaF. The children consumed 0.2 ml milk daily during 250 days per year. Our investigations were carried out on approximately the 200th day. Urinary spot samples were collected between 7—8 hours in the morning. In 21 girls the 24 hour urinary excreted fluoride amount and the fluoride/creatinine ratio of the spot samples were determined simultaneously.

Fluoride concentration was determined by fluoride-sensitive electrode (Radelkis OP-7443), while the creatinine concentration was measured by a method based on the Jaffe reaction using Centrifichem 600 (Union Carbide), a centrifugal analyzator system. The fluoride/creatinine ratio was expressed as the quotient of molar concentrations in mol/mol $\times 10^{-3}$. TISAB buffer from Radelkis (Budapest) and "Test Combination Creatinine" from Boehringer (Mannheim) was used, other chemicals were purchased from Reanal (Budapest).

Table I

The fluoride concentration of drinking water and urinary fluoride/creatinine ratio (Q) of children living in different children's homes in Hungary

Home No	(F) of drinking water μmol/l	No of children investigated	(F)/(Cr) (Q) mol/mol $\times 10^{-3}$	
			$\bar{X}$	S.D.
1	6.0	14	1.39	0.63
2	5.0	32	1.44	0.60
3	3.6	16	0.91	0.40
4	7.0	21	1.51	0.48
5	4.0	46	1.25	0.63
6	9.0	32	1.48	0.70
7	20.0	17	2.88	1.74
8	4.2	14	1.25	0.63

Results

Linear correlation was obtained between the 24 hour urinary excreted fluoride quantity and the fluoride/creatinine ratio (Q) of morning urine samples, $r = 0.68, p < 0.001$ (Fig. 1.).

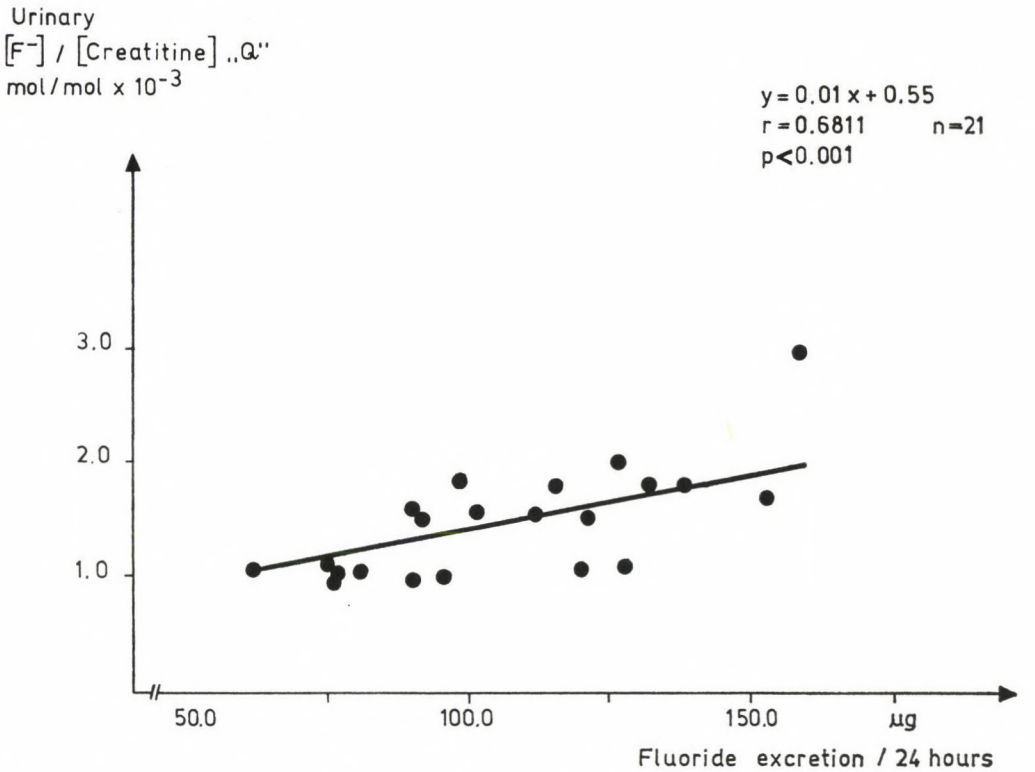

Fig. 1. Correlation analysis between the 24 hour urinary fluoride excretion and fluoride creatinine ratio (Q) of urinary spot samples taken in the morning

The mean value of fluoride/creatinine ratio (Q) in groups of children from eight different parts of Hungary, also showed linear correlation with the measured fluoride concentration of drinking water, $r = 0.9720$, $p < 0.001$ (Fig. 2.).

No singnificant difference was found in the fluoride/creatinine ratio (Q) according to sex, in any of the homes.

Out of the three children's homes, where fluoridated milk consumption was called for, an elevated fluoride/creatinine ratio was measured in home "C", only (Fig. 2. and Table II.).

Table II

The fluoride concentration of drinking water and urinary fluoride/creatinine ratio (Q) from homes where fluoridated milk consumption was prescribed

Home	(F) of drinking water µmol/l	No of children investigated	(F)/(Cr) mol/mol × 10^{-3} $\bar{X}$	(Q) S.D.
"A"	3.0	73	1.47	0.58
"B"	5.0	27	1.64	0.80
"C"	7.0	34	2.56	1.52

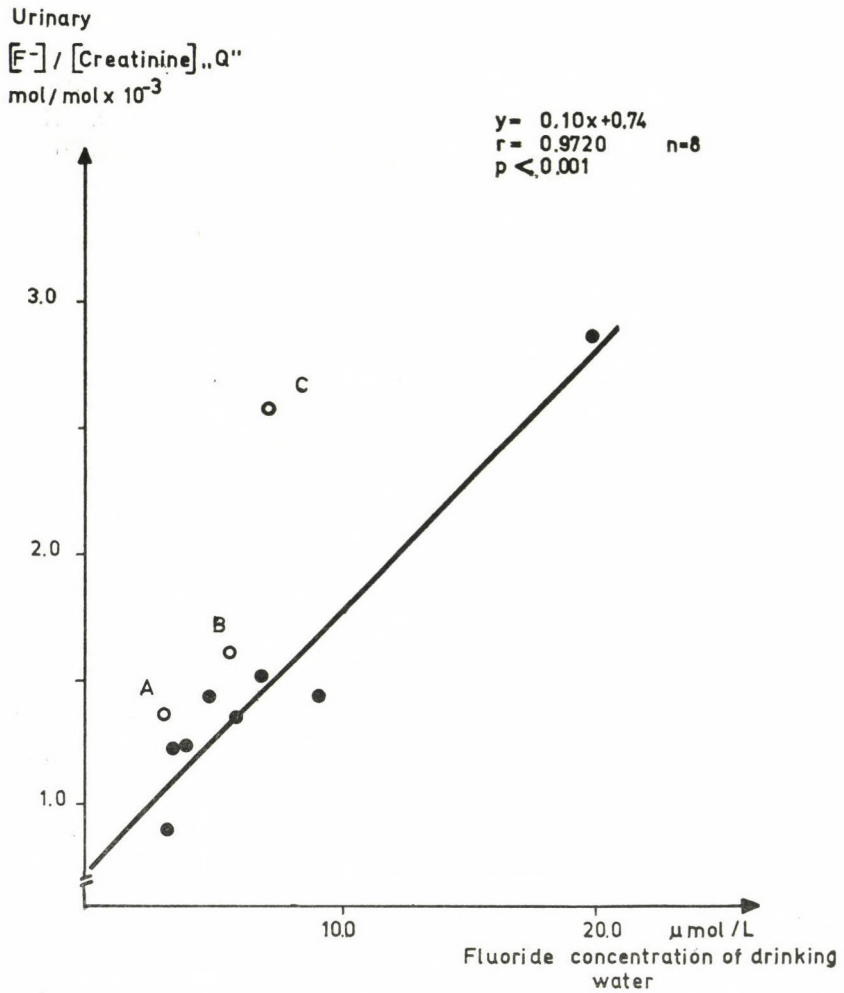

Fig. 2. Correlation analysis between the fluoride concentration of drinking water and the fluoride creatinine ratio (Q) of urinary spot samples taken in the morning. Regression line was calculated on the basis of filled circles (●—●), while the discrete circles (○ ○ ○) represent the mean of homes "A", "B", and "C", where the control of fluoride intake was performed

Discussion

In healthy objects significant correlation was obtained between the fluoride and creatinine clearance [17], just as between the plasma fluoride and plasma creatinine concentrations in the age group [6] investigated in the present study. In consequence the fluoride/creatinine ratio (Q) of a spot sample can be regarded as constant in the course of the examination. This suggests that the

urinary fluoride/creatinine ratio (Q) may be more informative of the fluoride intake than the fluoride concentration of one or several urine fractions because this index (Q) is independent of the actual diluting or concentrating function of the kidney.

Between the 24 hour urinary fluoride excretion and the fluoride/creatinine ratio (Q) of the morning urine sample a highly significant relation was found. However, the absolute value of the correlation coefficient (0.6811) indicates that, for individual tests, the fluoride amount excreted with the 24 hour collected urine can not be replaced by the fluoride/creatinine ratio. On the other hand, the close linear correlation ($r = 0{,}9720$, $p < 0.001$) observed between the fluoride concentration of drinking water and the mean value of fluoride/creatinine ratio (Q) of children from different parts of Hungary, confirm that the fluoride/creatinine ratio (Q) on group level, is an adequate method for monitoring the fluoride intake, due to the highly significant correlation between the fluoride concentration of drinking water and urinary fluoride excretion [literature cited in Ref. 8].

The observation, whereby the same fluoride intake results in a lower urinary fluoride output in females than in males [6, 7, 10, 16] seems to be contradictory with the finding that there was no difference between the fluoride/creatinine ratio (Q) of girls and boys. It can be explained and resolved by the fact that males excrete higher amount of creatinine than females, and so the difference in the urinary fluoride excretion according to the sexes disappears when the fluoride/creatinine ratio is calculated.

Two out of three children's homes where the milk fluoridation programme was introduced, in homes "A" and "B", the mean values of the measured fluoride/creatinine ratios and their relation to the regression line of Fig. 2. enabled us to demonstrate that these groups had not consumed fluoridated milk as regularly as called for by the protocol. It was also confirmed by the management of the homes, but only after the urine analysis had been carried out and evaluated. In home "C" the fluoride/creatinine ratios were higher, corresponding to the expectations.

Our results seem to indicate that the simultaneous determination of the fluoride/creatinine ratio (Q) and fluoride concentration of drinking water makes it possible to monitor the fluoride administration within the frame of a caries preventive field study with adequate certainty.

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INTERACTION BETWEEN THE Ca^{2+}-ATPase AND THE PROTEOLIPID IN ARTEFICIAL MEMBRANES

I. JÓNA+, A. MARTONOSI

Department of Biochemistry, Suny, Upstate Medical Center, Syracuse, New York, 132101 USA

Received October 10, 1987
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The interaction between the Ca^{2+} transport ATPase and the proteolipid of rabbit sarcoplasmic reticulum was analyzed by fluorescence energy transfer, using the following donor : acceptor combinations: Ca^{2+}-ATPase tryptophan→IAEDANS-proteolipid; IAEDANS-ATPase→IAF-proteolipid; IAEDANS-proteolipid→IAF-ATPase. The observed energy transfer may indicate weak interaction between the Ca^{2+}-ATPase and proteolipid, but collisional energy transfer definitely contributes. The energy transfer was abolished by deoxycholate or sodium dodecylsulfate at concentrations sufficient to solubilize the membrane. In view of the low proteolipid content of sarcoplasmic reticulum and the weak interaction suggested by the energy transfer, at best only a small fraction of ATPase molecules could exist in the form of ATPase-proteolipid complexes.

Keywords: proteolipid, Ca^{2+}ATPase, fluorescence, energy transfer

In addition to the Ca^{2+} transport ATPase sarcoplasmic reticulum membranes contain a proteolipid with an estimated molecular weight of 12,000 [9, 10]. The proteolipid copurifies with the ATPase, which led to speculations about its possible involvement in the Ca^{2+} transport [10]. Racker and his coworkers suggested that the proteolipid may serve as ion channel during the Ca^{2+} translocation [13, 4].

The proteolipid fraction of sarcoplasmic reticulum contains at least four distinct components [4, 12]. The major component, which accounts for 65—70% of the total proteolipid, contains tyrosine as the C-terminal amino acid, and its N-terminal sequence is Met-Glx-Arg-Ser-Thr-Arg-Glx-Leu-Cys-Leu--Asp-Phe [12]. The proteolipid is characterized by a relatively high tyrosine, phenylalanine and apolar amino acid content and the absence of tryptophan.

The purpose of the studies presented in this report was to analyze by fluorescence energy transfer, whether interaction exists between the Ca^{2+} transport ATPase and the proteolipid in reconstituted sarcoplasmic reticulum vesicles. Several approaches were utilized:

Correspondence should be addressed to (and present adress)
István JÓNA
Central Research Laboratory, University Medical School, Debrecen
H-4012 Debrecen Nagyerdei krt 98, Hungary

1. Energy transfer between the tryptophan groups of the Ca^{2+}-ATPase and dansyl-proteolipid or IAEDANS-proteolipid incorporated into the reconstituted ATPase vesicles. These experiments are feasible because the proteolipid does not contain tryptophan and the emission spectrum of tryptophan overlaps with the absorption band of the dansyl or IAEDANS chromophores which serve as energy acceptors.

2. Energy transfer between IAEDANS-ATPase and IAF-proteolipid.

3. Energy transfer between IAEDANS-proteolipid and IAF-ATPase.

Energy transfer was measured by analyzing the ratio of donor and acceptor fluorescence intensities and by measuring the lifetime of donor fluorescence in the presence or absence of acceptor.

The observations indicate fluorescence energy transfer between the Ca^{2+}-ATPase and the proteolipid under conditions when the proteolipid concentration in the membrane was relatively high. While these observations are consistent with close proximity between the ATPase and proteolipid molecules in the membrane, collision is likely to contribute to the energy transfer. There is no clear indication that stoichiometric Ca^{2+}-ATPase -proteolipid complexes exist in the membrane under the conditions of these experiments.

Materials and methods

Rabbit microsomes isolated as described earlier [11] were used for the preparation of Ca-ATPase and proteolipid. Ca-ATPase was prepared according to MacLennan [9]. The reconstituted preparations had ATPase and Ca transport activity comparable to those reported in the literature [3]. Proteolipid was isolated by a combination of two methods (10, 15), and further purified by the procedure of Ohnoki and Martonosi [12].

Phosphatidylcholine vesicle were prepared as follows: 20 mg phosphatidylcholine in ethanol solution was dried onto the wall of a small Potter homogenizer under vacuum. Two ml buffer were added and the suspension was dispersed by homogenization. Sonication was carried out under N_2 atmosphere at 0 °C using a Heat System, Ultrasonics, Inc., Model W185E (Plainview, N. Y.) sonicator, for 10 times 10 seconds, with 5 second interruptions, to allow the temperature to equilibrate. The preparation was stored at 4 °C and used within five hours. Measurement of ATPase activity was carried out as described earlier [12]. For labelling with dansyl-Cl, the proteolipid was dissolved in a medium containing n-butanol, 2.5 mM $NaHCO_3$, 2.5% water and dimethylaminonaphthalenesulfonylchloride (2.4 to 48 mole/mole proteolipid). The final concentration of proteolipid was 2.1×10^{-5} M (249 μg/ml). After reaction for 2—5 h at 37 °C, the reaction mixture was diluted with 50 volumes of 5 mM $NaHCO_3$ and dialyzed against several changes of 500 volumes of 5 mM $NaHCO_3$ and finally against water. The labelled proteolipid was lyophilized and dissolved in n-butanol. The concentration of bound dye was evaluated from the fluorescence spectra. The absence of free dye was tested by thin-layer chromatography on silica gel G using a solvent system of chloroform:methanol:NH_4OH = 65:35:5 (v/v), or chloroform:methanol:1 M HCl = 66:33:1 (v/v).

For labelling with IAEDANS the proteolipid was dissolved in n-butanol (440 μg/ml) and incubated for 2 h at 37 °C under nitrogen in the presence of 10-fold molar excess dithiothreitol in a 4% water, 94% butanol mixture. Solid IAEDANS was added in 100-fold molar excess and the incubation continued for 5 hours under continuous stirring. The sample was dried under a stream of nitrogen, dispersed in 0.1 M KCl, 10 mM Tris, pH 7.0; with vigorous stirring and dialyzed against several changes of 0.1 M KCl, 10 mM Tris-HCl, pH 7.6, followed by dialysis again water. The sample was lyophilized, and dissolved in n-butanol. Free IAEDANS was removed by chromatography on Sephadex LH-20 column (40 × 0.8 cm) equilibrated with n-butanol.

For labelling the proteolipid with IAF, the proteolipid (355 μg/ml) was reacted with 100-fold molar excess of IAF in a medium containing 98% n-butanol, 2% water and 2 mM NH_4HCO_3. After centrifugation at 2,000 g for a few minutes, the solution was loaded onto a Sephadex LH-20 column equilibrated with n-butanol. Aliquots of the proteolipid fractions were evaporated to dryness under vacuum, dissolved in 0.1% SDS and the protein content was determined according to Lowry et al. [8]. The peak fractions were pooled, drie d under vacuum and dissolved in n-butanol.

The labelling of purified ATPase (10 mg protein/ml) was carried out essentially as described earlier [17] in a medium of 1 M KCl, 250 mM sucrose, 50 mM Tris-HCl, and 10 mM imidazole, ph 8.0. IAEDANS or IAF were added to 4.4 to 44-fold molar excess over ATPase and the labelling continued with stirring at 5 °C overnight. The free dye was removed by chromatography on Sephadex G-50 column (70 × 1 cm) equilibrated with 1 M KCl, 250 mM sucrose, 50 mM Tris-Cl and 10 mM imidazole, pH 8.0 and 0.02% Na-azide. The turbid fluorescent fractions containing the labelled ATPase were clearly separated from the free dye. The ATPase was collected by centrifugation at 65,000 g for 1 h and the pellet was resuspended in buffer of appropriate composition as described in Legends. The ATPase activity of the labelled preparations was only slightly lower than that of the control. The amount of protein-bound label was calculated from absorption and fluorescence date corrected for light scattering, in comparison with known concentration of free dye measured under similar conditions.

For the incorporation into ATPase vesicles the required amount of proteolipid dissolved in n-butanol was evaporated in a small glass tube under nitrogen. The appropriate buffer solution containing purified Ca^{2+}-ATPase vesicles at a final concentration of 0.1—0.9 mg/ml was added and the mixture was vigorously stirred for 1 h at 25 °C and continued with gentle stirring at 5 °C for 12—24 h.

For fluorescence quenching studies to labelled ATPase preparations (55 μg/ml) suspended in 0.1 M NaCl and 10 mM Tris, pH 7.6, NaI or iodobenzene were added in increasing amounts up to final concentrations of 1.9 M and 5 mM, respectively. Stock solutions of NaI were dissolved in the same buffer as the ATPase with 0.2 mM Na-thiosulfate added while iodobenzene was dissolved in ethanol. Corresponding volumes of buffer or ethanol were added to control samples to correct for optical and dilution effects. The relationship of fluorescence to the concentration of quencher was evaluated according to the Stern-Volmer (16) equation:

$$\frac{F_0}{\Delta F} = \frac{1}{K_Q C_Q g(a)} + \frac{1}{g(a)}$$

where g(a) describes the accessibility of the fluorophore to the quencher K_Q is the quenching constant, C_Q the concentration of quencher, F_0 the fluorescence intensity in the absence of quencher, and ΔF is the change in fluorescence due to quenching after correction for absorption of the fluorescence by the quencher. K_Q was calculated by least square fitting and g(a) by iterative computer analysis of a linear form of the Stern-Volmer equation.

Absorption spectra were recorded on an Aminco DW-2 spectrophotometer. Fluorescence measurements were carried out on an Aminco-Bowman spectrophotofluorometer equipped with a thermostated cell holder. For all the measurements 1 × 1 cm quartz cuvettes were used with magnetic stirring at 20 °C.

N-iodoacetyl-N^1-(5-sulfo-1-naphthyl) ethylenediamine (IAEDANS) and 5 iodoacetamidofluorescein (IAF) were obtained from Molecular Probes, Inc., Plano, Texas. Sephadex LH-20, dithiothreitol, glycogen, and deoxycholate were purchased from Sigma Chemical Co., St. Louis. Deoxycholate was recrystallized twice from ethanol before use. Iodobenzene, acrylamide, NN^1-methylene bisacrylamide and tetramethylethylene diamine were obtained from Eastman-Kodak, Rochester, N.Y., n-butanol from Mallinckrodt, St. Louis, Mo., and phosphatidylcholine from Sylvana Chemical Co., Grand Island, N.Y.

Results

The proteolipid was labelled with dimethylaminonaphthalene sulfonyl chloride (dansyl chloride) and its fluorescence emission spectrum was compared with that of several dansyl amino acids (Fig. 1). The spectrum of proteolipid contains significant contribution from O-dansyl-l-tyrosine. The number of dansyl groups introduced into proteolipid increases with reactant concentration

but even with 48 moles of dansyl chloride per mole of proteolipid in the reaction mixture only 0.59 moles of DANS were covalently bound per mole of proteolipid. The fluorescence intensity at 500 nm increases with the bound DANS content upon excitation at 350 nm, and to a lesser extent, at 280 nm indicate that intramolecular energy transfer from tyrosine to bound DANS residues within the proteolipid is minimal. Reconstituted vesicles containing the Ca^{2+} transport ATPase and DANS-proteolipid at a weight ratio of 100:1 were excited at 280 nm, and the fluorescence emission was measured at 500 nm. The intensity of fluorescence was significantly greater in reconstituted ATPase-DANS proteolipid vesicles than the sum of the fluorescence intensities of the Ca^{2+}-ATPase and DANS-proteolipid measured separately. This suggests energy transfer from chromophores on the Ca^{2+} transport ATPase to the DANS-residue bound to the proteolipid. The difference fluorescence attributable to energy transfer is much

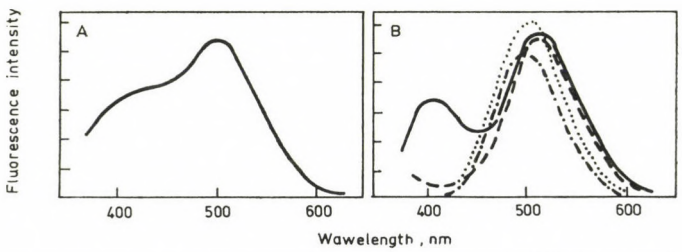

Fig. 1. Fluorescence emission spectra of dansyl proteolipid and dansyl derivatives of various amino acids. The emission spectra were recorded at an excitation wavelength of 350 nm. All compounds were dissolved in n-butanol. A. Dansyl proteolipid (1.17 μM); B. Dansyl-L-tyrosine (—, 1.15 μM); dansyl chloride (— — —, 1.0 μM); 0-dansyl-L-lysine (. . ., 1.25 μM); dansyl-L-methionine (·—·—·, 1.2 μM).

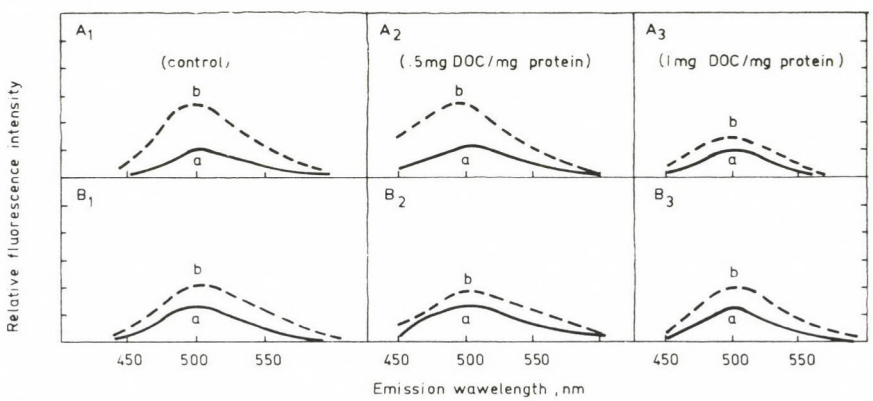

Fig. 2. Effect of deoxycholate on energy transfer between Ca^{2+}-ATPase and proteolipid. The fluorescence emissions of the samples were measured at excitation wavelengths of 280 nm (A) and 350 nm (B) at the following deoxycholate concentrations: 0 (A_1, B_1); 0.5 mg deoxycholate per mg protein (A_2, B_2); and 1 mg deoxycholate per mg protein (A_3, B_3). Line "a" represents the emission spectrum of proteolipid (Sample 2) after correction for blank (Sample 1). Line "b" represents the difference emission spectrum of the ATPase+proteolipid system (Sample 4) minus the ATPase (Sample 3).

smaller upon excitation at 350 nm (Fig. 2B) which is the optimum wavelength for direct excitation of the dansyl chromophore. The deoxycholate (0.375—0.75 mg/ml) solubilized the membranes and greatly reduced the fluorescence of DANS-proteolipid in systems containg Ca^{2+} transport ATPase excited at 280 nm (Fig. 2A and Table II, column 1 and 3). The deoxycholate had no effect upon the fluorescence of DANS-proteolipid in the absence of ATPase using either 280 nm (Fig. 2A, Table II, column 2) or 350 nm for excitation. The fluorescence intensity of DANS-proteolipid was also unaffected by deoxycholate with or without ATPase present using light of 250 nm for excitation (Fig. 2B, Table II, column 4). The solubilization of membranes by deoxycholate decreased the absorbance of the suspension at 280 nm. These observations are consistent with the interpretation that efficient fluorescence energy transfer requires the proxi-

Table I

Reaction of proteolipid with dansyl-Cl

Sample	Moles of dye per mole proteolipid in the reaction mixture	Moles of dye bound per mole proteolipid	Relative fluorescence intensity at 500 nm	
			excited at 280 nm	excited at 350 nm
n-BuOH control	—	—	0.5	1.1
Proteolipid #1	—	—	2.5	3.0
Proteolipid #2	2.4	0.17	5.0	6.5
Proteolipid #3	12.0	0.32	5.5	9.5
Proteolipid #4	48.0	0.59	5.0	17

The proteolipid was dissolved in n-butanol containing 2.5% H_2O and 2.5 mM $NaHCO_3$ to final concentrations of 126—248 μg/ml, and labelled with dansyl-chloride (2.4—48 moles per mole of proteolipid) for 2 h at 37 °C as described under Methods and Materials. The amount of bound dye was determined from the fluorescence emission at 500 nm using light of 350 nm for excitation.

Table II

Effect of deoxycholate upon energy transfer between tryptophan residues of Ca^{2+}-ATPase and dansyl proteolipid

	Fluorescence intensity ratio				
	$(4—3)\lambda_{280}$ / $(4—3)\lambda_{350}$	$(2—1)\lambda_{280}$ / $(2—1)\lambda_{350}$	$(4—3)\lambda_{280}$ / $(2—1)\lambda_{280}$	$(4—3)\lambda_{350}$ / $(2—1)\lambda_{350}$	Absorbance at 280 nm as per cent of Control
Control	1.41 (100%)	0.7 (100%)	2.39 (100%)	1.6 (100%)	100%
0.375 mg DOC/ml	1.35 (95%)	0.8 (114%)	2.2 (92%)	1.5 (94%)	85%
0.75 mg DOC/ml	0.87 (62%)	0.8 (114%)	1.45 (61%)	1.6 (100%)	75%

Medium: 0.1 M KCl, 10 mM Tris-HCl, pH 7.4, with deoxycholate (DOC) added in the indicated final concentrations. The dansyl proteolipid contained 0.74 mole dansyl per 12,000 g of protein. The fluorescence intensity was measured at 500 nm using light of 280 nm or 350 nm for excitation. The difference fluorescence ratios given in the Table refer to the following sample numbers: 1, protein-free control; 2, dansyl proteolipid (7.6 μg/ml); 3, purified ATPase (0.75 mg/ml); 4, dansyl proteolipid (7.6 μg/ml) incorporated into ATPase vesicles (0.75 mg/ml).

mity of Ca^{2+}-ATPase and DANS-proteolipid molecules in the membrane; solubilization of the membrane with deoxycholate increases the average distance between donor (ATPase) and acceptor (DANS-proteolipid) molecules and thereby causes a decrease in the efficiency of energy transfer.

As shown in Fig. 3, the fluorescence intensity of IAEDANS-proteolipid excited at 290 nm is much greater in the presence (Fig. 3B) than in the absence (Fig. 3A) of the Ca^{2+}-ATPase, indicating that the excitation of tryptophan resi-

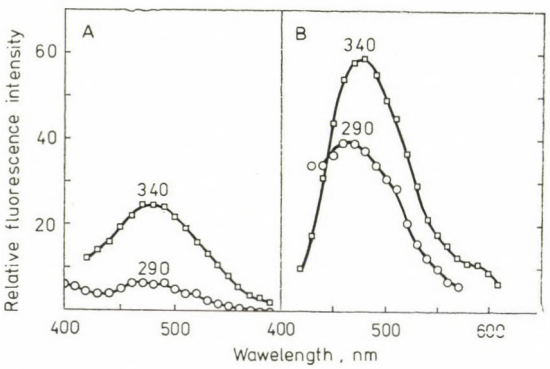

Fig. 3. Energy transfer from tryptophan groups of unlabelled ATPase to IAEDANS-proteolipid. The IAEDANS-proteolipid was prepared as described under Materials and methods and in Legend in Table 3. The medium contained 100 mM KCl, and 10 mM Tris-HCl, pH 7.4. Sample 1 served as control. Sample 2 contained IAEDANS-proteolipid (9.6 μg/ml); Sample 3 contained unlabelled ATPase (950 μg/ml). Sample 4 was a mixture of unlabelled ATPase and IAEDANS proteolipid at final concentrations of 950 μg/ml and 9.6 μg/ml, respectively. The ATPase/proteolipid molar ratio was 8.3. The proteolipid contained 1.75 mole dye per 12,000 g protein. Measurements were performed at room temperature 24 hours after mixing. A. Difference fluorescence of Sample 2 minus Sample 1. B. Difference fluorescence of Sample 4 minus Sample 3. Excitation wavelengths were 290 nm (0—0) and 340 nm (□—□)

dues in the Ca^{2+}-ATPase contributes to the fluorescence emission of IAEDANS residues on the proteolipid. This difference fluorescence is much reduced in the presence of 1—2 mg deoxycholate per mg protein (Table III, column 1), which brings the membrane into solution. The fluorescence of Ca^{2+}-ATPase-IAEDANS-proteolipid systems excited at 340 nm as compared with 290 nm was much less sensitive to deoxycholate (Table III, column 2). The fluorescence of IAEDANS-proteolipid in the absence of ATPase was essentially unaffected by deoxycholate at either 290 or 340 nm excitation (Table III, columns 3 and 4). These observations are consistent with energy transfer between the tryptophan residues of the Ca^{2+}-ATPase and the IAEDANS-proteolipid in reconstituted ATPase vesicles; after solubilization with deoxycholate the energy transfer is abolished due to the dilution of donor and acceptor fluorophores. The absorption spectra of ATPases labelled with IAEDANS and IAF are characterized by absorption maxima at about 340 nm and 490 nm, respectively (Fig. 4). The

Table III

Effect of deoxycholate upon the difference fluorescence spectra of systems containing unlabelled ATPase and IAEDANS-proteolipid

	Difference Fluorescence Emission			
	Sample #4—Sample #3		Sample #2—Sample #1	
	λ_{290}	λ_{340}	λ_{290}	λ_{340}
Control	39.5 (100%)	59 (100%)	6.1 (100%)	24.5 (100%)
0.5 mg DOC per mg protein	39 (98%)	56.2 (96%)	5,6 (93%)	23.5 (96%)
1 mg DOC per mg protein	33 (83%)	54 (92%)	5.7 (95%)	23.9 (98%)
2 mg DOC per mg protein	29.1 (74%)	54 (92%)	5.9 (96%)	23.7 (97%)

To samples prepared as described in Fig. 6, deoxycholate (DOC) was added to final concentrations of 0.5, 1.0 and 2.0 mg per mg protein; the fluorescence emission was measured at 470 nm using light of 290 or 340 wavelength for excitation. The difference data are given in terms of the following sample numbers: 1, protein-free control; 2, IAEDANS-proteolipid; 3, Ca^{2+}-ATPase; 4, Ca^{2+}-ATPase + IAEDANS-proteolipid.

amount of bound label was 1.8—4.3 mole/mole of IAEDANS ATPase and 0.4—1.2 mole/mole of IAF-ATPase (Table IV). The ATPase activity of labelled preparations was in the range of 0.9—1.5 μmole P_i/mg/min, at 25 °C.

The fluorescence emission spectrum of IAEDANS-proteolipid overlaps with the absorption spectrum of IAF-ATPase, permitting energy transfer measurements between the two proteins. In mixtures of IAEDANS-proteolipid and IAF-ATPase, the fluorescence emission of IAF measured at 540 nm is greatly enhanced compared with IAF-ATPase in the absence of proteolipid. At the same time, the fluorescence emission of IAEDANS-proteolipid is reduced in

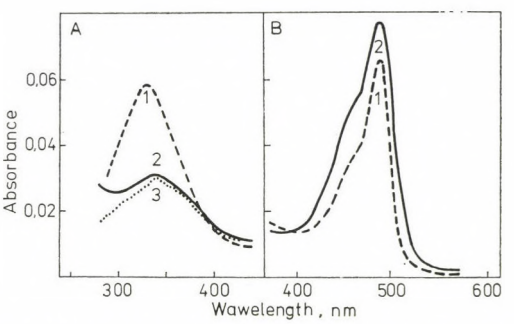

Fig. 4. Absorption spectra of IAEDANS-ATPase and IAF-ATPase. ATPase was labelled with IAEDANS and IAF as described under Materials and Methods. Measurements were carried out in a medium of 20 mM KCl, 2.5 mM Tris-Cl, pH 8.0 and 5 mM sucrose. A. 1. Free IAEDANS (10 μM); 2. IAEDANS-ATPase (0.2 mg protein per ml containing 23.5 nmole dye per mg protein); 3. Sample 2 after correction for the absorption of unlabelled ATPase. B. 1. Free IAF (1 μM); 2. IAF-ATPase (0.21 mg protein per ml containing 4.19 nmole IAF per mg protein).

Table IV

Labelling of Ca^{2+}-ATPase with IAEDANS and IAF

Label	Moles of dye per mole of ATPase in reaction medium	Incubation time, hours	ATP Concentration, mM	Moles of bound dye per mole of ATPase	ATPase Activity μmole P_i/mg/min
IAEDANS	4.4	14	—	2.1	1.3
IAEDANS	4.4	14	10	1.8	1.3
IAEDANS	44.3	19	—	4.3	1.0
IAEDANS	44.3	19	10	3.4	0.9
IAF	4.4	14	—	0.4	1.4
IAF	4.4	14	10	0.5	1.5
IAF	44.4	19	—	1.2	1.3
IAF	44.4	19	10	1.0	1.4

The labelling was carried out as described under Methods and Materials in a medium of 1 M KCl, 250 mM sucrose, 50 mM Tris-Cl, pH 8.0. The amount of protein-bound dye was determined from the absorption spectrum of the labelled derivatives using an extinction coefficient of 5.9×10^3 for IAEDANS (at 335 nm) and 7.2×10^4 for AIF (at 495 nm). A similar value for the number of moles of dye bound per mole of protein can also be obtained from the fluorescence emission measured at 470 nm using 360 nm excitation for IAEDANS and at 525 nm using excitation at 460 nm for IAF.

the presence of IAF-ATPase (Fig. 5). This is consistent with energy transfer from IAEDANS-proteolipid to IAF-ATPase. The energy transfer is abolished in the presence of 0.1% sodium dodecylsulfate, which solubilizes the two proteins and presumably increases the distance of their separation. The ratio of donor: acceptor fluorescence intensities is only moderately influenced by changing the excitation wavelength of the IAEDANS-chromophore between 320 and 340 nm (Fig. 5).

In order to establish the energy transfer between ATPase and proteolipid with greater certainty, the measurements given in Fig. 5 were repeated using IAEDANS-ATPase as energy donor and IAF-proteolipid as energy acceptor (Fig. 6). The presence of acceptor diminished the fluorescence intensity of donor and the presence of donor increased the fluorescence intensity of the acceptor; this is consistent with energy transfer between them (Fig. 6). The energy transfer was abolished by deoxycholate (2 mg/ml) which solubilized the membranes and inhibited the ATPase activity. It was also reduced by the addition of unlabelled proteolipid to the system containing IAEDANS-ATPase and IAF-proteolipid. Unlabelled proteolipid by itself had no influence upon the fluorescence emission of IAEDANS-ATPase under conditions similar to Fig. 6 but it did with IAF-proteolipid omitted (not shown). The lifetime of IAEDANS-ATPase fluorescence was measured with and without IAF-proteolipid in a nanosecond fluorescence spectrometer. In the presence of IAF-proteolipid, the rate of decay of IAEDANS fluorescence was slightly but consistently enhanced supporting the occurrence of energy transfer (Fig. 7). The effect was dependent upon the

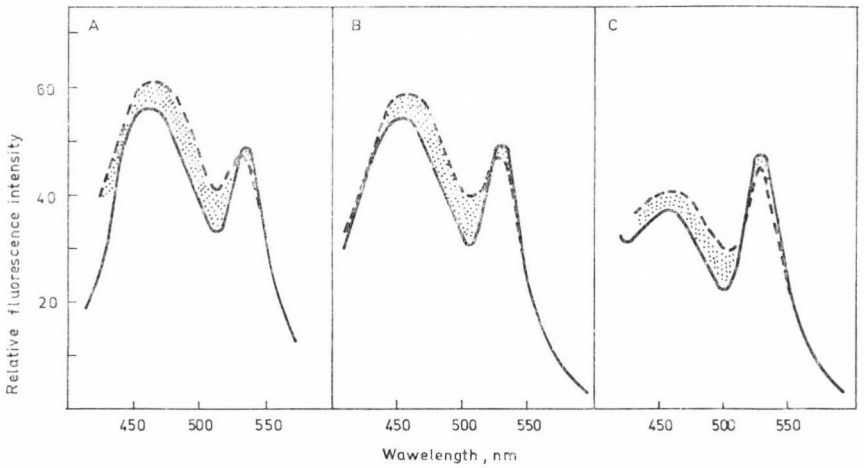

Fig. 5. Fluorescence emission spectra of systems containing IAEDANS-proteolipid as energy donor and IAF-ATPase as energy acceptor. The molar ratio of ATPase/proteolipid was 5.2. The excitation wavelength was 320, 330, and 340 nm in A, B, and C, respectively. Protein concentrations were: IAEDANS-proteolipid, 40 μg/ml (Sample 2 and 4); IAF-ATPase, 0.71 mg/ml (in Samples 3 and 4). Broken lines represent the algebraic sum of fluorescence emissions of IAEDANS-proteolipid (Sample 2) and IAF-ATPase (Sample 3); solid lines give the emission intensity of the mixture of IAEDANS-proteolipid and IAF-ATPase corrected for background (Sample 4)

Table V

Effect of IAF-proteolipid on the fluorescence lifetime of IAEDANS-ATPase

IAEDANS-ATPase, μg/ml	IAF-proteolipid μg/ml	$\frac{T_1}{T_{10}}$	$\frac{T_2}{T_{20}}$	$\frac{a_2}{a_1 + a_2}$	Mole ratio IAEDANS-ATPase / IAF-proteolipid
100	—	1.04	1.03	0.73	—
100	1.2	0.97	0.94	0.7	10
100	2.5	1.05	0.94	0.69	5
100	4.2	1.05	0.93	0.67	3
100	6.2	0.83	0.84	0.58	2

IAEDANS-ATPase and IAF-proteolipid were prepared as described in Methods and Materials. Samples containing the indicated components were incubated for 4 hours at 4 °C and fluorescence lifetimes measured as described in the legend of Fig. 11. The decay curves were measured, lifetimes and amplitudes were calculated assuming that the decay curve has two components, one with a shorter (T_{10}) and one with a longer lifetime (T_{20}). After this first incubation, all samples gave similar lifetimes, with an average of $T_{10} = 7.5$ nsec, and $T_{20} = 19.3$ nsec. After measurements were completed, incubation was continued for 4 hours at 25 °C and for another 12 hours at 4 °C. The measurement of lifetime was repeated; the two components corresponding to the shorter and longer lifetimes are denoted as T_1 and T_2, respectively, with the corresponding amplitudes given as a_1 and a_2. In the Table, T_1/T_{10} shows the ratio of the calculated lifetimes after 20 and 4 hours of incubation, respectively, for the first component; T_2/T_{20} has a similar meaning for the longer life component. With increasing proteolipid concentration the T_2/T_{20} decreased from 1.03 to 0.84 indicating a decrease in the lifetime of the longer component. The measurement of the shorter lifetime component was less reliable, therefore, the results of T_1/T_{10} are not interpreted. The ratio of amplitudes, $a_2/(a_1+a_2)$, indicates that the majority of the counted photons correspond to the longer component.

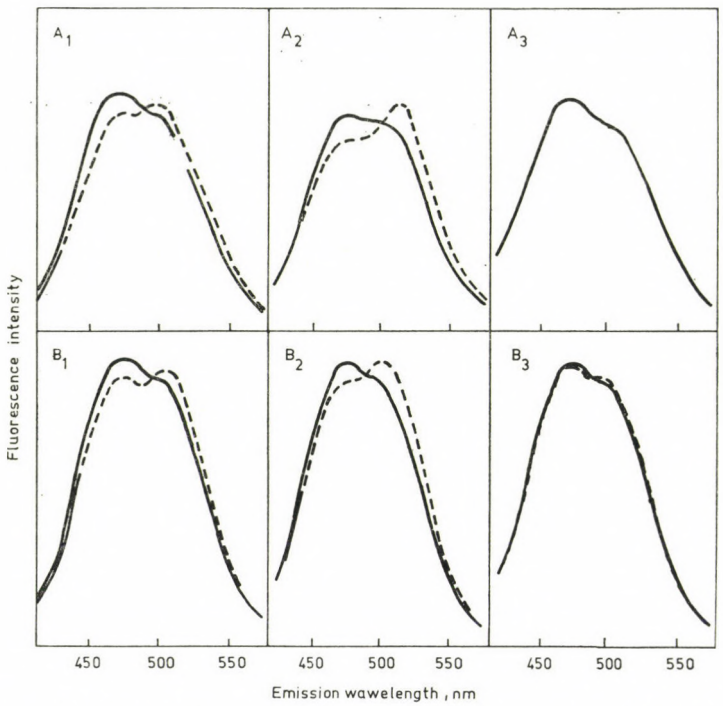

Fig. 6. Energy transfer measurements using IAEDANS-ATPase as energy donor and IAF-proteolipid as energy acceptor. Fluorescence emission was measured using excitation wavelengths of 320 nm (A), and 340 nm (B), respectively. Sample 1 served as control; Sample 2 contained 7.3 μg/ml IAF-proteolipid; Sample 3 contained 290 μg/ml IAEDANS-ATPase (290 μg/ml) were mixed at a molar ratio of 1:5. Buffer composition: 1 M KCl, 10 mM Tris-HCl, pH 7.4. Solid line represents the sum of the emission intensities of Samples 2 and 3. Broken line is the emission intensity of Sample 4 corrected for backgound. A_1, B_1, control samples; A_2, B_2, the same samples 3 hours after the addition of deoxycholate to a final concentration of 0.5 mg/ml; A_3, B_3, the same samples 4 hours after the addition of deoxycholate to a final concentration of 2 mg/ml

concentration of IAF-proteolipid added to the system (Table V) and could be abolished with excess unlabelled ATPase or unlabelled proteolipid (Table V). Egg lecithin (5 mg/mg ATPase) added to dilute the lipid phase of the system had little or no effect on the energy transfer.

The accessibility of IAEDANS and IAF covalently bound to the Ca^{2+}-ATPase was measured using a water-soluble (NaI) and a water-insoluble (iodobenzene) quencher. The decrease in fluorescence intensity with increasing concentration of quencher was evaluated according to the nonlinear Stern-Volmer equation after correction for dilution and self-absorption. The fact that the accessibility of the fluorophore in IAEDANS-ATPase is much greater to I than to iodobenzene, suggesting localization in a relatively hydrophilic region of the membrane (Table VI). The accessibility of IAF in the IAF-ATPase to I and iodobenzene is similar. Therefore, IAF and IAEDANS are apparently bound in a different environment in the Ca^{2+}-ATPase.

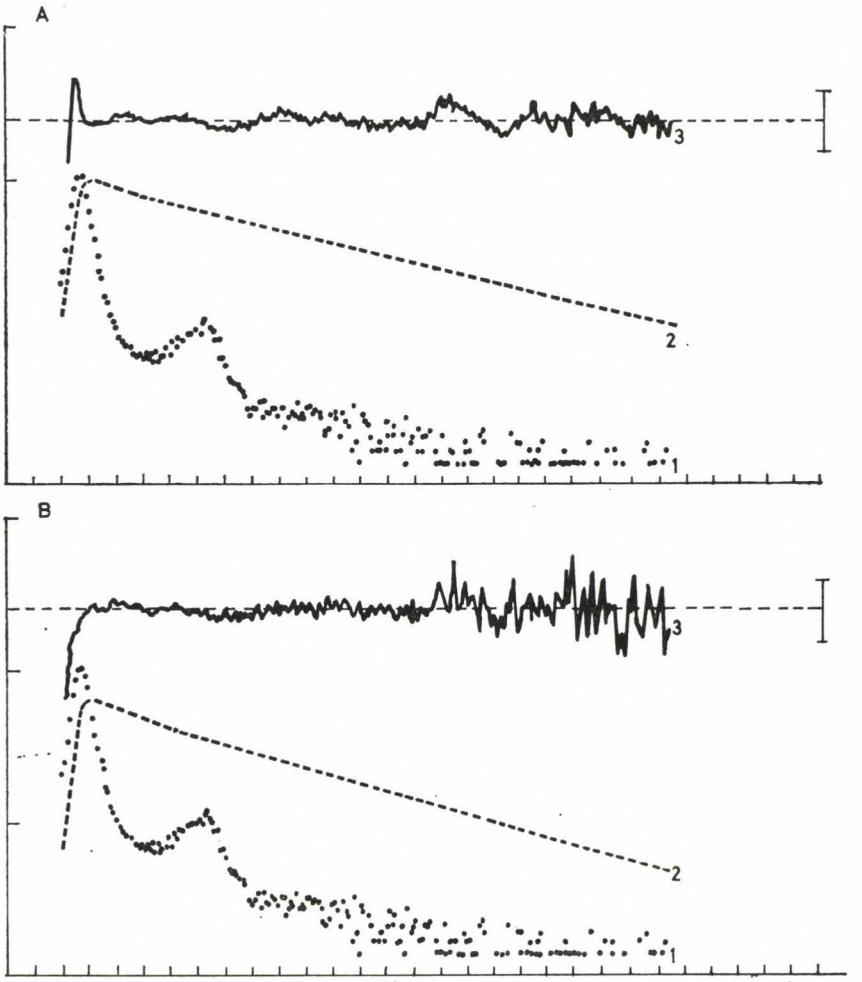

Fig. 7. Nanosecond decay of IAEDANS-ATPase fluorescence in the presence of IAF-proteolipid. The control sample (panel A) contained 0.1 mg/ml IAEDANS-ATPase (4 mole IAEDANS per mole of ATPase). In the experimental sample (panel B) 0.1 mg/ml IAEDANS-ATPase was mixed with 6.3 μg/ml IAF-proteolipid. The ATPase/proteolipid molar ratio was 2.0. Medium contained 0.1 M KCl, 10 mM Tris-HCl, pH 7.4. Measurements were carried out 21 h after mixing in an Ortec Model 9200 nanosecond fluorescence spectrometer equipped with a 6240 B Multichannel Analyzer using UV 360 and SS 4700 interference filters to isolate the IAEDANS emissions. The data were processed via punched tape through an HP 9300 calculator and analyzed by the method of moments. Line 1 represents the lamp-profile, line 2 the decay curve of IAEDANS -ATPase fluorescence and line 3 the deviation of experimental data from calculated values. The scale bar on the right of the deviation plot is 20%. The following lifetimes (T) and amplitudes (a) were obtained for the short (T_1 and a_1) and for the long lifetime components (T_2 and a_2): panel A, $T_1 = 7.61$ nsec, $T_2 = 19.1$ nsec, $a_1 = 0.0826$, $a_2 = 0.119$. IAF-proteolipid decreased the lifetime of the short (T_1) as well as the long (T_2) components, indicating energy transfer from IAEDANS-ATPase to IAF-proteolipid

Table VI

Quenching of fluorescence of IAEDANS-ATPase and IAF-ATPase by NaI and iodobenzene

Preparation	NaI		Iodobenzene	
	K_Q	g(a)	K_Q	g(a)
IAEDANS-ATPase	1.12	.40	445	.18
IAF-ATPase	1.18	.36	780	.36
IAEDANS (free dye)	3.40	>.88	—	—
IAF (free dye)	10.10	>.94	—	—

$K = \frac{\Delta F}{F_0 \cdot C_Q \cdot g(a) - \Delta F \cdot C_Q}$; g(a) represents accessibility to quencher as defined by the equation: $g(a) = \frac{F_0}{\Delta F} \cdot \frac{C_Q \cdot K_Q}{1 + C_Q + K_Q}$

Discussion

The purpose of these studies was to analyze the spectral properties of fluorescent conjugates of the Ca-ATPase and the proteolipid of sarcoplasmic reticulum. An attempt was made to establish energy transfer measurements by means of fluorescence whether interaction occurs between the Ca^{2+} transport ATPase and the major component of proteolipid isolated from the sarcoplasmic reticulum membranes. The energy transfer was analyzed by the determination of donor:acceptor fluorescence intensity ratios or by fluorescence lifetime measurements.

Indications of fluorescence energy transfer between fluorophores covalently attached to the Ca^{2+}-ATPase and the proteolipid have been obtained in the following systems:

1. Ca^{2+}-ATPase tryptophan (donor) to dansyl proteolipid or IAEDANS-proteolipid as acceptors;
2. IAEDANS-proteolipid (donor) to IAF-ATPase (acceptor);
3. IAEDANS-ATPase (donor) to IAF-proteolipid (acceptor).

The energy transfer in all systems was reduced or abolished in the presence of deoxycholate or sodium dodecylsulfate, parallel with the solubilization of the membrane. The solubilization is expected to increase the average distance between the ATPase and the proteolipid molecules beyond the range where efficient energy transfer is possible. In addition, both detergents cause conformational changes in the Ca^{2+}-ATPase with eventual loss of enzymatic activity that could contribute to the loss of energy transfer.

The energy transfer implies close proximity between Ca^{2+}-ATPase and proteolipid molecules in the membrane. The actual distance between donor and acceptor molecules cannot be determined since the position and orientation of

the fluorophores on the two proteins and their disposition in the membrane is unknown, therefore, random collision between them could significantly contribute to the observed energy transfer.

The proteolipid content of sarcoplasmic reticulum is not known with certainty. Based on a yield of 2—3 mg proteolipid per g microsomal protein (i.e., about 700 mg Ca^{2+} transport ATPase) and molecular weight of 12,000 for the proteolipid, the Ca^{2+}-ATPase: proteolipid mole ratio is of the order of 28—41. Therefore, it is unlikely that in the sarcoplasmic reticulum the proteolipid and the ATPase would form stoichiometric complexes. Low fluorescence intensity prevented measurements at these low proteolipid concentrations. Most of the experiments reported here were performed at ATPase: proteolipid molar ratios of 3—10. Under these conditions the energy transfer increased with increasing proteolipid concentration up to and probably beyond proteolipid: ATPase molar ratios of 0.5 and there was no clear evidence for the formation of tight stoichiometric ATPase -proteolipid complexes. ATPase-proteolipid interaction is readily disrupted by detergents as indicated by the loss of energy transfer; extraction of phospholipids with Triton X-100 also readily removes the proteolipid from Ca^{2+}-ATPase preparations [4, 2].

Proteolipids are generally defined as hydrophobic membrane lipoproteins characterized by their solubility in organic solvents [5, 14] are generally used for isolation. Although Loomis published two brief abstracts [6, 7] on an alternative procedure using detergents and Sephacryl S-100 chromatography no detailed report is available for this method. Therefore, the possibility cannot be excluded that the proteolipid used in these experiments was altered by the preparation procedure or that the reconstituted vesicles did not provide adequate environment for the interaction of proteolipid with the Ca^{2+} transport ATPase. However, hydrophobic proteins such as the C_{55} isoprenoid alcohol phosphokinase retain the activity after isolation in organic solvents and with small hydrophobic proteins such as the proteolipids denaturation in organic solvents is even less likely. Development of new techniques for the isolation of proteolipid and for the assessment of its functional integrity will be necessary in order to set aside the remaining doubts about the possible interaction of proteolipid with the Ca^{2+}-ATPase.

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Abbrevations: The following abbrevations were used: IAEDEANS, N-iodoacetyl-N'-(5-sulfo-1-naphthyl)-enthylene diamine; IAF, 5-iodoacetamidofluorescein; DT T,dithiothreitol; DANSYL-dimethyl-amino-naphtalene-sulphonic.

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THE EFFECTS OF CLONIDINE ON THE KIDNEY FUNCTION IN THE ANESTHETIZED DOG

G. KÖVÉR, Hilda TOST, Anna DARVASI

Department of Physiology, Semmelweis University Medical School, Budapest, Hungary

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Studies were performed to determine the mechanism by which the antihypertensive agent clonidine increased urine flow. The response of the kidney has been examined in four combinations. The parameters of renal function have been compared during volume expansion by 1.5—2.0% body weight Ringer solution.

In the control animals, volume expansion by 2% body weight, resulted in a slight increase in sodium excretion and urine flow.

In 10 anesthetized dogs 1.0 μg/kg/min of clonidine infused i.v. during 30 minutes (the total amount of clonidine infused was 30 μg/kg) decreased the arterial blood pressure from 136$\pm$13 mmHg to 127$\pm$12 mmHg and elevated urine flow from 2.95$\pm$1.65 ml/min to 4.34$\pm$1.77 ml/min while the urine osmolality diminished from 399$\pm$107 mosm/l to 265$\pm$90 mosm/l and the glomerular filtration remained constant.

In 5 animals 0.1 μg/kg/min of clonidine was infused into the left renal artery (this dose is corresponding to the renal fraction of the cardiac output) without any effects in the left kidney.

1.0 μg/kg/min of clonidine infused directly into the left renal artery produced vasoconstriction in the ipsilateral kidney, decreased the glomerular filtration rate and the urine flow. By contrast in the right kidney the urine flow rose without hemodynamic changes, and the urine osmolality became hypoosmotic compared to the plasma.

In ten dogs 1.0 μg/kg/min of clonidine and 1 mU/kg/min of arginine-vasopressin were infused intravenously. The vasopressin infusion superimposed on the clonidine could not inhibit the increase of the urine excretion, and the fall of the urine osmolality.

The results suggest that the clonidine increases the renal medullary blood flow possibly via a direct mechanism, decreases the sympathetic outflow to the kidney and via an indirect pathway, mediated by the renin-angiotensin system. The renal medullary flow increase produces a washout of the medullary osmotic gradient, and the water reabsorption diminishes.

Keywords: clonidine, urine excretion, urine osmolality, vasopressin.

The imidazoline derivative clonidine (2-(2,6-dichlorphenylamino)-2-imidazoline-hydrochloride) is a sympathomimetic agent with marked cardiovascular effects. It lowers mean arterial pressure through inhibition of central vasomotor and cardiac center [2, 33]. When administered intravenously it produces a transient increase in blood pressure, before its more sustained hypotensive effects are noted [16]. It also causes renal vasoconstriction and may decrease sodium excretion [21]. In anesthetized dogs [12] and rats [19] cloni-

Correspondence should be addressed to
George KÖVÉR
Department of Physiology, Semmelweis University Medical School
1088 Budapest, Puskin u. 9., Hungary

dine increases urine flow. In addition to lowering blood pressure, clonidine has a variety of endocrine effects. In dogs, these include, increased growth hormone secretion, decreased ACTH secretion, decreased vasopressin secretion and decreased renin secretion [25, 27].

It was found that clonidine increased urine flow and decreased urinary osmolality and solute free water reabsorbtion when given intravenously to intact hydropenic dogs, but it does not when given to acutely hypophysectomized animals receiving a constant infusion of ADH. These findings indicate that clonidine suppresses the release of vasopressin and thus increases water excretion [13].

The blood pressure lowering action of clonidine is generally considered to be a stimulation of central- adrenergic receptors, causing a reduction in the peripheral sympathetic outflow [14].

Because the activation of the sympathetic nervous system is a stimulus for renin release [36], it is not surprising that some investigators have tried to link increased activity of the sympathetic system with increased activity of the renin system [3]. Clonidine appears to exhibit inhibitory effects on both these mechanisms, for it supresses sympathetic outflow from the central nervous system, as well as it inhibits the renal release of renin [25]. Although its antirenin action may be secondary, at least in part, due to its reducting effect on neural one [36] there is also some evidence that clonidine through its actions at renal- adrenergic receptors [24] has a direct suppressive effects on renin release.

To determine which of these effects normally predominates we further examined the effects of clonidine on the renal function in anesthetized dogs.

Materials and methods

The experiments were performed on mongrel dogs of either sex weighing between 15 and 27 kg. For 24 hours prior to the experiment the animals were kept on water only. Anesthesia was induced by intravenous administration of pentobarbital sodium (Nembutal, Abott 30 mg/kg body wt.) and maintained with periodic additional administration. After the anesthesia each animal received an infusion of Ringer solution equal to 1% of the body weight, containing para-aminohippuric acid (PAH) and inulin, ensuring a plasma concentration of 2 mg and 30 mg per 100 ml respectively, followed by an intravenous infusion of 0.25 ml/kg of Ringer solution containing 154 mmol/l NaCl, 4 mmol/l KCl, 1.78 mmol/l $NaHCO_3$, 2.16 mmol/l $CaCl_2$ per min for the rest of the experiment.

Cannulation of the femoral arteries and veins of both sides was performed in all animals for arterial blood collections and for giving infusions. Mean arterial blood pressure was measured by a Statham strain range transducer connected to a polyethylene catheter in the left femoral artery and recorded on a RADELKIS OH 814/1 recorder.

From a lower midline incision the bladder was exposed and the ureters were catheterized supravesically using fine polyethylene cannulas.

A short segment of the left renal artery was exposed by a flank incision and cleared of adhering tissue to accomodate positioning of an electromagnetic flow probe. Mean renal blood flow (RBF) was measured by connecting the flow probe to a flow meter (NYCOTRON 375) and recorded by relaying the signal to a RADELKIS OH 814/1 recorder.

A thin canula was inserted in the left renal artery and during the experiment 0.5 ml Ringer solution per min was infused by means of a pump directly in the blood flowing to the left kidney.

Approximately 40 min were allowed to elapse following surgery for equilibration of the preparation.

About 60 minutes after starting the intravenous infusion urine was collected separately from both kidneys in 20 min periods. Blood was taken at the midpoint of each clearance period. After centrifugation the supernatant plasma was collected and the erythrocytes were reinfused in Ringer solution. In the middle of the periods the values of the arterial blood pressure and the renal blood flow were registered. In all groups the data of the first period served as controls.

The animals were divided into five groups.

Group I.: Time control experiments

After the control period (period 1) following a 10 minute interval the second 20 min period was made (period 2). After the second period there was a 10 minute interval again, followed by the third 20 min period (period 3), this was the post infusion control period.

The experimental protocol was the same in all the five groups except that in the other groups the clonidine or clonidine plus vasopressin were infused.

Group II.: Effects of i.v. administration of clonidine in anesthetized animals

In ten animals following the control period (period 1) 1.0 μg/kg of clonidine was infused intravenously per minutes, dissolved in 0.5 ml Ringer solution (the volume of the constant infusion (0.25 ml/kg/min) did not change). 10 min was allowed for the equilibration and the renal parameters were examined during the second 20 min period (period 2). At the end of the second period the clonidine infusion was stopped and again 10 minutes were allowed for the equilibration. Following the 10 min interval the post infusion control period was made (period 3).

Group III.: Effects of 0.1 μg/kg/min of clonidine infusion into the left renal artery

In five animals after the control period (period 1) 0.1 μg/kg of clonidine was infused per minute into the left renal artery, which was dissolved in 0.5 ml Ringer solution (it was given to the Ringer solution infused in constant infusion into the left renal artery).

Following a 10 min equilibration period the renal parameters separately in both kidneys were examined during 20 minutes (period 2). At the end of the second period the clonidine infusion was stopped and after a 10 minutes interval the renal parameters were again studied during a 20 min period (period 3).

Group IV.: Effects of 1.0 μg/kg/min of clonidine infusion into the left renal artery

In ten animals the experiments were conducted under the protocol of the Group III. But in these experiments during the second period (period 2) 1.0 μg/kg/min of clonidine was infused into the left renal artery.

Group V.: Effects of i.v. administration of clonidine and arginine-vasopressin on the renal parameters.

In ten animals after control period (period 1) 1.0 μg/kg of clonidine and 1 mU/kg arginine-vasopressin (SIGMA) together dissolved in 0.5 ml Ringer solution were infused intravenously per minute. After a 10 minutes equilibration time the renal parameters were examined during a 20 min period (period 2).

At the end of the second period the clonidine infusion was stopped while the arginine-vasopressin infusion was sustained during the 10 minutes interval and the postinfusion 20 min control period (period 3).

PAH concentration in urine and plasma was determined by the method of Smith et al. [34] that of inulin by the method of Little [20]. Urinary and plasma sodium and potassium concentration was measured by flame photometry. Total osmolality of urine and plasma was measured by the method of freezingpoint depression in a Fiske osmometer. Hematocrit was determined by means of Hawskley microhematocrit centrifuge, plasma protein concentration by the biuret method [9].

The clearance of PAH (C_{PAH}) and the clearance of inulin (C_{inulin}) were determined by the usual formules. The data for C_{PAH}, C_{inulin}, urine flow, sodium excretion ($U_{Na}xV$), potassium excretion ($U_K x V$), free water clearance (C_{H_2O}) and renal blood flow (RBF) were referred to 100 g kidney tissue weight. The total renal vascular resistance ($R_{kidney/kg}$) was calculated for 1000 g kidney weight. Statistical evaluation of the data was made by paired and unpaired analysis using Student's t test. A p value of less than 0.05 was considered significant.

Results

The base-line control values for the renal parameters of dogs used in these experiments are listed in Table I—VI.

There were no significant differences in the left kidney parameters in the five groups during the first period.

In addition we could not observe any differences between the data of the left and right kidneys meaning that the preparation and the cannulation of the left renal artery did not disturb the left kidney function.

The first series of studies concerns the effects of Ringer solution infusion in anesthetized dogs. The results of the group are summarized in Table I.

In our experiments isosmotic extracellular hypervolaemia was induced by the infusion of Ringer solution. During the control period (period 1) the extracellular volume increased by about 1—2% of body weight.

Blood pressure in the control animals was 128±13 mmHg and remained constant during the experiment. The renal blood flow (RBF) during the initial period was 612±165 ml/min, fell slightly to 513±147 ml/min at the end of the

Table I

The renal parameters of the left kidney ($\bar{x}\pm SD$) in the time control experiments ($n = 10$) and during 1.0 μg/kg/min of clonidine infusion intravenously ($n = 10$)

		Period 1.	Period 2.	Period 3.
Blood pressure mmHg	control	128± 13	126± 11	124± 10
	clonidine	136± 13	127± 12	116± 12
Renal blood flow (RBF) ml/min/100 g	control	612±165	557±162	514±147
	clonidine	615±165	524±162	485±171
Renal vascular resistance $mmHg.s.ml^{-1}.kg^{-1}$	control	1.25±0.30	1.47±0.36	1.58±0.54
	clonidine	1.33±0.41	1.65±0.62	1.67±0.68
C_{PAH} ml/min/100 g	control	331± 51	301± 70	280± 57
	clonidine	336± 69	286± 66	267± 63
C_{inulin} ml/min/100 g	control	87± 8	82± 24	85± 17
	clonidine	88± 15	85± 26	82± 26
Urine flow ml/min/100 g	control	2.50±1.47	2.83±1.53	3.18±1.41
	clonidine	2.95±1.65	4.43±1.77	4.71±1.98
Sodium excretion (U_{Na}x V) μmol/min/100 g	control	311±243	264±234	267±177
	clonidine	352±213	342±159	216±147
Potassium excretion (U_Kx V) μmol/min/100 g	control	88± 40	95± 30	90± 30
	clonidine	90± 50	96± 33	86± 39
Urine osmolality mosm/l	control	383± 93	357±129	299±114
	clonidine	399±107	265± 90	183± 62
C_{H_2O} ml/min/100 g	control	−0.27±0.90	−0.19±1.80	+0.09±1.56
	clonidine	−0.69±0.81	+0.81±1.50	+2.02±1.56

experiment. We observed a small decrease of the C_{PAH} too. Despite the progressive decline in RBF, the glomerular filtration (GFR) was relatively stable.

With infusion of Ringer solution the urine flow rose, urinary osmolality fell slightly and there was a small increase of the free water clearance (C_{H_2O}). The sodium excretion ($U_{Na}x\ V$) was relatively high in the first period (331 ± 243) μmol/min) and did not change during the experiment. The above described changes in urinary osmolality and electrolyte concentrations, occurred with no changes in the plasma osmolality, and sodium concentration.

In these experiments the haematocrit value remained constant, and during the infusion of Ringer solution the plasma protein concentration decreased by about 0.4 g per 100 ml.

Ten dogs of the Group II. were prepared as in Group I. except that after control measurements were taken, clonidine was added to the intravenous infusion to deliver 1.0 μg/kg/min. After 10 minutes, a 20 minutes clearance period was obtained, Table I. and Table II. summarize the results of this group. During the control period (period 1) the renal parameters did not differ from the data measured in the time control animals.

During the experimental period the arterial blood pressure decreased slightly from 136 ± 13 mmHg to 127 ± 12 mmHg and the glomerular filtration did not change significantly in either kidney; however significant elevation of

Table II

The renal parameters of the right kidney ($\bar{x} \pm SD$) in the time control experiments ($n = 10$) and during 1.0 μg/kg/min of clonidine infusion intravenously ($n = 10$)

		Period 1.	Period 2.	Period 3.
C_{PAH} ml/min/100 g	control	342± 56	308± 69	288± 60
	clonidine	346± 72	291± 72	264± 58
C_{inulin} ml/min/100 g	control	92± 7	90± 9	95± 13
	clonidine	85± 15	90± 13	80± 15
Urine flow ml/min/100 g	control	3.11±1.40	2.81±1.62	3.56±1.44
	clonidine	3.60±3.07	4.98±2.08	5.29±2.44
Sodium excretion ($U_{Na}x\ V$)	control	375±228	291±237	292±181
μmol/min/100 g	clonidine	410±364	379±208	246±192
Potassium excretion ($U_K x\ V$)	control	68± 32	72± 28	83± 24
μmol/min/100 g	clonidine	87± 55	100± 38	89± 42
Urine osmolality	control	399±119	385±156	314±126
mosm/l	clonidine	377±116	251± 86	177± 60
C_{H_2O} ml/min/100 g	control	−0.62±0.92	−0.22±1.10	+0.41±1.35
	clonidine	−0.40±1.07	+1.01±1.51	+2.28±1.53
Haematocrit %	control	39± 5	38± 5	38± 5
	clonidine	40± 5	42± 5	43± 5
Plasma protein conc.	control	6.03±0.79	5.88±0.78	5.60±0.75
g/100 ml	clonidine	5.43±1.07	5.10±1.01	5.17±0.89

the urine flow was noted in both kidneys. During the clonidine infusion period urinary volume, osmolality and sodium excretion were similar in the right and the left kidney.

After the intravenous clonidine infusion (period 3) the blood pressure decreased further from 127±12 mmHg to 116±12 mmHg and the urine volume was significantly greater than that in the period 2.

The decrement in urinary osmolality, and the increase of the C_{H_2O} during clonidine infusion were markedly greater than those observed in the control animals.

Table III. and table IV. summarize the clearance and hemodynamic measurements received in Group III. and Group IV. before and after clonidine infusion into the left renal artery.

During the control period (period 1) urinary volume, osmolality and sodium excretion as well as GFR and RBF were similar in both groups.

In the Group III. after the control period 0.1 μg/kg/min of clonidine was infused into the left renal artery. As the data of the Table III. and IV. show GFR, and C_{PAH}, the urine volume and osmolality and the sodium excretion were similar in the right and left kidney, and the renal parameters did not

Table III

The renal parameters of the left kidney ($\bar{x}$±SD) during 0.1 μg/kg/min of clonidine infusion (n = 5), and 1.0 μg/kg/min of clonidine infusion into the left renal artery

		Period 1.	Period 2.	Period 3.
Blood pressure mmHg	0.1 μg	125±7	124±12	127±12
	1.0 μg	125±15	134±14	122±16
Renal blood flow (RBF) ml/min/100 g	0.1 μg	610±60	512±64	524±75
	1.0 μg	629±70	406±64	451±76
Renal vascular resistance mmHg.s.ml^{-1}.kg^{-1}	0.1 μg	1.28±0.22	1.48±0.25	1.49±0.29
	1.0 μg	1.32±0.24	2.03±0.35	1.67±0.35
C_{PAH} ml/min/100 g	0.1 μg	340±78	278±92	287±103
	1.0 μg	351±71	256±50	257±62
C_{inulin} ml/min/100 g	0.1 μg	102±32	94±30	93±29
	1.0 μg	82±20	76±12	66±11
Urine flow ml/min/100 g	0.1 μg	2.45±0.78	2.70±1.08	4.09±1.63
	1.0 μg	2.27±0.95	1.75±1.06	4.49±1.77
Sodium excretion (U_{Na}x V) μmol/min/100 g	0.1 μg	286±135	307±154	329±140
	1.0 μg	254±154	106±100	162±148
Potassium excretion (U_Kx V) μmol/min/100 g	0.1 μg	76±25	93±45	118±61
	1.0 μg	67±35	50±29	66±34
Urine osmolality mosm/l	0.1 μg	494±50	415±60	301±66
	1.0 μg	425±105	366±133	166±41
C_{H_2O} ml/min/100 g	0.1 μg	−1.18±0.54	−0.90±0.59	+0.16±0.96
	1.0 μg	−0.57±0.65	−0.04±0.64	+1.96±1.00

differ from those observed in the time control experiments. These results suggest that this clonidine dose is without a specific effect on the renal function.

Table III. and Table IV. depict the changes in renal parameters in both kidneys in Group IV. After 1.0 μg/kg/min of clonidine infusion into the left renal artery the arterial blood pressure increased from 125±15 mmHg to 134±14 mmHg and the RBF decreased significantly in the left kidney. As a result of the increase in the renal vascular resistance, the glomerular filtration fell slightly and a decrease in the urine volume as well as in the sodium excretion could be observed. In the right kidney the C_{PAH}, C_{inulin} did not change and the urine flow was significantly higher than that in the left kidney. The decrement in urine osmolality in the right kidney was greater than that in the left kidney. The increment in free water clearance (C_{H_2O}) in the right kidney was significantly higher than that in the left kidney. The results obtained in the Group IV. demonstrate, that the clonidine in higher dose produces vasoconstriction in the kidney.

The data of the table V. and VI. summarize the clearance and hemodynamic measurements observed in the Group II. and Group V.

The hemodynamic parameters, the glomerular filtration rate, the urine flow, sodium excretion and urine osmolality were similar in the two groups

Table IV

The renal parameters of the right kidney ($\bar{x} \pm SD$) during 0.1 μg/kg/min of clonidine infusion ($n = 5$), and 1.0 μg/kg/min of clonidine infusion into the left renal artery

		Period 1.	Period 2.	Period 3.
C_{PAH} ml/min/100 g	0.1 μg	320±56	310±38	292±50
	1.0 μg	341±51	306±48	279±54
C_{inulin} ml/min/100 g	0.1 μg	107±16	100±15	100±20
	1.0 μg	85±15	90±13	80±15
Urine flow ml/min/100 g	0.1 μg	2.20±0.83	2.95±0.92	4.04±0.90
	1.0 μg	2.74±1.09	4.41±1.60	5.97±1.53
Sodium excretion (U_{Na}x V)	0.1 μg	273±149	346±161	332±128
μmol/min/100 g	1.0 μg	329±142	310±205	205±154
Potassium excretion (U_Kx V)	0.1 μg	91±39	117±49	133±66
μmol/min/100 g	1.0 μg	79±35	85±31	81±30
Urine osmolality	0.1 μg	526±64	419±50	303±70
mosm/l	1.0 μg	401±101	261±91	161±61
C_{H_2O} ml/min/100 g	0.1 μg	−1.44±0.84	−1.14±0.73	+0.07±0.93
	1.0 μg	−0.59±0.64	−0.97±1.25	+3.14±0.99
Haematocrit %	0.1 μg	40±3	39±3	39±3
	1.0 μg	38±5	40±6	41±7
Plasma protein conc.	0.1 μg	5.77±0.85	5.72±1.18	5.50±1.04
g/100 ml	1.0 μg	4.58±1.41	4.49±1.37	4.52±1.44

during the control period (period 1). The data received in the Group II. of the animals, were discussed before. It was demonstrated that 1.0 μg/kg/min of clonidine infused intravenously increased the urine flow, and decreased the urine osmolality.

In the Group V. following the control period 1.0 μg/kg/min of clonidine and 1.0 mU/kg/min of arginin-vasopressin were infused together intravenously.

Table V

The renal parameters of the left kidney ($\bar{x} \pm SD$) during 1.0 μg/kg/min of clonidine infusion i.v. ($n = 10$), and 1.0 μg/kg/min of clonidine plus 1.0 mU/kg/min of arginine-vasopressin infusion intravenously ($n = 10$)

		Period 1.	Period 2.	Period 3.
Blood pressure mmHg	clo.	136±14	127±13	116±13
	clo.+vas.	136±12	142±20	133±19
Renal blood flow (RBF) ml/min/100 g	clo.	615±173	524±170	485±179
	clo.+vas.	618±150	576±123	529±135
Renal vascular resistance $mmHg.s.ml^{-1}.kg^{-1}$	clo.	1.33±0.43	1.65±0.65	1.67±0.71
	clo.+vas.	1.32+0.39	1.55+0.36	1.60±0.39
C_{PAH} ml/min/100 g	clo.	336±73	286±69	267±66
	clo.+vas.	309±75	258±39	225±36
C_{inulin} ml/min/100 g	clo.	88±27	85±27	82±27
	clo.+vas.	98±25	102±23	96±26
Urine flow ml/min/100 g	clo.	2.95±1.74	4.43±1.87	4.71±2.10
	clo.+vas.	3.24±1.53	5.65±2.97	5.56±2.22
Sodium excretion ($U_{Na}x$ V) μmol/min/100 g	clo.	352±225	342±168	216±156
	clo.+vas.	374+237	566+393	378±240
Potassium excretion ($U_K x$ V) μmol/min/100 g	clo.	90±52	96±36	86±42
	clo.+vas.	74+27	108±39	98±42
Urine osmolality mosm/l	clo.	399±113	265±96	183±65
	clo.+vas.	340± 63	279±75	220±78
C_{H_2O} ml/min/100 g	clo.	−0.69±0.84	+0.81±1.59	+2.02±1.63
	clo.+vas.	−0.24±0.81	+0.73±1.59	+1.80±1.89

The aim of these experiments has been to demonstrate whether the vasopressin inhibits the diuretic effect of clonidine or not. Comparing the renal parameters during the experimental period (period 2) in the left and in the right kidney, in the two groups (the effects of the clonidine and the effects of the clonidine superimposed vasopressin) we can see that the renal parameters did not differ significantly in either kidney. Those data suggest that the increase of the plasma vasopressin concentration can not decrease the effects of the clonidine infusion on the renal function.

Table VI

The renal parameters of the right kidney ($\bar{x} \pm SD$) during 1.0 μg/kg/min of clonidine infusion i.v. ($n = 10$), and 1.0 μg/kg/min of clonidine plus 1.0 mU/kg/min of arginine-vasopressin infusion intravenously ($n = 10$)

		Period 1.	Period 2.	Period 3.
C_{PAH} ml/min/100 g	clo.	346±72	291±72	264±58
	clo.+vas.	297±55	258±38	220±33
C_{inulin} ml/min/100 g	clo.	85±15	87±31	81±29
	clo.+vas.	94±21	106±18	98±21
Urine flow ml/min/100 g	clo.	3.60±3.07	4.98±2.08	5.29±2.44
	clo.+vas.	2.78±1.58	5.91±3.37	5.33±1.48
Sodium excretion (U_{Na}x V) μmol/min/100 g	clo.	411±364	379±208	246±192
	clo.+vas.	318±228	585±432	352±249
Potassium excretion (U_Kx V) μmol/min/100 g	clo.	89±55	100±38	89±42
	clo.+vas.	69±25	114±42	94±41
Urine osmolality mosm/l	clo.	377±116	251±86	177±60
	clo.+vas.	381+81	292+85	230±88
C_{H_2O} ml/min/100 g	clo.	−0.40±1.07	+1.01±1.51	+2.28±1.53
	clo.+vas.	−0.38+0.74	+0.68±1.66	+1.60±1.80
Haematocrit %	clo.	40±5	42±5	43±5
	clo.+vas.	41±3	45±3	45±4
Plasma protein conc. g/100 ml	clo.	5.43±1.07	5.10±1.21	5.17±0.89
	clo.+vas.	4.85±1.15	4.70±1.12	4.59±1.20

Discussion

The present studies confirm that intravenous administration of clonidine increases urine flow in anesthetized, Ringer solution loaded dogs, and in addition they show that this rise is associated with a decrease in urine osmolality. Other investigators reported increased urine volume after clonidine injection, but did not determine the mechanism responsible for this effect [12, 18, 25].

In the present studies, the rise in renal water excretion did not occur as a result of increased arterial blood pressure because in our experiments, during clonidine infusion, the blood pressure rather diminished as increased. The intravenous clonidine infusion did not alter the glomerular filtration rate, so these factors could not account for the enhanced water excretion.

The observation of Humphrey and Reid [13] suggested that the increased water excretion induced by clonidine was not hemodynamically mediated, but rather occurred as a result of central inhibition of ADH release or through inhibition of the action of antidiuretic hormone on the renal tubule.

It is now well-established that numerous non osmotic stimuli influence the release of ADH [32]. Factors leading to decreased vasopressin secretion

include left atrial distension [11] and increased activity of carotid and aortic baroreceptors [31]. Hemorrhagic hypotension [8] and diminished blood volume [30] all increase ADH release.

The set of the osmoregulatory system can also pharmacologically be altered. When administered to rats in sub-hypotensive doses opiates like morphine and butorphanol transiently inhibit vasopressin secretion by raising the set of the osmotat [15]. The effect of opiates on the osmoregulation of vasopressin suggest that endogenous peptides may have a similar effect. This effect cannot be accounted for by hemodynamic variables and appears to be due to the agonist action of the drugs. However the site and mechanism of the hemodynamic and pharmacologic actions must be different because, unlike the agonists, diprenorphine (an opioid antagonist) does not affect the osmoregulation of vasopressin under normovolaemic, normotensive conditions. The past decade has witnessed continuing advances in our understanding of the physiology of vasopressin secretion. Despite some empirical and theoretical objections, linear regression analysis of the relationship between plasma vasopressin and plasma osmolality or sodium to provide a simple and useful way to describe the major functional properties of the osmoregulatory system [26].

It is known that norepinephrin increases renal water excretion by inhibiting anti-diuretic hormone secretion [28]. It was found that clonidine increased urine flow and decreased urinary osmolality and solute free water reabsorbtion when given intravenously to intact hydropenic dogs, but it did not when given to acutely hypophysectomized animals receiving a constant infusion of ADH. These findings indicate that clonidine supresses the release of vasopressin and thus increases water excretion by the same mechanism as norepinephrine [13]. These studies do not however rule out other possible pathways. The changes of the glomerular filtration (the rise during norepinephrine infusion or the diminution following hypophysectomie) modify the vasopressin effects on the distal nephron segments.

In our experiments the exogenous vasopressin infusion did not inhibit the clonidine induced increases in the urine flow during extracellular isosmotic hypervolaemia. The results of the present studies do not support the hypothesis that the decrease of the vasopressin secretion is the cause of the clonidine induced diuresis and urine hyposmolality.

In spite of the relatively high vasopressin plasma concentration the clonidine infusion elevated the urine flow and the urine became hyposmotic as compared to the plasma.

Previous micropuncture and microcatheterization studies have shown that during acute saline loading sodium reabsorption in the juxtamedullary nephron is inhibited more than in the superficial nephron, with net addition of sodium between the superficial late distal tubule and the papillary collecting duct [35]. One explanation for this phenomenon is a fall in passive sodium-

transport in the thin ascending limb of Henle due to the dissipation of the sodium gradient which normally exist between this nephron segment and ascending vasa recta [22]. The decreased osmotic gradient could result from medullary solute washout secondary to augmented medullary blood flow during acute saline loading in normal animals [5].

It is conceivable that the clonidine infusion increases the renal medullary blood flow, thereby leading to a greater diuretic response in our experiment. Since inner medullary blood flow constitutes only 1% of total RBF in anesthetized dogs [23], changes in RBF may not be detected even through a profound alteration in renal medullary flow occurs. Dissociation between total RBF and medullary blood flow has been reported in a variety of circumstances [4] and these data suggest independent regulation of the cortical and inner medullary circulation. It is widely recognized that angiotensin II plays a pivotal role in the regulation of renal hemodynamics, salt and water balance, arterial blood pressure [10, 17, 18]. and experimental data suggest that angiotensin regulates regional blood flow in the medulla [6].

The location and nature of the receptors in the brain on which clonidine acts to decrease renin secretion have been investigated in dogs. The results suggest that the renin-regulating receptors are located in the brain stem in a region different from the receptors mediating the depressor response, that they are α_2-adrenoceptors, and that they are postsynaptic in location [7]. Clonidine is generally considered to be an agonist with a high affinity for α_2-adrenoceptors. According to Schmitt's hypothesis [29] clonidine is an agonist of central α-adrenoceptors. The stimulation of these receptors induces a decrease in peripheral sympathetic outflow and hence, a fall in blood pressure, simultaneously plasma cathecolamines and plasma renin activity are reduced.

In our experiments 1.0 μg/kg/min of clonidine infused intravenously increased the urine flow while 0.1 μg/kg/min of clonidine infused into the left renal artery was ineffective (the latter dose is equal to the renal cardiac output fraction). These data suggest that clonidine modifies the renal urine excretion by a central nervous system pathway.

The results of the present studies demonstrate, that 1.0 μg/kg/min of clonidine infused directly into the left renal artery produces a fall of renal blood flow, glomerular filtration rate, and urine flow in the left kidney, while in the right kidney the effects of the clonidine were the same as observed after intravenous clonidine infusion.

Clonidine is a potent α-adrenergic agonist, producing cardiovascular effects which mimic in part those of norepinephrine, and which are prevented by the α-blocking agent phentolamin [1]. Thus, it is possible that higher clonidine dose (the smaller dose 0.1 μg/kg/min was ineffective) by the fall of the GFR, resulted in a decrease in renal water excretion. Consistent with

this suggestion is the finding that in the right kidney without glomerular filtration changes the urine flow increased and the urine compared to the plasma became hypoosmotic.

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EFFECTS OF STIMULUS PARAMETERS ON CARDIOVASCULAR RESPONSES TO MEDULLARY STIMULATION IN THE CAT

L. ÁNGYÁN

Institute of Physiology, University Medical School, Pécs, Hungary

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The results of this study indicated that when stimulus intensity, pulse frequency and train duration were varied the basic topography of the cardiovascular responses to medullary stimulation did not change, but was merely increased or diminished in magnitude. Pressor responses were usually obtained in conscious cats, and also narcosis produced reversal effect on rare occasions. It is suggested that the reversal in cardiovascular responses is probably locus specific, and the medullary loci yielding reversal effect are more limited than those eliciting consequently pressor responses.

Keywords: anaesthetization, blood pressure, heart rate, medulla oblongata, respiration rate, stimulus parameters

Electrical stimulation of the medullary autonomic nuclei produces a variety of somatomotor and autonomic effects [3, 4, 5, 7, 12]. Because of the significance of these medullary nuclei in the central control of circulation, the cardiovascular responses have been the subjects of numerous investigations [1, 2, 5—11, 13, 14, 16, 17]. However, several conflicting reports have been published. For example, electrical stimulation of the areas regarded as depressor regions by the earlier investigators mapping the medullary pressor and depressor points [2, 7, 8, 14, 17], consistently elicited a rise in blood pressure in unrestricted cats [5, 6]. However, anaesthetized or decerebrate cats were previously used and also the stimulus parameters were different. It thus appears that to determine whether other kinds of changes might be obtained with different parameters is a logical step in investigating the cardiovascular responses to medullary stimulation both in conscious, behaving, and in anaesthetized cats.

Correspondence should be addressed to
Lajos ÁNGYÁN
Institute of Physiology, University Medical School,
H-7643 Pécs, Szigeti út 12., Hungary

Methods

Eight adult cats implanted with bipolar electrodes were used. The electrodes were constructed of two insulated nikrothal wires (0,3 mm in diameter, bared 0,5 mm at the tip) having tip separation of 0,5 mm. The tip resistance varied between 0,1 and 0,2 megohm. In each animal one pair of electrodes was placed into the rostral region of the nucleus of the tractus solitarius (NTS) and another pair of electrodes into the region of the paramedian nucleus (NRP) using the coordinates of the Snieder and Niemer's brain atlas [15]. To compare the effects obtained from these loci with those elicited by stimulation of a somatic nucleus, a third pair of electrodes was implanted into the cuneate nucleus (NC). After seven to ten days recovery a PVC canula was inserted into one of the common carotid arteries under pentobarbital sodium (30 mg/kg, i.v.) anaesthesia for recording arterial blood pressure. One or two days after this second operation the stimulation effects on blood pressure, heart rate and respiration rate were recorded on freely moving cats. The following stimulation parameters were systematically varied: stimulus intensity, pulse frequency and pulse train duration. The pulse duration was 0,3 ms. The interstimulation interval was 20 s or longer. All stimulations were repeated on three consecutive experimental days. Finally, the cats were anaesthetized either with chloralose (40 mg/kg, i.v.), or with pentobarbital sodium, and the stimulation effects were tested under anaesthesia. A more detailed description of the stimulation and recording techniques is given elsewhere [5, 6].

Upon completion of the experiment, all sites of stimulation were verified histologically, and the points which were localized outside the target regions were omitted from this study. Figure 1. shows the localization of the electrode tips in the medullary nuclei from which the present results were obtained.

Results

Effects of stimulation parameters. To obtain an *intensity function* at least three trials at each of three levels of intensity were administered employing a 100 cps and 10 s pulse train duration stimulation. The threshold intensity was determined at each electrode site as the intensity which produced the first observable somatomotor effect. This intensity was between 0,1 and 0,5 mA.

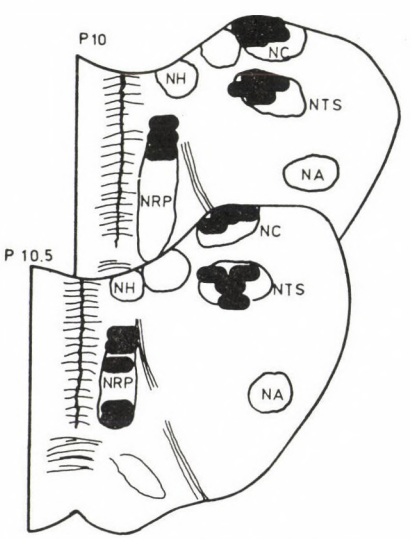

Fig. 1. Diagram showing the electrode tip localizations in the medullary nuclei

The higher the intensity of stimulation the higher the elevation in blood pressure was obtained (Fig. 2.). However, both the magnitude and the shape of the blood pressure curves were different at the three regions (Fig. 3.). A continuous rise in blood pressure was obtained from the region of NRP, maximal responses amounting to 50 mm Hg or more. Decrease in blood pressure did not occur. Both the heart rate and the respiration rate increased parallel with the blood pressure (Fig. 2.). The stimulation of a single locus in the NRP region with suprathreshold intensities produced the arrest of respiration.

NTS stimulations elicited a moderate rise in blood pressure, up to about 30 mm Hg. Some variations in the shape of blood pressure curves might be observed. In most cases after an initial raising phase an apparently steady state

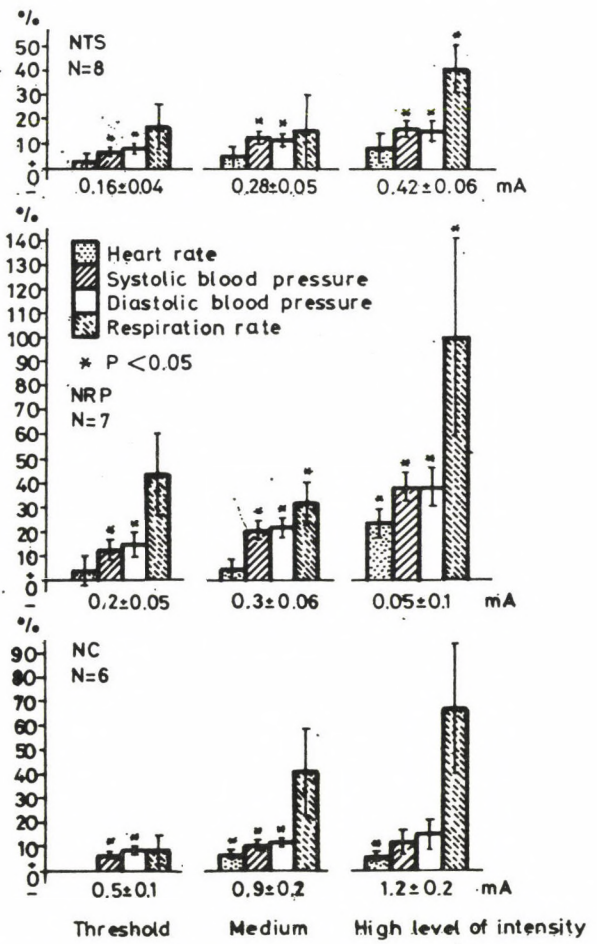

Fig. 2. Effects of stimulations at different stimulus intensities in percent of prestimulation control

level was maintained during stimulation. At two out of eight loci the blood pressure increased only after an initial decrease. This initial decreasing phase lasted about 1 s. The heart rate and respiration rate effects were variable: both increase and decrease occurred, but decrease in these functions was predominant during stimulations at threshold intensity.

As it was expected, stimulation of the cuneate region elicited moderate increase in blood pressure and heart rate only at high level of intensity. Inter-

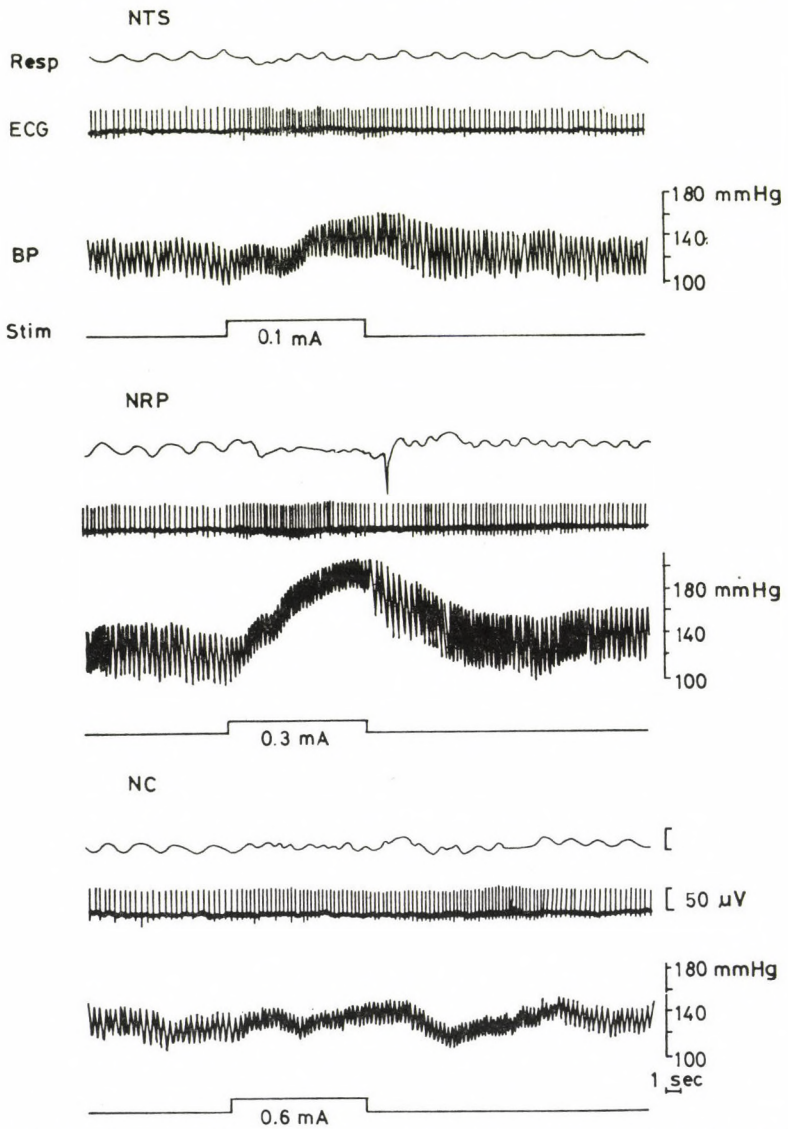

Fig. 3. Respiratory (Resp), ECG and blood pressure (BP) responses to stimulation of the three medullary regions at the intermediate intensity and 150 cps

estingly, the respiration rate consistently increased during stimulations at suprathreshold intensities.

The frequency function was obtained at the intermediate intensity. The frequencies used were: 10, 20, 30, 50, 100, 150 and 200 cps. Stimulations at frequencies lower than 50 cps produced no change or slight effects. Stimulations at higher frequencies elicited pressor responses (Figs 4 and 5). At one of the seven NRP loci high frequency stimulations elicited the arrest of respiration (Fig. 5.). It occurred only at one electrode placement in the region of NTS that stimulations at frequencies lower than 50 cps elicited a depressor response, i.e., a fall in blood pressure and bradycardia, associated with retching and salivation. Under stimulations with higher frequencies the retching did not appear and the blood pressure increased after a short initial decreasing phase. Under chloralose anaesthesia, stimulation of the same locus at all frequencies produced rise in blood pressure and no retching occurred.

The pulse train durations (1, 2, 3, 5 and 10 s) were tested at the intermediate intensity and 100 cps. The shortest pulse train duration to obtain a complete response pattern was from 3 to 5 s. Shorter stimulations at the NTS and NC produced no noticeable effects. Such stimulations of the NRP region elicited pressor responses reaching their peak amplitude after the termination of stimulation. Evidently, the magnitude of these effects was smaller than that obtained during longer lasting stimulations.

The effects of anaesthesia. In the course of testing these medullary stimulations under anaesthesia a higher threshold for cardiovascular response was found than that was determined on conscious, unrestricted animals. The increase in blood pressure might be reversed to depressor response by both pentobarbital sodium and chloralose anasthesia only at a single electrode placement in the region of NRP (Fig. 6.) At all the other loci the general form of the response patterns obtained under anaesthesia were similar to those observed on freely behaving animals.

Discussion

The results of this study show that when stimulation parameters were varied the basic topography of the cardiovascular responses did not change, but was merely increased or diminished in magnitude. Also the anaesthetization was effective in producing changes in stimulation-induced blood pressure effects only at two out of 15 electrode placements in the NTS and NRP regions altogether. It seems logical to argue that it is not possible to generalize a conclusion from these results, because the effects of pulse frequencies and pulse train durations were tested only at the intermediate intensity, and also because the level of anaesthesia was not controlled, eg. by recording EEG or EMG. However,

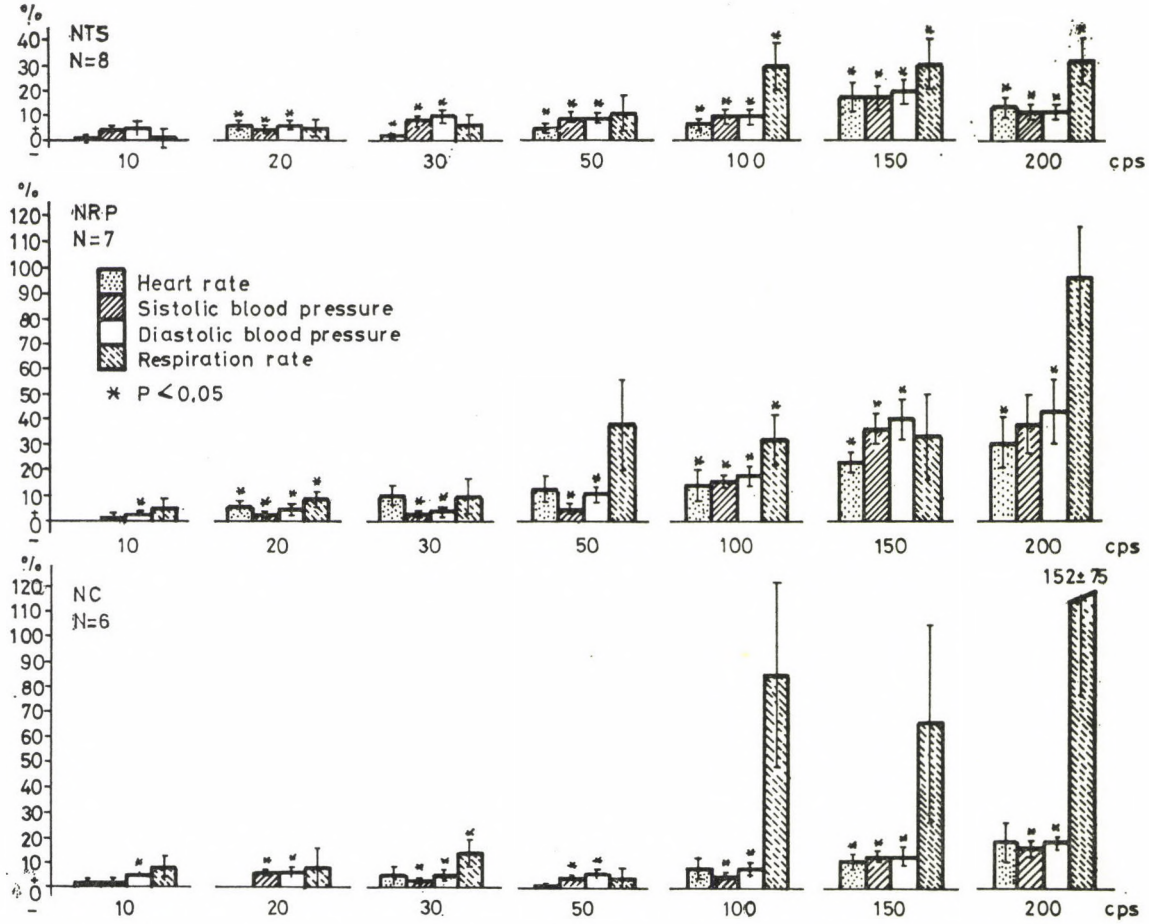

Fig. 4. Effects of stimulations at different pulse frequencies in percent of prestimulation control

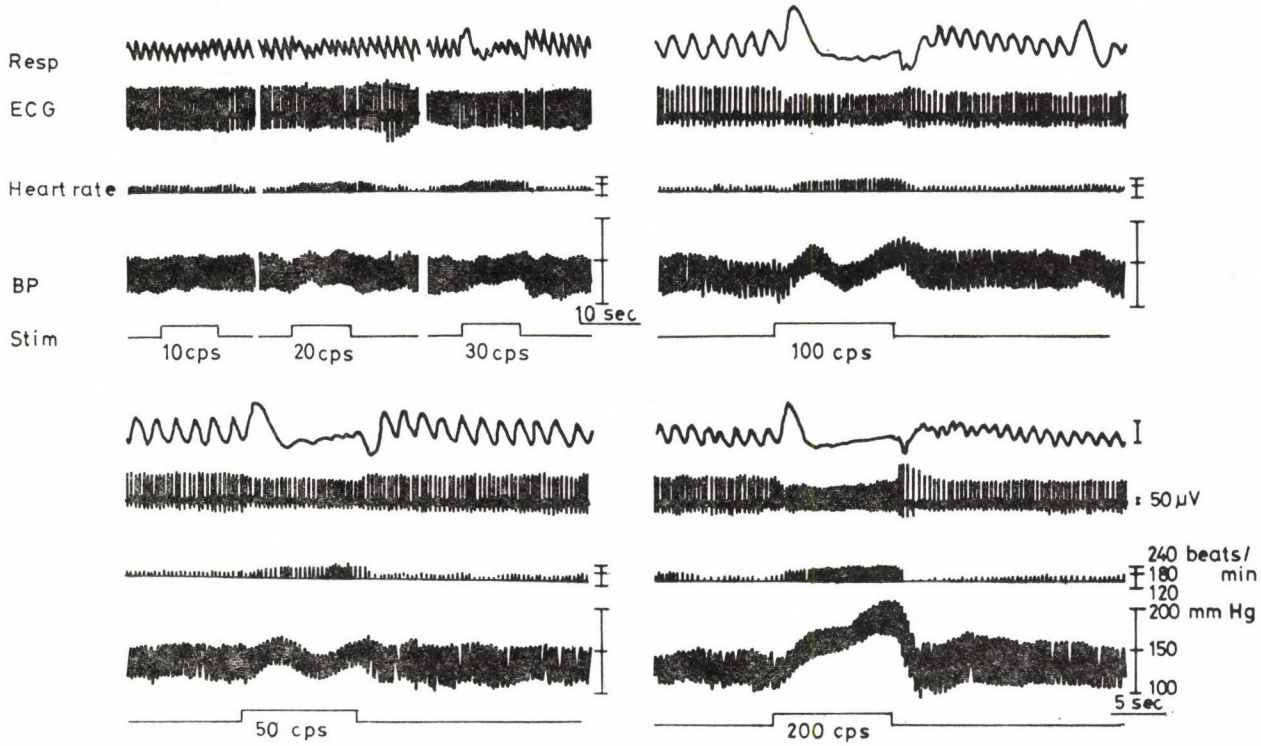

Fig. 5. Respiratory (Resp), ECG, heart rate (HR) and blood pressure (BP) responses to NRP stimulation at medium intensity. Stimulation at 50 cps or higher frequencies produced changes of remarkable magnitude, but the basic topography of these changes was unchanging at the different pulse frequencies

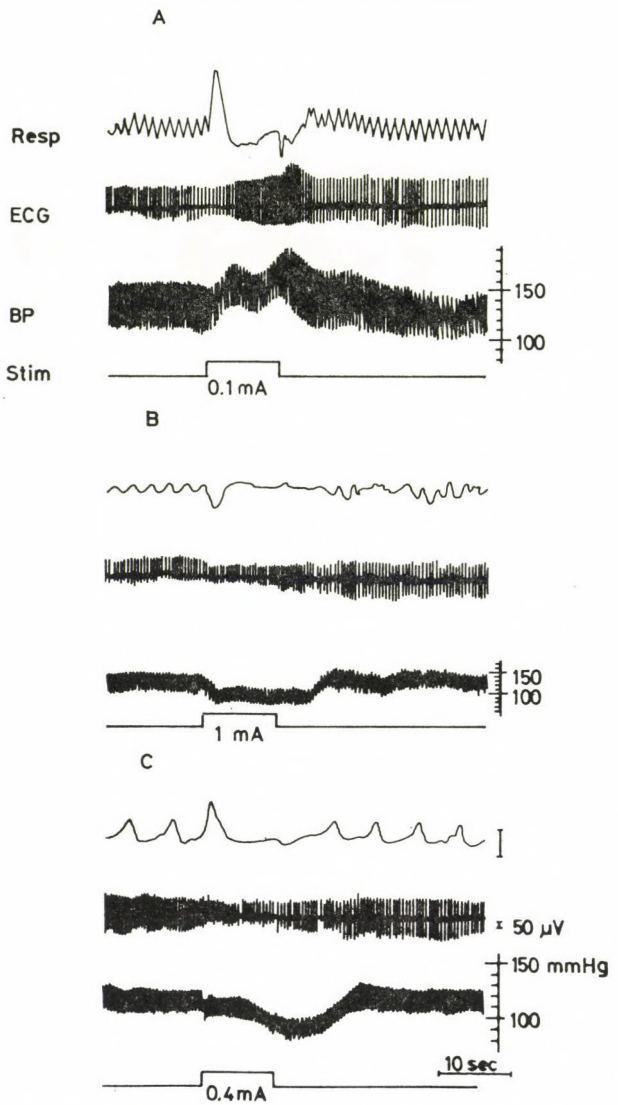

Fig. 6. The increase in blood pressure (BP) elicited by NRP stimulation at 100 cps on conscious, freely moving cat (A) was reversed to decrease in blood pressure both by pentobarbital sodium (B) and chloralose (C) anaesthesia

the evidences obtained from this study indicate convincingly that neither the stimulation parameters nor the anaesthetization can offer a simple explanation for the opposite cardiovascular responses to medullary stimulation mentioned in the introduction. The present data indicate that whatever the experimental conditions are, a pressor point usually remains pressor point, and the depressions are only obtained on rare occasions. It seems probable that the reversal effect of narcosis is locus specific, and the number of such points is much smaller than of those producing consistently pressor responses.

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ACTIVE AND PASSIVE IMMUNIZATION AGAINST LUTEINIZING HORMONE IN PIGS

Bożena SZAFRAŃSKA, A. ZIĘCIK

Institute of Animal Physiology, University of Agriculture and Technology, Olsztyn—Kortowo, Poland

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Active immunization of four adult pigs with highly purified porcine luteinizing hormone (pLH)—using method of multiside intradermal injections—has been performed and resulted in the production of specific antibodies. Immunization caused prolongation of estrous cycle to 47—49 days in two gilts and to 26 days in the other ones.

Obtained anti-pLH pig serum was administered intravenously to 40 day pregnant gilt during 5 days (10 ml of serum, twice daily). Blood plasma progesterone (P_4) concentrations decreased significantly from 8—13 to 2—4 ng/ml after two days of infusion and remained at this level for the next 5 days.

Administration of this anti-pLH pig serum to gilt in the luteal phase of the estrous cycle caused the inhibition P_4 to undetectable amounts.

The different results were found after the passive immunization of 40 day pregnant gilt with rabbit anti-pLH globulin preparation (5 days, equivalent to 3 ml of original undiluted serum, twice daily). Although after two days of infusion P_4 concentration decreased, in the next days P_4 level slowly increased to pretreatment concentrations.

The data suggest the possibility of specific anti-pLH antibody production in pigs by using active immunization, and the repeated utilization of such obtained antiserum in the same species for the inhibition of corpus luteum (CL) function.

Keywords: antiserum pLH, pigs, luteinizing hormone, immunization, estrous cycle, progesterone

Mechanisms controlling P_4 production by the CL of pregnancy in the pig and in other CL-dependent species, such as cow and goat, have not been fully elucidated.

Du Mesnil du Buisson and Leglise [2, 3] have initiated studies to clarify the role of LH during estrous cycle and pregnancy in pigs. Spies et al. [7] used rabbit LH antiserum to inhibit ovulation and luteal function in gilts for the same purpose. The present experiments were designed to test the possibility of raising porcine LH antiserum by active immunization and the repeated utilization of such antiserum in the same species for the inhibition of CL function.

Correspondence should be addressed to
Bożena SZAFRAŃSKA
Institute of Animal Physiology, University of Agriculture and Technology
10-718 Olsztyn-Kortowo, Poland

Materials and methods

Highly purified porcine LH GPZ-1 (potency 0,63x NIH-LH-S_1) obtained in our laboratory [13] was used as immunogen. The following hormones of the porcine pituitary frontal lobe were used for cross-reactions: porcine follicle-stimulating hormone (pFSH-B1), porcine prolactin (pPRL B-1) and porcine growth hormone (pGH B-1) provided by USDA Hormone Program. Purified human chorionic gonadotropin (hCG CR-121) was provided by the Center for Population Research at the NICHHD. Bovine thyroid-stimulating hormone (bTSH, No.T-2026) was supplied by Sigma.

Four regularly cycling gilts weighting about 100—120 kg were immunized intradermally at 30—40 sites behind the ears according to Vaitukaitis et al. [11] with certain modification — one pig received 1 mg of the hormone in the first injection and immunization was repeated after 6 and 9, 11, 14 weeks with lower hormone doses (50 and 30% respectively). Additionally booster injection was performed in two gilts after 17 weeks. The gilts were checked once daily for behavioural estrous by using vasectomized boar.

The hormone was dissolved in 1 ml saline and emulsified with an equal volume of Freund's complete adjuvant. The antibody titer was prepared by incubating dilutions of the antiserum under standard radioimmunoassay conditions [12] and expressed as the final dilution binding 30% of ^{125}I pLH. The cross-reactivity of antisera was defined as the percentage of cross-reacting material required to displace 50% ^{125}I — labelled pLH.

The rabbit pLH antiserum was obtained by multiside intradermal method as described previously by Zięcik et al. [12].

To examine the effect of neutralization of LH on progesterone production, two gilts at 40 day of gestation were injected i. v. via jugular vein with either 10 ml of pig serum during 5 days, twice daily or 10 ml solution of rabbit gamma-globulin [1] preparation (5 days, equivalent to 3 ml of original undiluted serum, twice daily). Additionally, the one gilt in the luteal phase was injected i.v. with 16 ml of pig anti-pLH serum during 6 days, twice daily.

Blood samples were collected every two hours from a canula inserted into the vena via the cephalic vein. Progesterone was determined following the method described by Hotchkiss et al. [6].

Results

The length of estrous cycles before and after immunizations and antibody titers in serum from four gilts actively immunized with pLH are shown in Fig. 1.

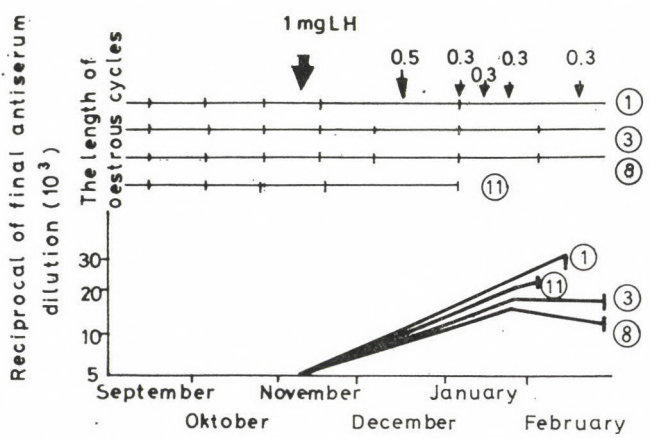

Fig. 1. The length of estrous cycles before and after immunizations and antibody titers in serum from four gilts actively immunized with pLH. The small arrows indicate booster injections following primary immunization. Numerals in circles denote the number of the pigs

All pigs showed detectable antibody titers 7 weeks after primary immunization. Frequent booster immunizations were able to maintain relatively high titers of antibodies in two animals. Sera taken from these pigs have not shown differences in antibody affinity to pLH and low (2.0—0.1%) cross-reaction with pGH, bTSH and pFSH. No cross-reaction was detected with pPRL and hCG (Fig. 2a, b).

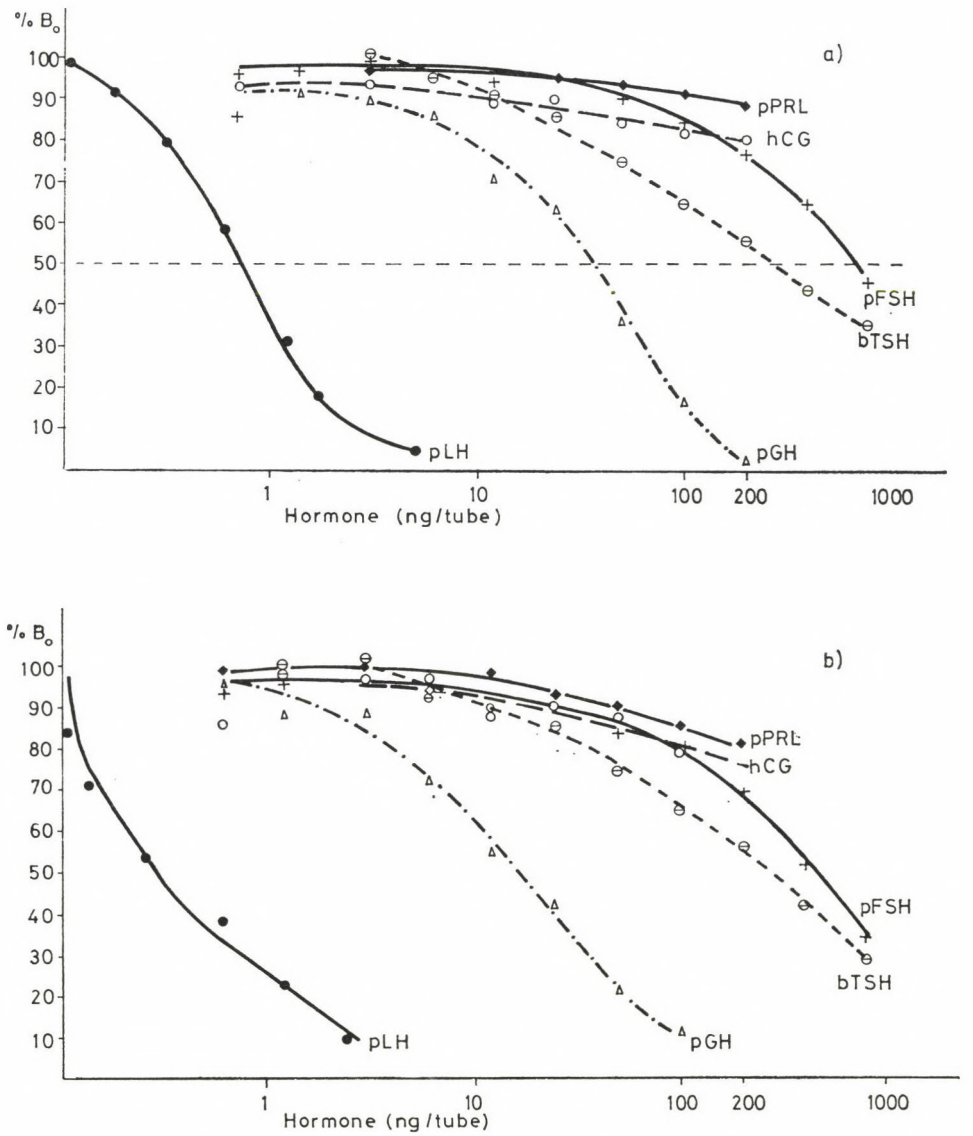

Fig. 2. Inhibition curves for pLH, pFSH, pPRL, pGH, hCG and bTSH in system ^{125}I-LH-(IWF-2,25 mg × LER 786-3) and LH pig antiserum: a) pig No 1 b) pig No 11

Administration of anti-pLH serum from pig No 1 to pregnant gilt A (Fig. 3) at day 42 decreased plasma P_4 concentrations from 8—13 to 2—4 ng/ml after two days of infusion. Different results were found after passive immunization with rabbit anti-pLH gamma-globulin preparation (gilt B, Fig. 3). After two days of infusion P_4 concentration decreased from 9—13 to 4—8 ng/ml, but in the next days increased to similar amounts as before the infusion of rabbit antiserum.

Administration of anti-pLH obtained from gilt No 1 to nonpregnant pig C in the luteal phase of the estrous cycle (Fig. 4) caused a change of the course cycle and inhibited secretion of progesterone to undetectable amounts.

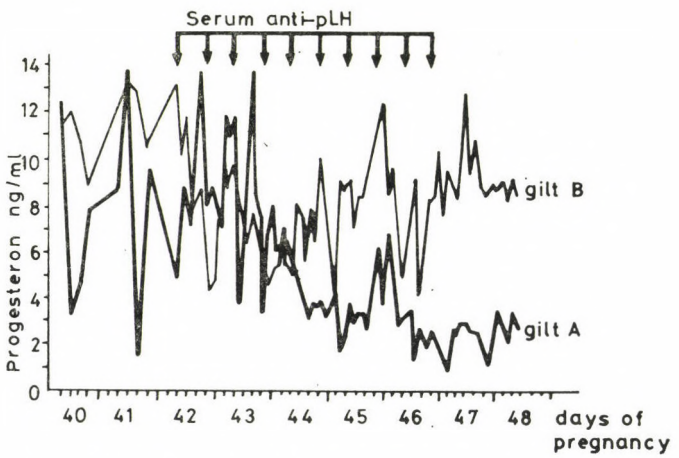

Fig. 3. Progesterone levels in the peripheral plasma of two pigs at 40—48 day pregnancy passively immunized with porcine (gilt A) anti-pLH serum and rabbit anti-pLH gamma-globulin preparation (gilt B). Arrows indicate antibodies injections

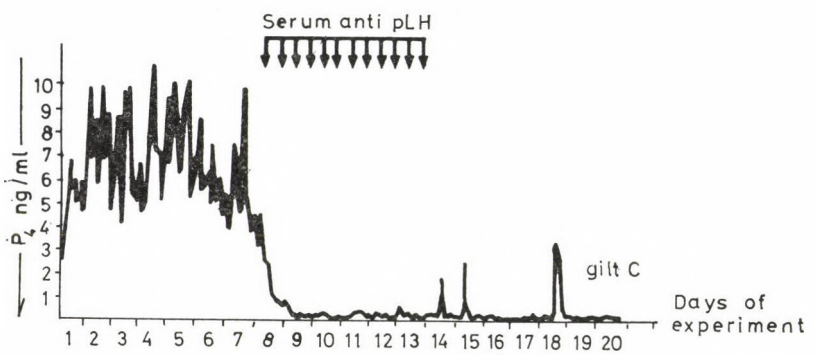

Fig. 4. Progesterone level in the peripheral plasma of pig C in the luteal phase of the estrous cycle passively immunized with porcine anti-pLH serum

Discussion

Actively immunized gilts, especially with high titer of antibodies, showed prolongation in the length of the estrous cycles (No 1 and 11, Fig. 1). After passive immunization against LH prolongation of estrous cycles in the sheep [4] and shortening of the estrous cycles in the monkey [5] was observed. Also active and passive immunization with beta-hCG has been used successfully to prevent or terminate pregnancy in primates [5, 8, 9, 10]. Treatment with rabbit antibodies to ovine LH caused the atrophy of CL and complete loss of embryos in early pregnant gilts [7]; probably as a result of the serologic problem after heterogenous serum. In our experiment we were able to use for the first time specific anti-pLH antibodies which were produced by pigs. Administration of these homologues for the same species antibodies to pregnant and nonpregnant gilts significantly decreased P_4 concentrations in blood plasma (Fig. 3, 4). Abortion did not occur in any of the pregnant pigs and in the next experiment the application of the same anti-pLH pig serum resulted in complete loss of embryos only in one of six early pregnant gilts (Bożena Szafrańska — unpublished information).

The data obtained in our study suggest the possibility of specific anti-pLH antibody production in pigs by using active immunization and the repeated utilization of such obtained homologous antiserum in the same species for the inhibition of corpora lutea function without serologic problems.

Acknowledgement

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RECOVERY OF MAO-B ENZYME ACTIVITY AFTER (—)DEPRENYL (SELEGILINE) PRETREATMENT, MEASURED IN VIVO

Júlia TIMÁR

Semmelweis University Medical School, Department of Pharmacology Budapest

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The recovery of MAO-B activity after a single dose of 0.25 mg/kg s.c. (—)deprenyl was measured in rat, by following the time course of the changes in the PEA (phenylethylamine)-induced hyperactivity. One hour after its administration (—)deprenyl enchanced both the PEA-induced locomotion and stereotypy, however, its effect on the latter was more marked. At 24 h and at later time-points only stereotypy was enhanced. The results show that a single small dose of (—)deprenyl which selectively blocks MAO-B, causes a long-lasting inhibition of PEA metabolism, and the enzyme activity needs more than one week to restore completely.

Keywords: —deprenyl, MAO-B enzyme, stereotyped behaviour, nigrostriatal system.

The enzyme monoamine oxidase exists in two forms, type MAO-A can be blocked selectively by clorgyline [9], while type B MAO by (—)deprenyl [14].

(—)Deprenyl is the only selective irreversible MAO-inhibitor isolated up to now which is widely used in the therapy of Parkinson's disease (for review see 1). It has also been shown to exert an antidepressant effect in the selective dose-range, and it is a promising drug in depression (18) and its beneficial effect in Alzheimer's disease has recently been demonstrated [25].

(—)Deprenyl and all the propargyl "suicide" irreversible inhibitors of MAO inactivate the enzyme by virtue of their covalent binding to the N-5 of the enzyme cofactor FAD [19, 23]. Thus MAO activity recovers only when replacing the irreversibly inhibited MAO by newly synthesized enzyme. The rate of recovery of MAO activity after irreversible inhibition has been widely investigated in different organs, using different MAO inhibitors and substrates [7, 17, 22, 24], however, all these investigations were carried out under in vitro circumstances.

The method to follow continuously the selective inhibition of MAO-B *in vivo* was first introduced by Knoll in 1976 [11]. He exposed the nictitating

Correspondence should be addressed to
Júlia TIMÁR
Department of Pharmacology, Semmelweis University of Medicine Budapest, Nagyvárad tér 4.
P.O.B. 370, Hungary

membrane of the cat and measured the PEA-induced release of noradrenaline in a dose-dependent manner prior to and after a single dose of 0.25 mg/kg (−)deprenyl, demonstrating thereby the selectivity of this drug on MAO-B, as very huge amounts of clorgyline left the effects of PEA unchanged. This is still the only rapid and highly reliable method for checking the selectivity of a B-type MAO inhibitor in vivo, and it is the best method for the rapid screening of the effect of an unknown compound on B-type MAO in vivo. Structure-activity relationship studies [12, 15] are good examples of the practical value of this method.

As the anaesthetized cat can be used in a one-day experiment only, Knoll's method cannot be used for following the recovery of MAO-B activity after a single dose of MAO-B inhibitor in vivo. The aim of this paper is to meet this need.

It has recently been demonstrated that PEA-induced stereotyped behaviour is a suitable method for the follow-up of MAO-B activity in rats in vivo [26]. PEA, one of the endogenous trace amines, induces stereotyped behaviour and hypermotility, similarly to amphetamine [2, 3], but its effect is weaker and shorter in duration. PEA, a specific substrate for MAO-B (28), is oxidized rapidly by this enzyme in the rat brain [5]. A single 0.25 mg/kg dose of (−)deprenyl potentiates the effect of PEA and the cessation of this potentiation indicates the recovery of MAO-B activity.

This method has now been used for the analysis of the in vivo recovery of MAO-B in the brain after single injection of (−)deprenyl.

Materials and methods

Experiments were performed in adult CFY (Sparague-Dawley strain) rats of both sexes, weighing 160—180 g. They were housed in groups of 10 at constant temperature (20—21 °C) under a standard 12 hr—12 hr light-dark cycle (light on at 6.00 a.m.). Water and food were available ad libitum. All the observations were made at comparable times of the day during the light period.

Locomotion, rearing and stereotyped head movements were measured simultaneously by the "Animal Activity Measurement System" designed and built by the Research Institute of the Electrical Industry (Budapest, Hungary). It consists of a testing box (50 × 50 × 30 cm) provided with a TV camera, a central unit based upon an Intel Z 80 microprocessor, and a printer. The testing box is a black painted open field area, where the animals are observed individually. It is set in an isolated dark room, illuminated by a standard laboratory lamp.

The movement of the animal is followed by the TV camera, by the aid of a mirror inclined in an angle of 45°. The central unit separates the different elements of activity, such as horizontal activity in any direction (locomotion), vertical activity (rearing) and stereotyped head movements (STHMV). The time spent on any of these activities during the whole observation period, the distance of locomotion, the number of rearing and the STHMVs are recorded, the resting time is considered to be the non-activity period.

The animals were pretreated with 0.25 mg/kg (−)deprenyl (Chinoin, Budapest, Hungary) 1, 24, 48, 96 or 168 hours before 40 mg/kg of PEA (Chinoin, Budapest, Hungary), or 1 h prior to different doses of PEA. Each experiment, carried out on the same day and compared to each other, consisted of 3 groups: saline+saline; saline+PEA and (−)deprenyl+PEA; six rats were in each group. The observation period started 10 min after PEA (without any adaptation) and

lasted for 30 min. Both PEA and (−)deprenyl were dissolved in distilled water and administered subcutaneously,

Statistical analysis was carried out by two-tailed Student's t-test, a p value of less than 0.05 was considered to be significant. The data are presented as mean values and standard error of the mean ($\bar{x} \pm$S.E.M.).

Results

PEA, 40 mg/kg, significantly decreased the non-activity period ($t = 3.28$, $p < 0.01$) and increased the time both of locomotion and STHMV ($t = 2.96$, $p < 0.05$; $t = 3.69$, $p < 0.01$ respectively). One hour pretreatment with 0.25 mg/kg (−)deprenyl potentiated the PEA-induced hyperactivity, the non-activity period decreased, the STHMV period and number increased significantly compared to the saline+PEA treated group ($t = 4.05$, $p < 0.01$; $t = 7.97$, $p < 0.001$, respectively), the locomotion also increased, but the difference was significant only for the distance and not for the time ($t = 2.12$, $p > 0.05$; $t = 2.22$, $p < 0.05$, respectively), Neither PEA, nor (−)deprenyl pretreatment influenced rearing (Table I).

Table I

Changes in the behaviour of PEA (40 mg/kg) and (−)deprenyl (0.25 mg/kg) + PEA (40 mg/kg) treated animals

	Saline[a] + saline	Saline[a] + PEA	(—)deprenyl[a] + PEA
Non-activity period[b]	26.6±0.5	21.5±1.3^{xx}	13.3±1.6^{yy}
Locomotion period	2.6±0.4	5.3±0.8^{x}	9.5±1.8
Locomotion distance (cm)	399±67	855±184^{x}	2023±492^{y}
Rearing period	0.7±0.2	1.3±0.5	1.1±0.5
Number of rearings	37.3±10.6	73.2±28.9	65.8±32.6
STHMV period	0.62±0.22	1.73±0.21^{xx}	6.10±0.51^{yyy}
Number of STHMVs	50±18	144±17^{xx}	577±47^{yyy}

[a] — pretreatment 1 hour prior to saline or PEA
[b] — all periods in minutes
^{x} $p < 0.05$; xx $p < 0.01$ compared to saline+saline group
^{y} $p < 0.05$; yy $p < 0.01$; yyy $p < 0.001$ compared to saline+PEA treated group

(−)Deprenyl pretreatment (0.25 mg/kg) facilitated also the smaller doses of PEA, which otherwise failed to change the behaviour, however, this potentiation could be demonstrated only for STHMV. The (−)deprenyl pretreated animals after 10 or 5 mg/kg PEA challenge displayed significantly higher STHMV ($t = 2.37$, $t = 2.58$, respectively; $p < 0.05$ for both). PEA, 2 mg/kg, had no effect even in the (−)deprenyl treated animals (Table II).

Table II

Potentiating effect of (−)deprenyl (0.25 mg/kg) on different PEA challenge[a]

	Locomotion period (min)	STHMV	
		period (min)	number
Sal+sal	3.0±0.6	0.53±0.10	43±8
Sal+PEA (10)[b]	3.3+0.7	0.50+0.07	41±5
(−)D+PEA (10)	4.6±1.1	2.05±0.65[x]	169±53[x]
Sal+sal	3.0±0.5	0.44±0.08	36±7
Sal+PEA (5)	1.9±0.5	0.49±0.20	40±16
(−)D+PEA (5)	2.4±0.7	1.70±0.42[x]	136±33[x]
Sal+sal	3.2±1.2	0.44±0.18	38±16
sal+PEA (2)	2.9±1.2	0.31±0.10	25±8
(−)D+PEA (2)	4.3±1.0	0.70±0.19	59±16

[a] — pretreatment 1 hour prior to saline or PEA
[b] — the dose of PEA in mg/kg
[x] $p < 0.05$ compared to sal+sal group
Sal—saline, (−)D—(−)deprenyl

Table III

Changes in the PEA-potentiating effect at different times after single dose of (−)deprenyl (0.25 mg/kg)

	Time between (—)D and PEA (hours)	STHMV		Ratio[a] of STHMV	
		period	number	period	number
Sal+sal	1	0.62±0.22	50±18		
Sal+PEA	1	1.73±0.21[xx]	144±18[xx]		
(−)D+PEA	1	6.10±0.51[yyy]	577±47[yyy]	3.52	4.0
Sal+sal	24	0.59±0.12	48±10		
Sal+PEA	24	2.61±0.58[xx]	217±48[xx]		
(−)D+PEA	24	6.97±1.57[y]	670±158[y]	2.67	3.08
Sal+sal	48	0.42±0.09	35±8		
Sal+PEA	48	1.81±0.49[x]	155±60		
(−)D+PEA	48	4.78±0.86[y]	430±79[y]	2.64	2.77
Sal+sal	96	0.43±0.08	34±6		
Sal+PEA	96	1.29±0.28[x]	109±24[x]		
(−)D+PEA	96	2.83±0.46[y]	243±41[y]	2.19	2.23
Sal+sal	168	0.43±0.08	36±7		
Sal+PEA	168	1.5±0.45[x]	125±37[x]		
(−)D+PEA	168	2.38±0.71[x]	211±62[x]	1.58	1.68

[a] ratio of the means of STHMV parameters in $\frac{(-)D+PEA}{sal+PEA}$ treated animals
[x] $p < 0.05$ [xx] $p < 0.01$ compared to sal+sal group
[y] $p < 0.05$ [yyy] $p < 0.001$ compared to sal+PEA treated group
sal—saline, (−)D—(−)deprenyl

In order to follow the cessation of the MAO inhibition caused by single (−)deprenyl (0.25 mg/kg), the facilitation of PEA's effect (40 mg/kg) was measured at different time intervals after (−)deprenyl. Twenty-four hr after its administration (−)deprenyl had no effect on the locomotion any more, while it significantly increased both the time and the number of STHMV ($t = 2.81$ and 2.72; $p < 0.05$). Similar results were obtained 48 h and 96 h after (−)deprenyl and even 1 week later the time and the number of STHMV were higher than in the saline-treated animals, however, the difference was not significant statistically (Table III).

To estimate the potentiation caused by (−)deprenyl we calculated the ratio of the means of both the time and the number of STHMVs displayed by the animals in the (−)deprenyl and in the saline treated groups. This ratio proved to be about 3.5—4 following one hour pretreatment, 2.5—3 in the case of twenty-four hr or forty-eight hr pretreatment, and only in the animals pretreated 1 week earlier did it decrease to values less than 2 (Table III).

Discussion

Single small doses of (−)deprenyl potentiated the hyperactivity-inducing effects of higher doses of PEA and induced hyperactivity after smaller, per se ineffective, PEA challenge. PEA is supposed to act as an indirect dopamine (DA) stimulant (16, 21). It is well known that the stimulation of DA receptors by various DA agonists may induce both stereotyped behaviour and hypermotility. Stereotyped behaviour is connected mainly to the DA neurons of the nigrostriatal system, whereas hypermotility is related to the mesolimbic DA-ergic system [4, 10].

Recently we have demonstrated that single doses of (−)deprenyl influence the amphetamine-induced stereotypy, while having a very weak effect on locomotion [27]. The present results with PEA agree with these data. While the STHMV-inducing effect of PEA was enhanced even 1 week after single (−)deprenyl treatment locomotion was enhanced only when (−)deprenyl was given 1 h before PEA. (−)Deprenyl seems to have only a weak and transient influence on the effect of PEA in the mesolimbic system, and a marked and long lasting influence in the nigro-striatum. Zsilla and Knoll reported on increased DA turnover in the striatum, but not in other parts of the telencephalon following repeated administration of (−)deprenyl [29]. On the other hand, (−)deprenyl has been demonstrated to cause a higher increase of endogenous PEA in the striatum than in the hypothalamus (8). All these results tend to support Knoll's conclusion that (−)deprenyl, when administered in small daily doses, facilitates the nigrostriatal DA-ergic system with utmost selectivity [13].

The applied dose of (—)deprenyl (0.25 mg/kg s.c.) blocks selectively type B MAO without influencing the type A enzyme (6, 11). The equivalent of this dose (5—10 mg/day) is generally used in the clinical practice. The strongest potentiation of PEA's effect could be seen one hour after (—)deprenyl, twenty-four hr and fourty-eight hr later the potentiation was equal, then it decreased gradually, but even 168 h after the administration it proved to be higher, although the difference was not significant statistically. (—)Deprenyl was reported to inhibit the DA uptake 1 h after a single dose of 0.25 mg/kg but failed to do this twenty-four hr later [30]. This may explain the more marked effect of (—)deprenyl on PEA one hour after its administration, when the DA uptake inhibition may indicate an additional effect to the PEA accumulation caused by MAO-B block. Concerning the other time intervals, the effect of a single small dose of (—)deprenyl on PEA might be considered as the consequence of MAO-B inhibition only.

The half-life of recovery of MAO enzyme activity to PEA was published to be 8.5 days both after 10 mg/kg s.c. (—)deprenyl [17] or 20 mg/kg tranylcypromine [7], as measured in the rat brain in vitro. When m-iodobenzylamine was used as substrate it was found to be 9.1 days after 10 mg/kg i.p. pargyline (22). All the mentioned doses were very high, nevertheless, our results prove that the single selective 0.25 mg/kg dose of (—)deprenyl, which inhibits the PEA deamination by about 70% [20], has a long-lasting effect as well.

Recently human platelet MAO activity to PEA was measured after a single 10 mg dose of (—)deprenyl, and the inhibition was found to be 81.8% at twenty-four hr, 27.9% on day 5, and 13% on day 16 (24). The MAO activity, measured by us as the magnitude of influencing the PEA's effect shows a good correlation with the time course of enzyme inhibition caused by (—)deprenyl in human platelets.

Earlier we reported that repeated (—)deprenyl (0.25 mg/kg s.c. daily for 8 weeks) administration facilitates the PEA's effect for more than two weeks [26]. This paper shows that by using our method the recovery of MAO-B in vivo after a single dose of a selective MAO-B inhibitor can be followed in a reliable manner.

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PARALLEL CHANGES IN BRAIN TISSUE BLOOD FLOW AND MITOCHONDRIAL FUNCTION DURING AND AFTER 30 MINUTES OF BILATERAL FOREBRAIN ISCHEMIA IN THE GERBIL

G. L. NÁDASY, L. MELA-RIKER*, M. REIVICH**, A. G. B. KOVÁCH

Experimental Research and 2nd Institute of Physiology, Semmelweis University, Medical School, Budapest, Hungary. *Department of Surgery, University of Pennsylvania, School of Medicine, Philadelphia, PA, USA. Present address: Department of Surgery, The Oregon Health Sciences University, School of Medicine, Portland, OR, USA **Cerebrovascular Research Center, University of Pennsylvania, School of Medicine, Philadelphia, PA, USA

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Forebrain ischemia was induced in Mongolian gerbils by bilateral occlusion of the common carotid arteries for 30 minutes. These animals do not have a complete circulus arteriosus Willisii. Mitochondria were prepared from the forebrain tissue at the end of the 30 minutes occlusion period as well as at different time points after the release of the occlusion. Tissue blood flow in the forebrain was also determined by measuring the brain tissue accumulation of ^{14}C-iodoantipyrine. Tissue blood flow in the forebrain decreased from a control level of 1.43 ± 0.03 ml/min/gr to 0.13 ± 0.03 ml/min/gr by the 30th minute of ischemia, increased to 1.12 ± 0.25 ml/min/gr after 5 minutes of reflow, but decreased again to 0.41 ± 0.07 ml/min/gr after $1^1/_2$ hours of reflow. Oxygen consumption rate of mitochondria prepared from the forebrain (glutamate+malate as substrates in the presence of ADP) was 98 ± 13 nmoles O_2/min/mg protein in control animals, decreased to 61 ± 9 nmoles O_2/min/mg protein after 30 minutes of occlusion, recovered to 106 ± 9 nmoles O_2/min/mg protein during the first 30 minutes of reperfusion. During extended reperfusion, mitochondrial respiratory activity declined reaching 20 ± 5 nmoles O_2/min/mg protein after 5 1/2 hours of reperfusion. Respiratory control ratio of the mitochondria (relative increase of respiration upon addition of ADP) was 9.2 ± 1.3 in control animals, 7.0 ± 1.5 after 30 minutes of carotid occlusion, 9.0 ± 1.2 after 30 minutes of reperfusion, and 5.8 ± 0.8 after 5 1/2 hours of reperfusion. Superoxide dismutase activity of the forebrain mitochondria was 5.10 ± 0.7 I.U./mg protein in control animals, decreased to 3.3 ± 1.6 I.U./mg protein after 30 minutes of occlusion and remained at this level throughout the reperfusion period. These data confirm earlier reports that deterioration of mitochondrial function may contribute to the development of ischemic and post-ischemic brain tissue damage. It also appears possible that postischemic damage of mitochondrial function develops secondary to postischemic deterioration of tissue blood flow.

Keywords: brain ischemia, gerbil, mitochondria, cerebral blood flow

Several investigators have demonstrated that mitochondrial function deteriorates when brain tissue is subjected to ischemia [2, 10, 11, 13, 17, 18, 19, 23, 24, 28, 30, 31, 37, 38, 44, 47, 50, 57, 58, 59, 62, 63, 66, 67]. Whether mitochondrial function returns to normal after the cessation of ischemia, however, has been questioned. Incidence of mitochondrial functional recovery to

Correspondence should be addressed to:
György NÁDASY
Experimental Research and 2nd Institute of Physiology, Semmelweis University, Medical School,
H-1082 Budapest, Üllői út 78/a., Hungary

near normal levels [17, 23, 24, 25, 35, 45, 51, 52, 54, 55, 58, 59, 62, 63], limited recovery or no recovery at all [13, 15, 23, 44, 59, 67] after brain ischemia have been described in different models, with different durations of the ischemic episode as well as with different times of reperfusion. In many cases after brain ischemia, a post-ischemic microcirculatory deficit has been observed ["no-reflow", 1, 3, 4, 5, 16, 26, 27, 29, 32, 33, 42, 43, 48, 53, 54, 56, 68]. The current investigation was undertaken to examine the effect of 30 min of total forebrain ischemia and the following recirculation on cerebral tissue blood flow and mitochondrial function in parallel measurements, using the gerbil bilateral carotid occlusion model [6, 7, 20, 34, 35, 39, 40, 49, 53, 74], which can be applied to unanesthetized animals following preparation of the experimental animals under light short-term anesthesia.

Methods

Animal preparation and bilateral carotid occlusion: All experiments were carried out on male Mongolian gerbils (Meriones unguiculatus) weighing 60—100 gr (Tumblebrook Farms N. J.). The experiments were done in 38 animals, from which the mitochondrial preparation and measurements were successful in 24 cases. The animals were anesthetized with ketamine (Ketalar, Park Davis, 50 mg/kg i.p.) and with 20% O_2 and 80% N_2O. Supplemental doses of ketamine (25 mg/kg i.p.) were occasionally administered. The left femoral vein and the tail artery were catheterized. Both carotid arteries were carefully separated and elastic threads were placed around them. The two ends of the elastic thread were funneled through a plastic tube. The tubing was sutured to the skin of the neck and the ends of the elastic threads fixed in loose position. All skin incisions were sutured, the anesthesia disconnected, and the animals were taped onto a plastic holder which allowed some movement for them. The animals quickly recovered from anesthesia and were alert before the carotic occlusion was initiated. Bilateral carotid occlusion was induced by pulling the elastic threads and fixing them in tight position. Previous studies in our laboratory proved that, in accordance with the literature, the connection between the carotid and vertebral arterial systems is absent in these animals [6, 7, 20, 34, 35, 39, 40, 49, 53, 74]. Blood flow to large areas of the forebrain essentially ceases during bilateral occlusion of the common carotid arteries. After 30 minutes of occlusion, the elastic threads were loosened. The reestablishment of circulation in the carotids could be immediately checked as the pupils which were wide and paralytic during occlusion narrowed at the beginning of the recirculation. Despite improvement in some reflexes, however, the animals never regained their original alert state.

Forebrain tissue blood flow: Before the scheduled time of decapitation, a slow intravenous infusion of 10—20 μCi ^{14}C-iodoantipyrine was given using a SAGE continuous flow pump. During the infusion arterial blood samples (10 μl) were collected for the arterial concentration curve of the isotope. Forebrain isotope concentrations were computed from the measured ^{14}C activities of the tissue homogenate used for mitochondrial isolation (see below). The isotope concentration measurements were done on a Tricarb (Packard) liquid scintillation counter. Tissue blood flow was comuted as described by Reivich et al. and Sakurada et al. [60, 61].

Mitochondrial preparations: After decapitation, the forebrain of the animals was quickly removed and placed in ice cold isolation medium. Mitochondria from the forebrain were prepared using the technique described by Clark and Nicklas [8] and modified by us [17, 59]. The isolation medium contained 0.225 M mannitol, 0.075 M sucrose and 1 mM EGTA. The forebrain was minced quickly and washed with ice cold isolation medium several times. Then Nagarse (0.5 mg/ml) and bovine serum albumin (2.5 mg/ml), dissolved in 10 ml of isolation medium were added. Following gentle homogenization in a hand operated Dounce homogenizator, the homogenized tissue was incubated for 60 seconds with the proteinase solution. The homogenate was centrifuged at 4 °C at 2200xg for 3 min. The supernatant containing the mitochondria was resuspended, and recentrifuged, the two supernatants pooled and centrifuged at 12,100xg for 8 min. The pellet was rehomogenized with a glass rod and suspended in 3% Ficoll solution. The 3% Ficoll solution containing the mitochondrial fraction was layered over a 6% Ficoll solution and centrifuged at 10,400xg for 30 minutes. The mitochondrial pellet was resuspended

in isolation medium and spun for 10 mint at 12,100xg to wash the mitochondria. The pellet containing the mitochondria was resuspended in 0.2 ml of isolation medium. All solutions were kept on ice, and all procedures were performed at 0—4 °C.

Mitochondrial function: The reaction medium contained 0.225 M mannitol, 0.075 M sucrose, 100 μM EGTA, 0.01 M H_3PO_4, 0.01 M HCl, neutralized to pH 7.40 with tris (hydroxymethyl aminomethane). Usually 25 μl of the mitochondrial suspension was added to 0.625 ml of reaction medium in a stirred chamber. The oxygen consumption of the mitochondria was measured with an oxygen electrode. The experiments were performed at room temperature (23 °C). State 4 respiratory activity (without ADP, but substrates added) was measured in the presence of 7.5 mM glutamate and 7.5 mM malate. State 3 respiratory activity (both substrates and ADP present) was measured in the repesence of 7.5 mM glutamate plus 7.5 mM malate and 460 μM ADP in the incubation medium. Superoxide dismutase activity of forebrain mitochondria was measured according to the method of Elstner and Heupel [14]. This assay of superoxide dismutase enzyme activity is based on the enzyme's ability to inhibit the formation of nitrates from hydroxylamine originating from a hypoxanthine-xanthine oxidase superoxide anion generating system.

Results

Physiological parameters of the animals during 30 minutes of bilateral carotid occlusion and varying periods of recirculation will be discussed in detail in a separate publication from our laboratories [53]. Blood pressure of the animals increased during the first minutes of bilateral carotid occlusion but quickly returned to near normal values. Reopening of the carotid arteries induced a drop in mean arterial pressure which was corrected by an I. V. infusion of Macrodex. Metabolic acidosis was corrected by bicarbonate given I. V. Blood gas parameters thus were normal at all points of time during the study. There was a time dependent decrease in the survival rate of the animals, about one third of them surviving 120 minutes after release of the bilateral common carotid occlusion of 30 minutes. *Forebrain tissue blood flow*: Changes in tissue blood flow of the forebrain as computed from the ^{14}C-iodoantipyrine curves as well as from the isotope concentration of the brain tissue homogenate, are in good agreement with autoradiographic tissue blood flow values measured in a separate set of similar experiments [53]. Forebrain tissue blood flow values measured parallel with mitochondrial function measurements are shown in Fig. 1. Tissue blood flow in the forebrain decreased from a control level of 1.43 ± 0.22 ml/min/gr to 0.13 ± 0.03 ml/min/gr during the 30 minutes ischemic period. This remaining blood flow possibly is a virtual one, labelled iodoantipyrine diffuses into the cerebrospinal fluid, as well as some diencephalic tissue with good blood flow from the vertebral system is included into the preparatum [53]. Tissue blood flow increased to 1.12 ± 0.25 ml/min/gr after 5 minutes of reflow, but decreased again to 0.41 ± 0.07 ml/min/gr after $1^1/_2$ hrs of reflow. Further blood flow measurements are not available since the deteriorating circulatory state of the animals made the ^{14}C-iodoantipyrine tissue blood flow measurements quantitatively unreliable. *Mitochondrial function*: Oxygen consumption rate of mitochondria prepared from the forebrain (glutamate plus malate as substrate, ADP added) was 98 ± 13 nmoles O_2/min/mg mitochondrial protein in control animals which decreased to 61 ± 9 nmoles O_2/min/mg protein after 30 minutes of occlu-

sion. After 30 minutes of reperfusion the oxygen consumption rate recovered to 106±9 nmoles/min/mg protein. A secondary decline occurred and the oxygen consumption rate dropped to 20±5 nmoles O_2/min/mg protein after $5^1/_2$ hours of reperfusion (Fig. 2.). Respiratory control ratio of the mitochondria (relative increase in respiratory rate upon addition of ADP), was 9.2±1.3 in control

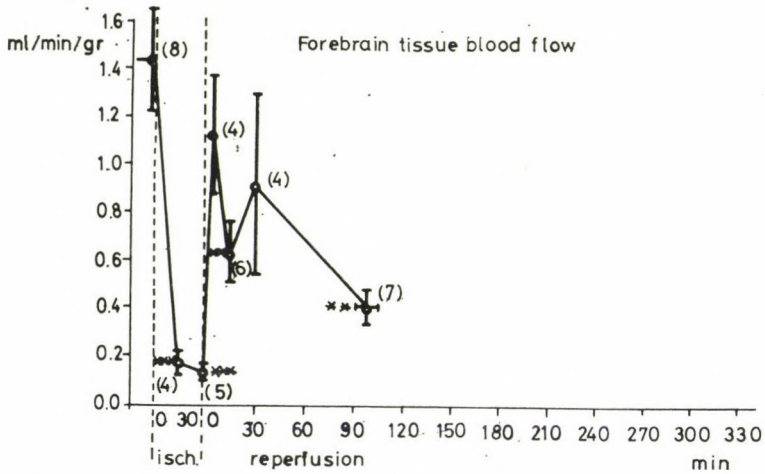

Fig. 1. Forebrain tissue blood flow measured by the ^{14}C-iodoantipyrine method in Mongolian gerbils, during 30 minutes of bilateral carotid occlusion and at different points of time of recirculation. Means±standard error. Significance level of unpaired t test (* < 0.05, ** < 0.01, *** < 0.005)

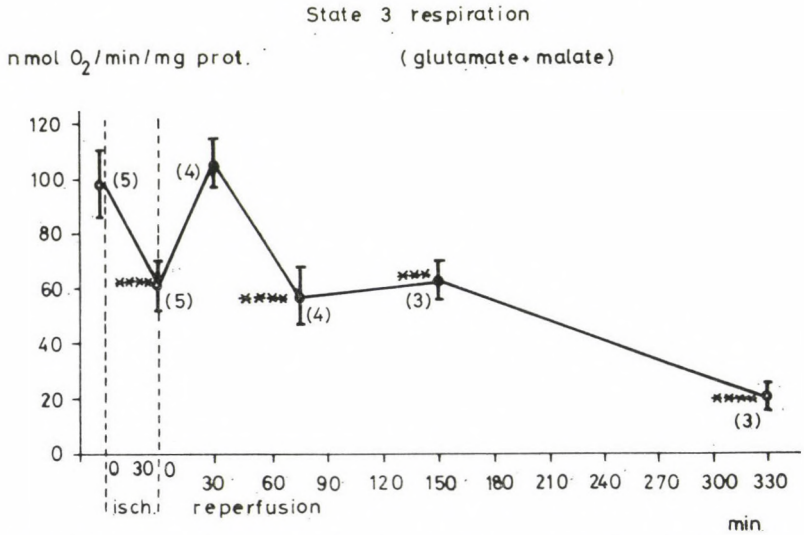

Fig. 2. State 3 respiration (glutamate+malate as substrates, ADP added) of mitochondria prepared from the forebrain of Mongolian gerbils during 30 minutes of bilateral carotid occlusion and at different time points of recirculation. Means±one standard deviation. Statistical significance levels are indicated in the Figure (Analysis of variance, * < 0.05, ** < 0.01, *** < 0.005, **** < 0.001)

animals, 7.0±1.2 after 30 minutes of occlusion, 9.0±1.2 after 30 min of reperfusion and 5.8±0.8 after 5 1/2 hours of reperfusion (Fig. 3.). Superoxide dismutase activity of the forebrain mitochondria was 5.1±0.7 international units/mg mitochondrial protein in control animals which decreased to 3.3±1.6 I.U./mg protein during the 30 minutes occlusion period. Superoxide dismutase activity remained at this low level throughout the reperfusion phase (Fig. 4.).

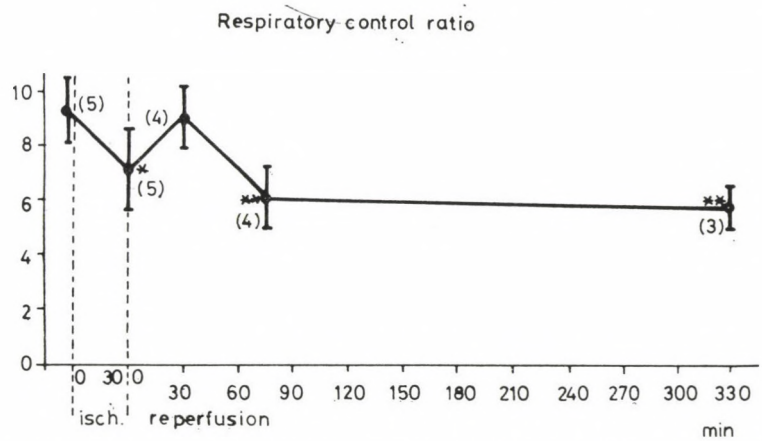

Fig. 3. Respiratory control ratio (ratio of oxygen consumption with ADP and without it, glutamate+malate as substrates) of mitochondria, prepared from the forebrain of Mongolian gerbils during 30 minutes of bilateral carotid occlusion and at different points of time of recirculation. Means±one standard deviation. Significance levels are indicated in the Figure (Analysis of variance, * < 0.05, ** < 0.01, *** < 0.005)

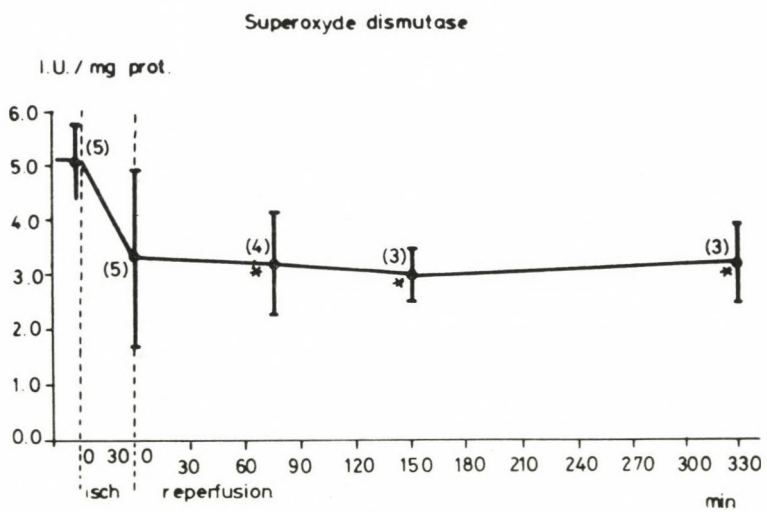

Fig. 4. Superoxide dismutase activity of mitochondria, prepared from the forebrain of Mongolian gerbils during 30 minutes of bilateral carotid occlusion and at different points of time of recirculation. Means±one standard deviation. Significance levels are indicated in the Figure (Analysis of variance, * < 0.05)

Discussion

Comparison of the time course of forebrain tissue blood flow (Fig. 1.) with the mitochondrial state 3 respiration (Fig. 2.) and respiratory control ratio (Fig. 3.), reveals some parallelism between these parameters. Mitochondrial respiration is reduced in preparations isolated from the forebrain at the end of the 30 minute period of ischemia. During the first minutes of reflow, forebrain tissue blood flow returns to values close to control levels (Fig. 1.). It should be noted, however, that the recovery of tissue blood flow is not homogenous. Regions of deficit remain as demonstrated by autoradiographic means [53]. The restored tissue circulation allows a characteristic improvement in mitochondrial function. Both state 3 respiration and respiratory control ratios (Figs 2. and 3.) reach preischemic control levels after 30 minutes of reperfusion. These findings suggest that the deterioration of mitochondrial function induced by 30 minutes of total ischemia is not an irreversible phenomenon. The time course of the forebrain tissue blood flow (Fig. 1.), however, shows a secondary deterioration of flow following the temporary improvement of this parameter. A large decline in blood flow, to 28% of the control, occurs after 100 min of reperfusion. At this point of time mitochondrial oxygen consumption is still 60% of control (Fig. 2.). A more rapid decline of mitochondrial respiratory function occurs after 150 min of reperfusion. A low level of mitochondrial activity, 20% of the control, is reached after 5 1/2 hours of reperfusion. These correlations, and the finding that mitochondrial functional recovery during early reperfusion phase is more substantial than the recovery of blood flow, suggest that the deterioration of mitochondrial function during reperfusion might occur secondary to the deficit in brain tissue perfusion.

The key element in irreversible ischemic-postischemic brain damage — if such key element really exists is still obscure. Current theories emphasize the significance of impaired tissue reperfusion ["no-reflow", 1, 3, 4, 5, 16, 27, 29, 32, 33, 42, 43, 53, 54, 56, 68], irreversible damage of mitochondrial function [13, 15, 23, 44, 59, 67], irreversible shifts in intracellular/extracellular Na^+ and K^+ [7, 9, 37, 44, 49, 64, 70, for review see 44], irreversible decrease in intracellular pH due to tissue lactic acidosis, and impaired ion pump mechanisms [6, 22, 23, 36, 44, 55, for review see 36], flooding of the intracellular space by Ca^{2+} ions [10, 13, 15, 18, 19, 28, 65, for review see 65], irreversible damage to blood brain barrier with ensuing edema [3, 29], membrane damage due to free radicals [12, 21, 44, 47, 69, 71, 72, 73], irreversible loss of such organic compounds as pyruvate and other citric acid cycle intermediates, adenine, NAD, membrane phospholipids [11, 41, 44, 46], and accumulation of free fatty acids [38, 44, 71, 72, for review see 44].

The temporary improvements of forebrain tissue blood flow and mitochondrial function after 30 minutes of total ischemia demonstrated in this study

are significant. It is important to note, however, that the neurological status of the animals (e.g. alertness, direction dependent movements, complex reflexes) did not return to normal. Autoradiographic measurements of regional cerebral blood flow in the same brain ischemia model [53] demonstrated serious deficits in tissue blood flow with inhomogenous columns and patches of underperfused areas in the forebrain, during the first 30 minutes of reflow. These deficits result in serious local and general neurologic symptoms. Such local circulatory and metabolic deficits could not be measured by the global parameters of tissue blood flow computed for the whole forebrain or function of mitochondria, prepared from the whole forebrain.

Ischemic damage to superoxide dismutase activity of mitochondria (Fig. 4.) and the inability of the brain rapidly resynthesize the enzyme may contribute to membrane damage caused by generation of free radicals.

In summary, we found a decrease of mitochondrial functional ability associated with 30 minutes of forebrain ischemia. During reperfusion only a temporary improvement in tissue blood flow and mitochondrial function was observed. With increasing time of reperfusion a parallel impairment of both tissue blood flow and mitochondrial functional ability was observed.

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EFFECTS OF SOMATOSTATIN ON INTESTINAL CIRCULATION AND OXYGEN CONSUMPTION

W. W. PAWLIK, P. GUSTAW, K. CZARNOBILSKI, R. SENDUR, S. J. KONTUREK

Institute of Physiology, Medical Academy, Krakow, Poland

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The effects of intraarterial administration of somatostatin upon intestinal blood flow, intestinal capillary surface area, oxygen consumption and intestinal motor activity were measured in anesthetized dogs. Blood flow to the segment of distal ileum was measured with an electromagnetic blood flow meter, and arteriovenous oxygen difference (AVO_2) was determined spectrophotometrically. Intestinal oxygen consumption was calculated as the product of AVO_2 and total blood flow. The clearance of ^{86}Rb was measured to estimate the density of the perfused intestinal capillaries. Changes in blood flow distribution were estimated from the distribution of radiolabelled microspheres. Intestinal motor activity was monitored from changes in intraluminal pressure.

Somatostatin induced a dose-related decrease in intestinal blood flow, capillary surface area and intestinal oxygen consumption. A significant increase in intestinal motor activity was also observed. The data of this study indicate that somatostatin acts on smooth muscle of both arterioles and precapillary sphincters and results in a potent vasoconstriction in the intestinal microcirculation.

Keywords: somatostatin, intestinal circulation, oxygen consumption.

Somatostatin (SS) has been shown to suppress the release of many hypophyseal and extra hypophyseal hormones including gastrointestinal hormones [3, 7, 13, 20]. The peptide was found to affect all basic functions of the digestive system such as secretion, absorption, motility and gastrointestinal circulation. SS is a potent inhibitor of gastric acid secretion [4, 13, 15, 17, 24] and pancreatic exocrine secretion [1, 11, 12]. In addition to suppressing gastric and pancreatic secretion, the peptide depresses gastric emptying [6] and gall bladder contractions [8]. It was also found to stimulate spontaneous gut motility [30].

SS administered systemically and locally in relatively low doses was found to affect gastrointestinal blood flow. The peptide has been used in clinical practice to treat upper gastrointestinal hemorrhage [9, 10, 16, 18, 25]. The mechanism of this therapeutic action of SS is not fully understood. To some extent these beneficial effects are brought about by vasocontrictive activity of SS in the splanchnic circulation. It has been shown that SS reduces the total and mucosal blood flow in the stomach [5, 17, 21] and in the pancreas [14].

Correspondence should be addressed to
Wieslaw W. PAWLIK
Institute of Physiology, Medical Academy
31-531 Krakow, Grzegórzecka 16, Poland

We have previously reported [14, 22] that in dogs intraarterial administration of SS consistently decreases the small intestinal blood flow in a dose-dependent manner. The finding that SS constricted the intestinal vessels points to the possibility that the peptide does not only decrease total intestinal blood flow via constriction of arterioles but it constricts the precapillary spincters reducing the nutrient portion of the intestinal microcirculation.

The aim of our study was to assess the effects of somatostatin on canine small intestinal blood flow, intramural blood flow distribution, intestinal capillary surface area and intestinal oxygen consumption. In addition we have investigated the effects of the neuropeptide on intestinal motor activity.

Materials and methods

Experiments were carried out on 15 anesthetized and heparinized dogs. A femoral vein was catheterized for injection of supplemental anaesthesia and another catheter was inserted into a femoral artery for measurement of systemic arterial blood pressure (AP) with a strain gauge transducer (Statham). After a midline laparotomy, a distal trunk of the superior mesenteric artery supplying a segment of distal ileum was exposed and a side branch of the artery was cannulated for infusion of drugs. The ends of the intestinal segment supplied by this trunk were ligated to block collateral circulation. The average weight of these segments was 120 g. An electromagnetic blood flow transducer of appropriate internal diameter was positioned around the mesenteric artery and connected with a blood flow amplifier (Scalar, Holland, MDL 503). A side branch of the mesenteric vein draining the intestinal segment and the femoral artery were cannulated. Following heparin administration a constant flow pump (Unipan) circulated blood at 7 ml/min from these vessels through the arterial and venous cuvettes of a photometric arteriovenous oxygen content difference analyzer (A-VOX System, San Antonio, Tex.) [29]. Arterial and venous effluents from the apparatus were then pumped back to the circulation via the femoral vein. Intestinal oxygen consumption (VO_2) was calculated as the product of the arteriovenous oxygen difference (AVO_2) and the total blood flow of the intestinal segment (BF).

Changes in blood flow distribution within the intestinal wall were determined using the radiolabelled microsphere technique [19]. Three different types of radiolabelled microspheres (^{141}Ce, ^{51}Cr and ^{85}Sr) of 15 ± 3 μm (3M Company) were used. Just prior to injection, the vials containing microspheres in 10% dextran were shaken and agitated on a vortex mixer for 10 min to insure homogenity. With each injection approximately 400.000—600.000 microspheres labelled with an isotope were introduced into the left ventricle. Injection of microspheres with different labels was made during the control period and during administration of the drug. After each experiment, control and experimental intestinal segments were removed, rinsed and weighed. Segments were opened along the mesenteric border and cut into portions about 10 cm long. The mucosal and submucosal layers were stripped from the muscular layer and counted in a gamma spectrometer. Fractional blood flow to the muscular compartment was calculated as the product of blood flow and the percentage of microsphere distribution to the muscle and expressed in ml/min-100 g.

Additional series of experiments were performed to evaluate the effects of somatostatin on the clearance of $^{86}RbCl$ measured by the method of Renkin and Rosell [26] and adapted to free flow preparation [22]. From the $^{86}RbCl$ clearance we calculated the PS product, which is an index of the effective capillary surface area and reflects the density of the perfused capillaries.

The intraluminal pressure of intestinal segment was measured with an open-tip catheter inserted into the lumen and connected with a pressure transducer (Statham). The mean motility index (MMI) was calculated by dividing the sum of the heights of all contractions during a 10 min period by the number of contractions in the same time period [32]. AP, BF, AVO_2 and intraluminal pressure were monitored in all experiments on a directwriting recorder (Dynograph Recorder R611, Sensor Medics).

Somatostatin (Peninsula Laboratories, San Carlos CA) was diluted in 0,9% NaCl immediately prior to use and was infused intraarterially (i.a.) into the intestinal vasculature in increasing doses; 0.5, 1.0, 2.0 and 4.0 μg/kg-min. Each dose was infused for a 10-minutes period.

The data from all experiments were evaluated statistically with Student's t-test for paired observations. Alterations from control were expressed as the percentage change. The significance of these changes was assumed at $p < 0.05$.

Results

After surgical preparation of the gut, 30-min period was allowed for BF, AVO_2 and pressure in the lumen to stabilize. Control values obtained from dogs were as follows: BF, 63.0 ± 6.7 ml/min-100 g tissue; AVO_2 3.2 ± 0.2 ml O_2/100 ml blood; VO_2, 2.4 ± 0.4 ml O_2/min-100 g, and MMI, 3.6 ± 0.4 mmHg. Intramural distribution of intestinal blood flow was $76.0 \pm 7.2\%$ for the mucosal-submucosal compartment and $23.0 \pm 2.0\%$ for the muscular layer. Control muscular fractional blood flow (FBF) was 26.7 ± 4.0 ml/min-100 g tissue. AP ranged between 130—160 mmHg. In seven of these dogs control PS-product was 2.7 ± 0.3. The weight of the intestinal segments ranged between 75—150 g.

The response to i.a. infusion of somatostatin included a significant dose-dependent decreases in BF, and VO_2. AVO_2 and MMI were significantly increased in all doses of somatostatin (Fig. 2.). A representative tracing illustrating these changes at 1.0 μg/kg-min somatostatin is shown in Fig. 1. All parameters reached a peak value at a dose of 4.0 μg/kg-min, when BF was decreased by $40.0 \pm 4.5\%$ ($p < 0.001$), VO_2 decreased by $32.4 \pm 4.6\%$ ($p < 0.001$), MMI

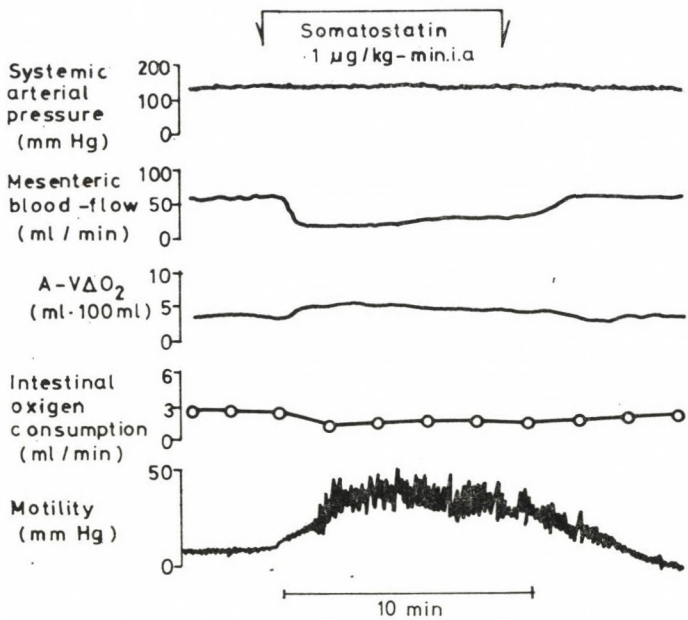

Fig. 1. Results of a single experiment in which one dose of somatostatin was infused into the mesenteric artery for 10 minutes. Blood flow decreased considerably and arteriovenous oxygen difference increased: hence, calculated intestinal oxygen consumption decreased. Intraluminal pressure increased and systemic arterial pressure was unaffected

increased by 95.0±17.0% ($p < 0.005$) and AVO_2 increased by 16.4±5.2% ($p < 0.005$) (Fig. 2.). Somatostatin at doses of 0.5 and 2.0 μg/kg-min decreased PS by 11.2±0.5% ($p < 0.005$) and by 21.8±2.3% ($p < 0.001$) respectively. Microsphere distribution was examined only at the end of the infusion of doses 1.0 and 4.0 μg/kg-min and showed a significant decrease in FBF by 25.6±4.0% ($p < 0.005$) and by 55.6±7.0% ($p < 0.001$) respectively (Table I).

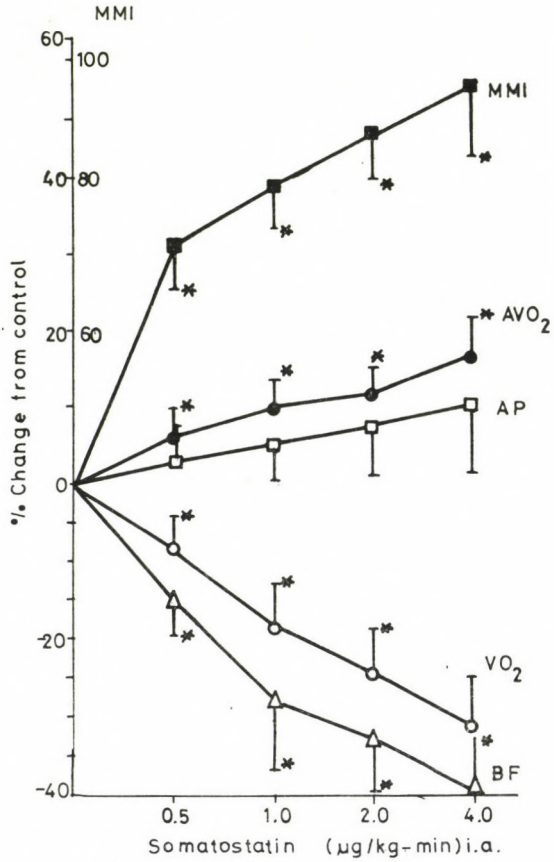

Fig. 2. Effects of intraarterial (i.a.) infusion of somatostatin on intestinal blood flow (BF), AVO_2, intestinal oxygen consumption (VO_2), intestinal motility (MMI), and systemic arterial pressure (AP). Each point represents a mean value in percentage of control±SEM. Asterisks denote statistical significance

Discussion

The wide distribution of somatostatin-producing (d) cells and the presence of somatostatin-like immunoreactivity in the enteric neurons in the wall of the gastrointestinal tract, suggest that this neuropeptide may exert its action both

Table I

Effects of somatostatin on the systemic arterial pressure (AP), intestinal blood flow (BF) the arteriovenous oxygen difference (AVO_2), the intestinal oxygen consumption (VO_2), the mean motility index (MMI) and the muscular fractional blood flow (FBF)

	Control	Dose of somatostatin			
		μg/kg—min i.a.			
		0.5	1.0	2.0	4.0
AP mm Hg	145±15	148±9	152±11	155±10	159±14
BF ml-min/100 g	63.0±6.7	53.6±2.6*	47.4±2.5*	42.9±2.2*	37.8±2.8*
AVO_2 ml/100 ml	3.2±0.2	3.4±0.1	3.5±0.1*	3.6±0.2*	3.7±0.2*
VO_2 ml-min/100 g	2.4±0.1	2.0±0.1*	1.9±0.1*	1.8±0.1*	1.6±0.1*
MMI mm Hg/min	3.6±0.4	6.2±0.5*	6.5±0.5*	6.7±0.6*	7.0±0.7*
FBF ml/min/100 g	26.7±4.0	NT	19.8±2.0*	NT	11.9±3.0*

NT = no tested, * $p < 0.05$

as a hormone and a neurotransmitter. Somatostatin may be released into the surrounding interstitial space, thereby acting locally on smooth muscle cells in the intestinal vessels.

In the present study we found that somatostatin is a potent vasoconstrictor of the canine intestinal circulation. Furthermore, there is a direct relationship between the dose of the neuropeptide administered intraarterially and the subsequent decrease in blood flow. The somatostatin induced reduction in blood flow through the small intestinal circulation observed in the current study corresponds to our previous reports showing that this agent when infused intraarterially consistently diminished the gastric and intestinal blood flow [11, 13, 14].

We also found that somatostatin evoked significant decrease in intestinal oxygen consumption. This reduction in VO_2 combined with decreasing BF could be due to either a direct metabolic effect of somatostatin, or a decrease in blood flow. The microcirculatory structure which regulates tissue perfusion is the precapillary sphincter. Closure of a large proportion of these sphincters in the gut by any means decreases the transport of oxygen from blood into the tissue [23, 27, 31]. The finding that somatostatin reduced the PS product indicates that the neuropeptide induced reduction in the intestinal oxygen consumption by a microvascular mechanism. Besides decreasing total intestinal blood flow,

somatostatin induced a redistribution of blood flow into the muscular compartment by a higher percent of decrease in the intestinal mucosal blood flow than in the total intestinal blood flow. This may be due to the stimulation of the intestinal smooth muscle. This agrees in part with earlier reports in which somatostatin has been described to reduce splanchic blood flow estimated by ^{133}Xe clearance and microsphere distribution techniques [25]. The knowledge about the mechanism by which somatostatin decreases intestinal blood flow is uncertain. The neuropeptide is secreted into the interstitial space of the intestinal wall, from which it enters the microcirculation. This close contact to the microcirculatory structures might imply a direct action on the vasculature. Somatostatin supressing the release of gastrointestinal hormones such as gastrin, cholecystokinin and vasoactive intestinal polypeptide and counteracting the vasodilatory action of local metabolites may also contribute to the contraction of arteriolar and precapillary phincters.

The observed vasoconstrictory action of somatostatin would also explain the favourable effect of the neuropeptide in the treatment of severe gastrointestinal haemorrhages.

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INVESTIGATION ON THE FREE RADICAL PRODUCING EFFECT OF HEMATOPORPHYRIN
(A spin trapping study)**

L. PÓTÓ[1], Timea BERKI*

Central Laboratory and *Institute of Pathology University Medical School, Pécs, Hungary

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Direct evidence was given concerning the free radical production of hematoporphyrin (HP) and its derivative, during illumination, by ESR spectroscopy. A spin trapping measurement was carried out using PBN spin trap for determination of this radical(s). Three different spin-adducts were found. Two of them are likely to be connected to the reactive free radical production of HP.

Keywords: HP, He-Ne laser, ESR, spin-trapping, PBN

Photosensitizers lead to severe damage of living cells after illumination. Hematoporphyrin (HP) and hematoporphyrin-derivative (HPD) are commonly used as photosensitizers in experiments and for phototherapy of tumours [2.].

Two different types of reaction mechanisms of photosensitization have been published so far [1,3]. The first step of both is the activation of the sensitizer to its triplet state [7]. During the type I reaction the most reactive compounds are the free radical form of the sensitizer, the superoxide anion (O_2^-) and the hydroxyl radical ($OH^\cdot$) [7], while during the type II reaction it is the singlet oxygen ($^1O_2$) [8]. All these compounds can damage cells by oxidation of membrane proteins or by lipid peroxidation.

It seemed reasonable to try to detect the above-mentioned free radicals by ESR spectroscopy. Unfortunately, the steady-state concentrations of these very reactive compounds are too low for the direct detection. To overcome this problem we used two indirect methods for the detection of free radicals. One of the numerous 'classical' reactions described in the literature [4] is the radical — radical combination using stable radicals as spin traps. The second is the spin trapping method. The first reaction does not provide information about the trapped radical. The decreasing amount of the stable free radical shows the

Correspondence should be addressed to
László PÓTÓ
Central Laboratory, University Medical School,
7643 Pécs, Szigeti út 12., Hungary

** Presented at the Membrane Symposium, May, 1988, Sümeg, Hungary.

production of the trapped radical. By the second method one can detect different spectra — depending on the spin trap used and the trapped radical. But generally the identification of the trapped radical is not easy.

Materials and methods

— Hematoporphyrin dihydrochlorid (HP) obtained from SIGMA and hematoporphyrin derivative (HPD, prepared according to Lipson at al [6]) were used as photosensitizers.
— Egg lecithin was prepared and tested by TLC in our laboratory.
— Stable free radicals: the lipid soluble 4-(2-n-undecyl-3-oxyl-4,4-dimethyloxazolidin-2-yl) butyric acid (HO-185) and the water soluble N-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)-maleimid (HO-16) were kindly provided by Prof. K. Hideg.
— The spin trap N-t-butyl-α-phenyl-nitrone (PBN) was from SIGMA.
— Liposomes were prepared by the micro-method of Kinsky et al [5]. Briefly, 5 mg of egg lecithin with or without 200 μg of HO-185 and HP at different concentrations (5—200 μg) were dissolved in 0.5 ml of chloroform and evaporated in a round flask under N_2-gas stream. The film was mixed with 1 ml Tris-HCl buffer of pH 7.4 containing 0.9% NaCl by a Vortex device for 3 minutes at room temperature.
— Spin trapping measurements were performed in a solution of 150 mM PBN in 70% ethanol-water with different concentrations of HP. The HP was added and the sample was taken into a cuvette in dark. Illumination was carried out by a 1 mW He-Ne laser ($\lambda = 632.8$ nm)
— Conventional ESR spectra were taken by an ERS 220 (ZWG, GDR) spectrometer.

Results and discussion

— Evidence for free radical production.

The decreasing amount of the stable free radical reflects the production of reactive radicals. The results show that the effect depends on the concentration of the sensitizer (HP or HPD) and the time of illumination. (Fig. 1). The HPD was less effective than the HP itself. No such effect has been observed in the absence of the sensitizer or the light.

— Spin adducts using PBN spin trap.

Spectra of three spin adducts have been detected (Fig. 2). Two of them (labelled as 1. and 2.) are double triplets, while the 3. is a single triplet. The hyperfine splitting constants are given in Table 1.

Table I

The hyperfine splitting constants of the three spin adducts using PBN spin trap

	a_N(mT)	a^H_β(mT)
1.	1.401	1.297
2.	1.545	0.176
3.	1.642	—

the accuracy of data is: 0.003 mT

By comparing our data to those of the literature [4] we could not identify the spin adducts. However, it is likely that the 3. adduct is a stable nitroxide radical while the 2. one arises by hydroxyl radical trapping. We have not been able to identify the 1. adduct so far.

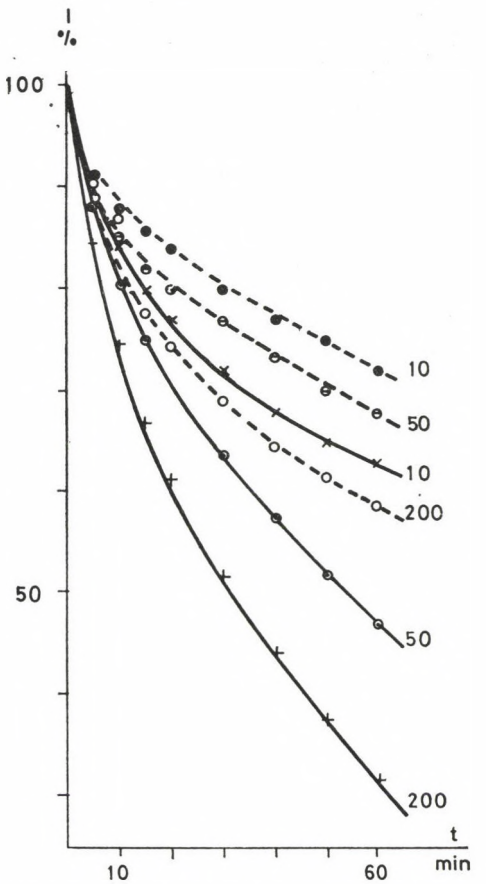

Fig. 1. Intensity changes of the stable free radical during illumination in the case of HP (solid lines) and HPD (dashed lines) in liposomes. The HP and HPD concentrations are given at the curves in μg/ml

The Figs 2/b show the time-kinetics of the spectra after the beginning of illumination: The $2/b_1$ spectrum illustrates that the 1. adduct arises first (illumination time $<$ 1 min) and is immediately followed by the appearance of the 2. adduct ($2/b_2$.). The quantity of both is increasing with the illumination time, but depends on the concentration of HP inversely (Fig. 3.). After a few hours of illumination the 3. adduct arrises in a comparable amount to 1. and 2. ($2/b_3$.). After half a day of illumination the 3. adduct becomes dominant.

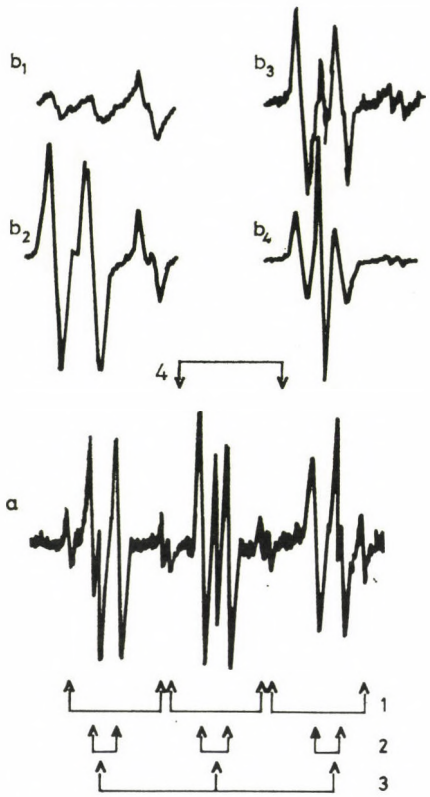

Fig. 2. a: The spectra of the three (1—3) spin adducts using PBN spin trap in 70% ethanol-water solution. 4: This part of the spectrumi s shown in the $2/b_1—b_4$ figures. $b_1—b_4$: The spectra of the adducts after 1 min, half an hour, 2—3 hours, half a day, respectively

The biological effects are observed after seconds or minutes of illumination, so the 3. adduct does not seem to have much significance at the moment.

The Fig. 4. shows the changes in the amounts of the 1. and 2. adduct after a few cycles of turning on/off the light. A possible explanation for the unexpected behaviour of the 2. adduct (Fig. 3. and Fig. 4.) can be as follows:

a, the production of the 1. adduct depends on the concentration of the HP and the illumination time; parallel to this there is a spontaneous decomposition of this adduct on the scale of minutes;

b, the production of the 2. adduct depends on the concentration of the 1. adduct; parallel to this there is a light-induced decay depending on the concentration of the HP; finally there is a spontaneous decomposition of the 2. adduct that has a characteristic time constant in range of 10 minutes.

Further extensive experimental work is needed to clarify these hypothetical processes.

— The role of the water.

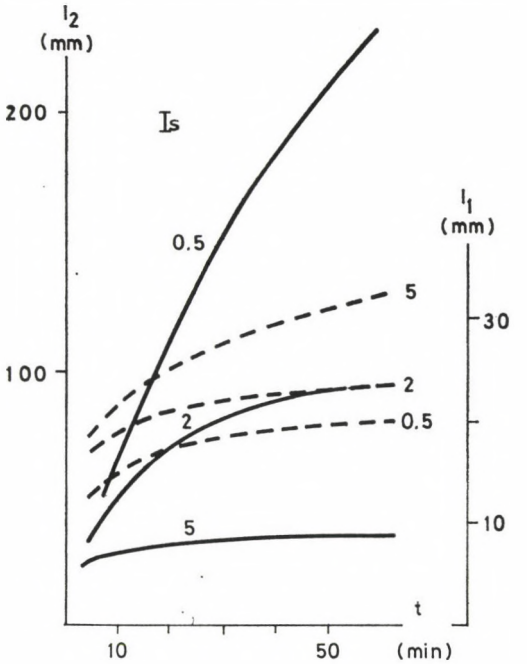

Fig. 3. Relative intensities of the 1. (I_1, dashed lines) and 2. (I_2, solid lines) adduct's spectra at different concentration of HP that is labelled at each curve in μg/ml. s: the measured intensities are within this region at each curve; the detection was carried out at each 1—1.5 minutes; the time was measured from the beginning of illumination

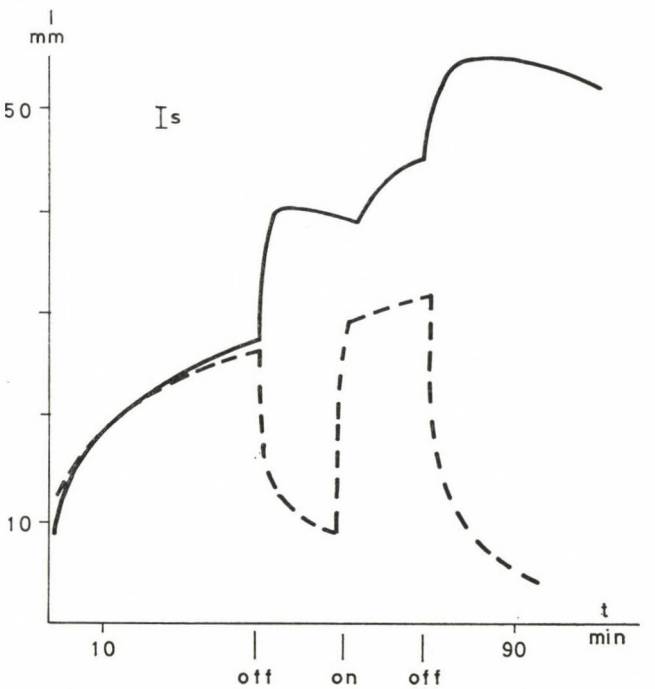

Fig. 4. Relative intensities of the 1. (dashed line) and 2. (solid line) adduct's spectra during some cycles of turning on (on) — switching off (off) the light. s: the same as in Fig. 3

The amount of the 1. adduct (at the same illumination time) depends on the water-content of the ethanol-water solution: In 2, 5, 15, 33% water-ethanol solution we got the relative intensities of the 1. adduct's spectra < 10, 13 ± 3, 21 ± 5 and 100 ± 10 respectively. We assume that in the case of the 1. adduct there is no connection with superoxide ion or singlet oxygen because the dissolved O_2 content does not change significantly if the water concentration is changing in the solution.

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SPIN-TRAPPING STUDIES WITH MTDQ, A DIHYDROQUINOLINE TYPE RADICAL SCAVENGER*

J. Belágyi, L. Pótó

Central Laboratory, University School of Medicine, Pécs, Hungary

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The effect of MTDQ, a dihydroquinoline type free radical scavenger was studied in biological membranes, and model systems. Using spin-labelling and spin-trapping methods, it was that MTDQ induced a decrease of the mobility of spin labels attached to the thiol sites of membrane proteins, and it was a strong free radical scavenger in hydroxyl and superoxide anion free radical generating systems. The experimental results suggest that the presence of MTDQ in the cell membranes may improve the protection of membrane components against free radical attacks.

Keywords: membrane proteins, oxygen free radicals, spin-trapping, free radical scavengers

The formation of free radicals, especially oxygen free radicals in biological systems is a consequence of aerobic life. The interest in this process rapidly increased because it has been established that peroxidative damage of biomembranes may play an important role in a number of diseases; the superoxide and hydroxyl free radicals may especially act as etiological factors in biological phenomena under pathological conditions [1, 2].

The potentially toxic products of oxygen metabolism are normally removed by ubiquitous endogenous scavengers. However, the imbalance between oxidative reactions and protective mechanisms indicates the basis of a number of diseases, this the use of natural and/or artificial antioxidants could help in the prevention of cell and membrane damages [3].

The aim of present work was to study the effect of recently developed dihydroquinoline type radical scavenger MTDQ in biological membranes and model systems using the spin-trapping technique in order to characterize its activity on the molecular level.

Correspondence should be addressed to
József Belágyi
Central Laboratory, University School of Medicine
7643 Pécs, Szigeti u. 12, Hungary

* Presented at the Membrane Symposium, May 1988, Sümeg, Hungary.

Methods

1. *Preparation and spin-labelling of nerve membranes* The nerves were isolated from frogs (Rana esculenta) and spin-labelled with N-(1-oxyl-2,2,6,6-tetramethylpiperidinyl)-maleimide as reported earlier [4].

2. *Spin-trapping* The preparation and the maintenance of the reagents in hydroxyl and superoxide free radical generating system were essentially the same as described by Floyd and Lewis (5), and Marklund (6). The samples were prepared in thermostat water bath at 37 °C and an aliquot was placed into the flat cell of Zeiss (GDR) and the spectrum recording was started within two minutes using an ERS 220 X-band spectrometer.

Results and discussion

1. *Characterization of the nerve membrane in the presence of MTDQ-DA.* The conventional and saturation transfer EPR spectra of the frog nerve indicated that the labels were attached almost exclusively to strongly immobilizing sites (apparent rotational correlation time was 62 ns).

MTDQ-DA induced the increase of the distance between the outermost hyperfine extrema evidencing the immobilizing effect of the drug upon the protein domain that holds the label. The distance 5.829 ± 0.023 mT changed rapidly with increasing concentration of MTDQ-DA, and attained to a constant level at about 50 mM concentration. Above 50 mM only insignificant variations in the hyperfine splitting were observed. In the case of fully immobilized labels the hyperfine splitting constant was calculated to be 6.013 ± 0.025 mT, therefore the change in the rotational correlation time after incubation with 50 mM MTDQ-DA corresponds to a 100% increase.

2. *The effect of MTDQ-DA on the hydroxyl free radical adduct of DMPO or PBN.* In hydroxyl free radical generating system the EPR spectrum at low concentrations of MTDQ-DA consists of a 1:2:2:1 quartet with hyperfine splitting constants of $a^N = a^H = 1.48$ mT, which was identified as the DMPO-OH radical adduct [7]. The presence of MTDQ-DA in the reaction mixture decreased the amount of DMPO-OH adduct demonstrating the hydroxyl free radical scavenger properties of MTDQ-DA. The free radical concentration of the DMPO-OH adduct at zero concentration of MTDQ-DA was about $10-20$ μM. Keeping the amount of DMPO and H_2O_2 constant in the reaction mixture, the relative intensity of the EPR spectrum decreased with increasing concentration of MTDQ-DA in a non-linear manner (Fig. 1). Similar results were obtained using PBN as spin-trapping agent. At higher concentrations of MTDQ-DA relative to DMPO or PBN, another free radical signal could also be observed in the spectrum (Fig. 2). The triplet showed that the diamagnetic compound was

Abbreviations:
MTDQ = 6.6'-methylene-bis-2.2.4-trimethyl-1.2-dihydroquinoline;
MTDQ-DA = 6,6'-methylene-bis-2,2,4-dimethyl-4-methane sulfonic acid sodium-1,2-dihydroquinoline;
DMPO = 5,5'-dimethyl-1-pyroline-N-oxide;
EPR = electron paramagnetic resonance
PBN = phenyltertiarybutylnitrone

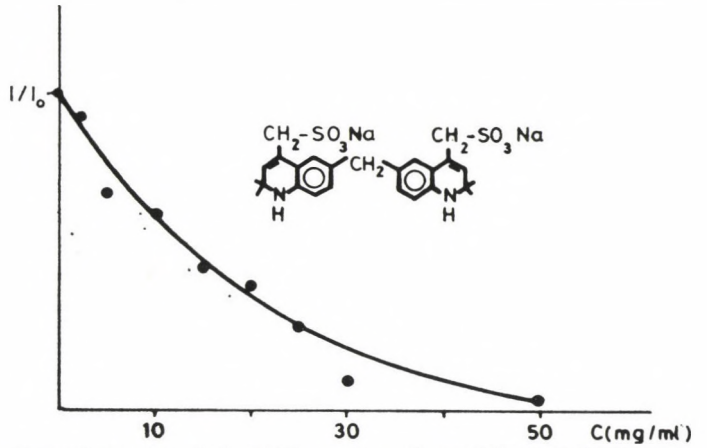

Fig. 1. The relative intensity of the EPR spectrum (second line) of DMPO-OH adduct vs. the concentration of MTDQ-DA in the reaction mixture. Spectra were recorded under identical conditions, only the concentration of MTDQ-DA was varied. I_0 is the intensity of the second line in the absence of MTDQ-DA. The inset shows the structural formula of MTDQ-DA. OH free radicals were generated in Fe^{2+}-ATP and H_2O_2 system

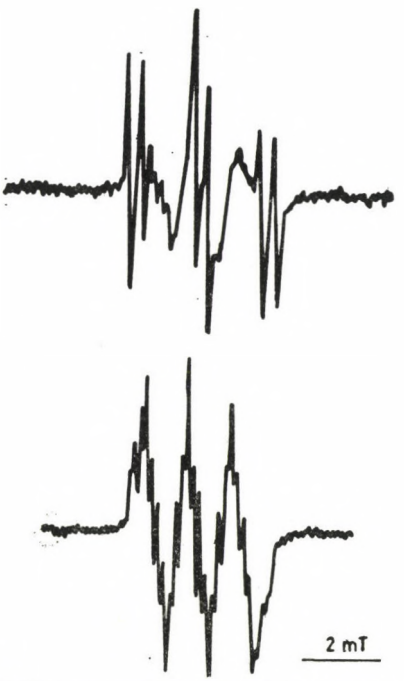

Fig. 2. EPR spectra of MTDQ-DA and PBM-OH adduct demonstrate the hydroxyl free radical scavenger property of MTDQ-DA. In the experiment the concentration of MTDQ-DA was 50 mM, whereas the final concentration of PBN was 15 mM in the sample. Bottom: EPR spectrum of MTDQ-DA in superoxide free radical generating system (100 mM MTDQ-DA, 15 mM KO_2 in 12 mM Na_2EDTA-Na_2CO_3 buffer, pH 10.2). The EPR spectra were taken within two minutes after the addition of the last component to the reaction mixture

converted into a free radical, the MTDQ free radical. Using the same reaction mixture and the sequence of the addition of the components without DMPO or PBN, a large EPR signal could be detected consisting of a triplet with a well-resolved superhyperfine structure. The free radical has the same structure as the one observed in superoxide free radical generating system.

It can be supposed that the amine group in MTDQ-DA was oxydized to a nitroxyl group [8], and the interaction of the unpaired electron with the ring protons gave rise to the observed pattern.

Conclusions

Under pathological conditions the increase in free radical activity represents an important factor in the injury of cells and membranes. Therefore, the idea of a therapy with artificial antioxidant agents seems to be a logical step to substitute for the imbalanced scavenger states [9].

According to our studies with EPR, we can conclude that

(i) MTDQ-DA decreases the mobility of the maleimide spin label attached to the thiol sites of the membrane proteins in the sciatic nerve prepared from frogs. At higher concentrations of the drug MTDQ-DA the immobilizing effect attained a saturated level.

(ii) The experimental results in hydroxyl free radical generating system proved that MTDQ-DA was a strong OH free radical scavenger, and the reaction rate constant of the formation of MTDQ-DA radicals was comparable with that of the DMPO-OH adduct.

(iii) Evidence was obtained in superoxide free radical generating system that MTDQ-DA participated in the free radical reactions. It can be assumed that the presence of MTDQ in the cell membranes may improve the protection of membrane components against free radical attacks.

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THE INFLUENCE OF PTH, CALCIUM GLUCONATE AND EDTA ON THE PAROTID SALIVA IN THE GOAT

M. D. Isac, A. Valverde, M. J. Lupiani, E. Martinez de Victoria, M. Mañas

Departamento de Fisiología. Facultad de Farmacia. Universidad de Granada. 18071 Granada (Spain)

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1. The role of exogenous parathyroid hormone (PTH) and stimulation or inhibition of endogenous hormone release, on the parotid gland of normal and thyroparathyroidectomized (t.x.p.t.x.) goats was studied.

2. The intravenous infusion of PTH and EDTA produced a transitory rise in saliva flow rate in intact animals. In t.x.p.t.x. goats the flow of saliva decreased transiently throughout the infusion.

3. The calcium levels in parotid saliva was unchanged throughout the infusion of PTH, EDTA, calcium gluconate both alone or with propranolol, in either intact or t.x.p.t.x. animals.

4. The parathyroid hormone infusion caused an increase in salivary phosphate concentration in both intact and operated goats. The effects of PTH upon the salivary flow and concentration of P are discussed.

Keywords: goat, PTH, calcium gluconate, EDTA, parotid gland

It is well known that the regulation of saliva secretion in mammals is eminently nervous, however in ruminants certain hormonal control seems possible. The reabsorption of phosphate by the renal tubules and the absorption of phosphate from the gut are known to be affected by PTH (parathyroid hormone) [1]. On the contrary ruminants usually excrete little phosphorus in their urine (3, 18, 24, 26, 29) and they secrete large amounts of phosphate by saliva into their gastrointestinal tract [10, 11, 16]. The phosphate plays an important role because microbial fermentation in the rumen has a high phosphorus requirement both for buffering and to satisfy microbial needs [6].

For some time phosphorus balance was thought to be regulated by intestinal absorption (8, 27); however some recent studies have led to the suggestion that the salivary glands may represent the major side of control (21, 22, 25) and

Correspondence should be addressed to
A. Valverde
Department of Physiology, Farmacological Faculty, University of Granada
18071 Granada, Spain

that the variation in faecal phosphorus excretion is achieved through an increase or decrease in the amount of phosphorus secreted into the gut via the saliva (22). Since the parotid glands secrete large quantities of phosphorus and since the secretion of phosphorus to the gut appears to play a major role in the regulation of phosphorus homeostasis in ruminants because these animals are more dependent on the digestive tract than on the kidneys as a pathway for phosphorus excretion [3, 23, 24], it seems plausible that PTH may exert an action on this secretion. The information presently available on the effects of PTH on parotid salivary secretion in ruminants is inconsistent. Some authors have found that PTH increase phosphate concentration in saliva [4, 23, 28] but others have not [14, 19]. The work described below was carried out in an attempt to resolve this discrepancy by investigating the effect that the administration of exogenous PTH or the endogenous release of this hormone exerts on various plasma and salivary parameters in normal and t.x.p.t.x. goats.

Materials and methods

Seven adult Granadinas goat weighing 35—40 kg were used in this study. During the experiments the animals were housed in cages and fed 1 kg per day a lucerne and broad bean diet, having free access to water and to a mineral salt lick. Any uneaten food was removed 3 hours before the experiment was begun.

Collection of saliva: Each animal was fitted with an unilateral parotid duct catheter according to the method described by Coats et al. (5). Saliva was led down a polyvinyl tube to a collecting bottle attached to a collar around the neck of the animal. It was usual to catheterize the jugular veins with a polyvinyl catheter, one for infusion and the others for the withdrawal of blood samples. The catheter was kept filled with 0.14 M sodium chloride containing 50 units of heparine per ml.

Thyroparathyroidectomy (t.x.p.t.x.): This operation was performed under halothane anaesthesia according to the method described by Care et al. [2]. Supplementation with 5 mg sodium L-tyrosine was given weekly by subcutaneus injection to establish an euthyroid state. It was necessary to remove both thyroid lobes because of the presence of an interior parathyroid gland in each lobe.

Intravenous PTH (Eli-Lilley) infusion: A priming dose of 100 USP of PTH was injected intravenously following the 30 min. control periods. This priming dose was followed by an intravenous infusion of PTH at a rate of 200 USP/h for 2 h.

Infusion of EDTA: After two 30 min. control periods, EDTA was infused at a rate of 6 g/h for 1 h. This test was only carried out obviously in normal goats.

Calcium infusion test: Calcium gluconate (15 mg Ca/Kg) was diluted in 216 ml of normal saline and infused at the of 1.8 ml/min. for 2 h.

Combined calcium and propranolol (Sumial, I.C.I. PHARMA) infusion test: After two 30 min. control periods calcium gluconate was infused by the same protocol as outlined above. At 1 h propranolol infusion (8.3 μg/Kg/min.) was superimposed so that the animal received calcium alone during the first hour and then calcium plus propranolol during the second hour.

In all the experiments, samples of saliva and blood were collected towards the end of each 30 min. period, until two h after the respective infusion was finished.

Analytical methods: Salivary flow rate was measured gravimetrically. Inorganic phosphate in saliva was measured by the method of Fiske-Subbarow [7]. Inorganic phosphate in plasma was measured using a phosphate test of F. Hoffman-La Roche and co. Ltd. (Switzerland). Total calcium in saliva and plasma was determined by atomic absorption spectrophotometry (Pye Unican SP 90 A).

Statistical procedures: Assays were expressed as means (SEM). Statistical significance was evaluated by Student's "t" test.

Results

PTH infusion: PTH infusion given to intact animals led to a significant transitory increase ($p < 0.01$) in the flow of saliva during the first half an hour of infusion, when the hormone was also administered via i.v. injection (100 USP). When given to t.x.p.t.x. animals however, PTH temporarily reduced the secretion of saliva. Calcium levels in parotid saliva were unchanged following infusion in both intact and operated animals. During infusion, a slight increase was noted in salivary phosphate levels in both intact and t.x.p.t.x. subjects (Fig. 1). No change was observed in plasma calcium or phosphate levels throughout the period of PTH infusion.

EDTA infusion: The infusion of EDTA in intact goats produced results similar to those recorded with PTH in terms of secretion rate and calcium concentration in parotid saliva. No change was noted in salivary phosphate levels following the administration of EDTA (Fig. 2).

Calcium gluconate infusion: Calcium gluconate, when given to both intact and t.x.p.t.x. animals, failed to modify parotid salivary secretion rates (Fig. 3). Joint administration of calcium gluconate plus propranolol to intact animals likewise had no effect on salivary flow rate (Fig. 4). Under the present experimental conditions no changes were seen in either salivary phosphate or calcium concentrations during calcium gluconate or propranolol administration. However the infusion of the former in intact animals was followed by a rise in salivary cation levels (Fig. 3), parallelled by a significant increase in calcemia ($p < 0.01$) (Table I).

Table I

Concentrations of calcium and inorganic phosphate in plasma from normal and t.x.p.t.x. goat before and during infusion of PTH ($n = 3$ $n = 3$), EDTA ($n = 11$), calcium gluconate ($n = 5$ $n = 8$) and calcium gluconate plus propranolol ($n = 4$)

		Normal		T.x.p.t.x.	
		Total calcium (mM/l)	Inorganic Phosphate (mM/l)	Total calcium (mM/l)	Inorganic Phosphate (mM/l)
Infusion of PTH	Basal	2.60±0.08	1.33±0.14	1.23±0.22	2.38±0.30
	During infusion	2.66±0.04	1.15±0.03	1.06±0.06	2.04±0.05
Infusion of EDTA	Basal	—	1.88±0.15	—	—
	During infusion	—	1.51±0.07	—	—
Infusion of calcium gluconate	Basal	2.49±0.12	2.42±0.17	1.81±0.08	1.45±0.22
	After 1h infusion	3.64±0.10	2.60±0.07	3.24±0.10	1.25±0.08
	After 2h infusion	4.01±0.06	2.60±0.10	3.38±0.09	1.27±0.08
Infusion of calcium gluconate plus propanolol	Basal	2.71±0.27	2.71±0.05	—	—
	Infusion of calcium gluconate	3.84±0.06	2.36±0.08	—	—
	Infusion of propanolol	4.32±0.06	2.25±0.05	—	—

(Mean values±S.E.M.)

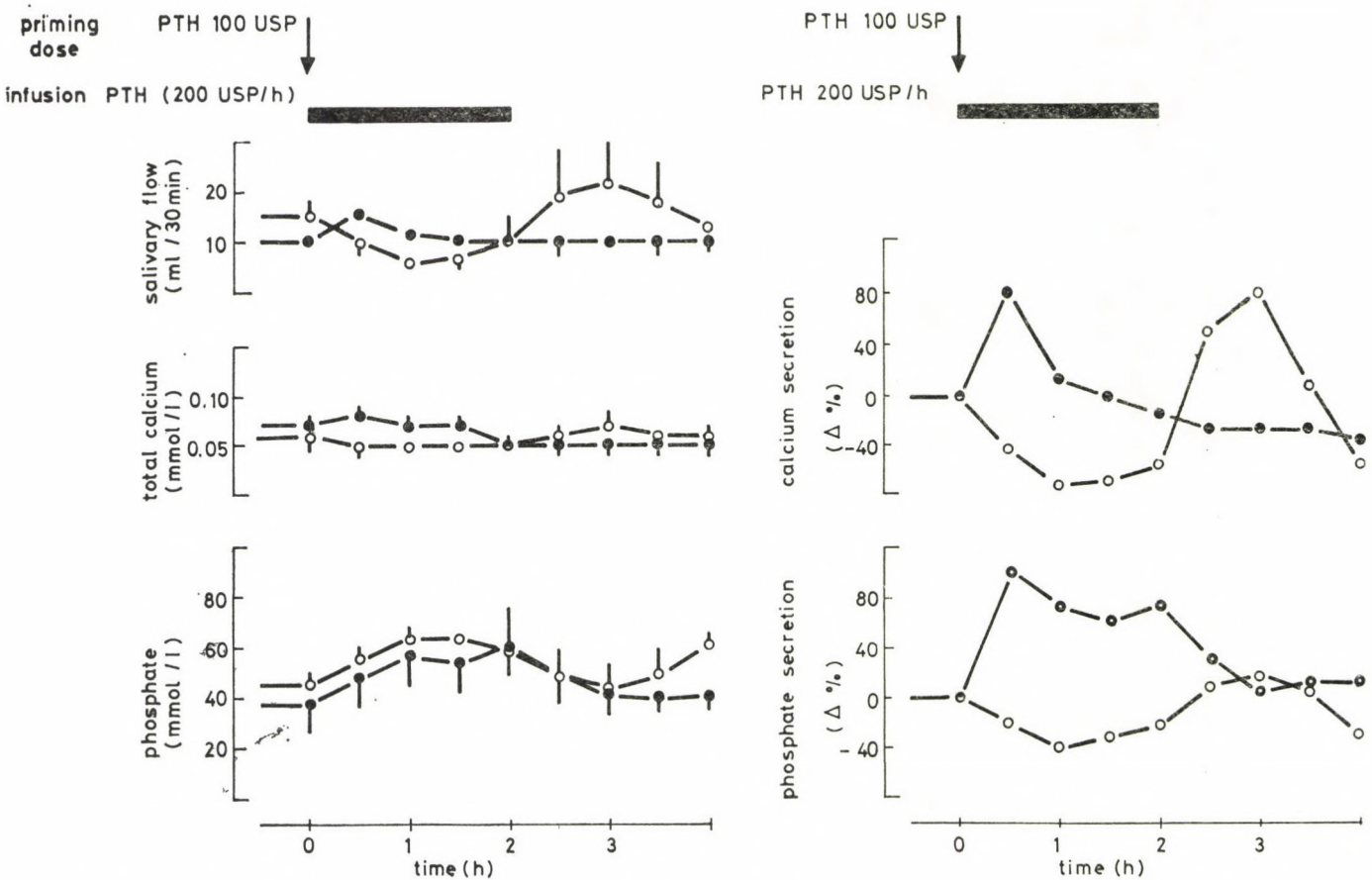

Fig. 1. Effects of PTH on the salivary flow rate and the concentrations of calcium and inorganic phosphate in normal (●——●) and t.x.p.t.x. (○——○) goat. The mean values are expressed ± S.E.M.

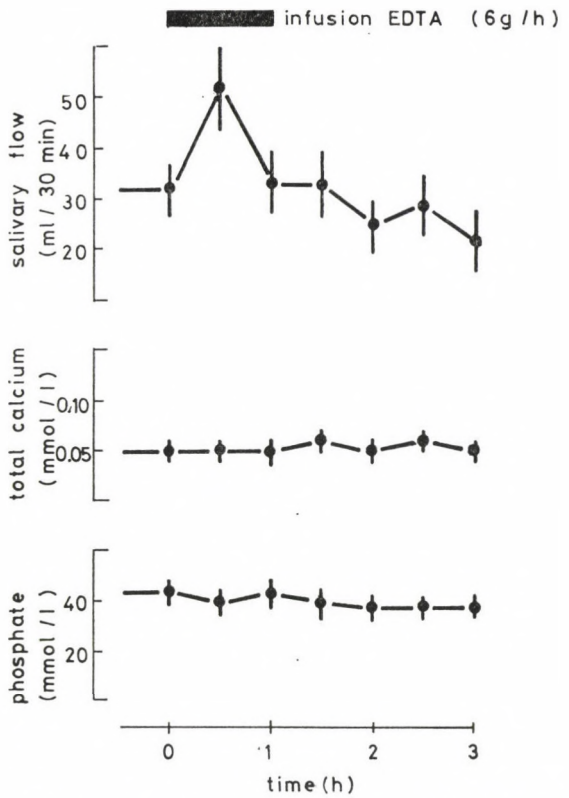

Fig. 2. Effects of EDTA on the salivary flow rate and the concentrations of calcium and inorganic phosphate. The mean values are expressed ± S.E.M.

Discussion

Salivary flow rate: As Figures 1 and 2 show, both exogenous (infused) and endogenous (released following the administration of EDTA) PTH produced a transitory rise in parotid salivary secretion in intact animals, similar to the effect described by Clark and al. [4] in conscious sheep. Paradoxically, the same dose of PTH in t.x.p.t.x. goats had the opposite effect of temporally reducing the flow of saliva. We can offer no clear explanation for this finding, although the drop seen in experimental animals may be a consequence of this parameter's characteristic variability in ruminants [17, 20], rather than a strictly hormonal effect. A further possibility is that PTH may increase blood flow to the parotid gland, as suggested by Wright et al. (29). Such an effect would go unnoticed in t.x.p.t.x. animals, in which lower plasma levels of PTH are reached.

In support of the above-mentioned concept, the administration of a calcemia enhancing dose of calcium gluconate (Table 1) which would theoretically

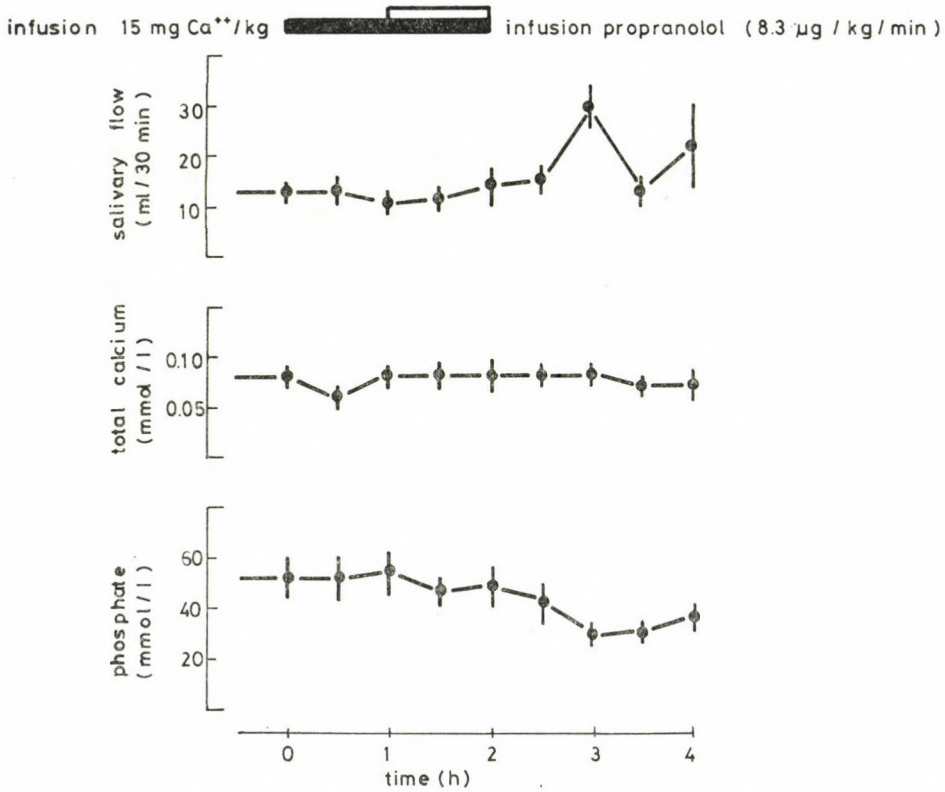

Fig. 3. Effects of calcium on the salivary flow rate and the concentrations of calcium and inorganic phosphate in normal (●———●) and t.x.p.t.x. (○———○) goat. The mean values are expressed ± S.E.M.

inhibit, at least partly, the release of endogenous PTH, produces no change in the rate of salivary flow (Fig. 3). Furthermore the joint administration of calcium gluconate plus propranolol (a beta-adrenergic blocker), which would be expected to drastically inhibit PTH release [12], also failed to influence salivary secretion.

These findings lead us to believe that PTH "per se", under normal conditions, has not effect on the flow of saliva. The rise in secretion recorded in the present study during PTH infusion or following EDTA-induced endogenous release are likely to be traceable to high circulating levels of the hormone.

Composition of the saliva secreted: In view of the importance of PTH in regulating the metabolism of phosphorus and calcium, the infusion of this hormone, or alternatively a change in plasma levels, might be expected to modify the mineral composition of parotid saliva, at least as regards calcium and phosphate content. Nevertheless PTH, EDTA, and calcium gluconate both

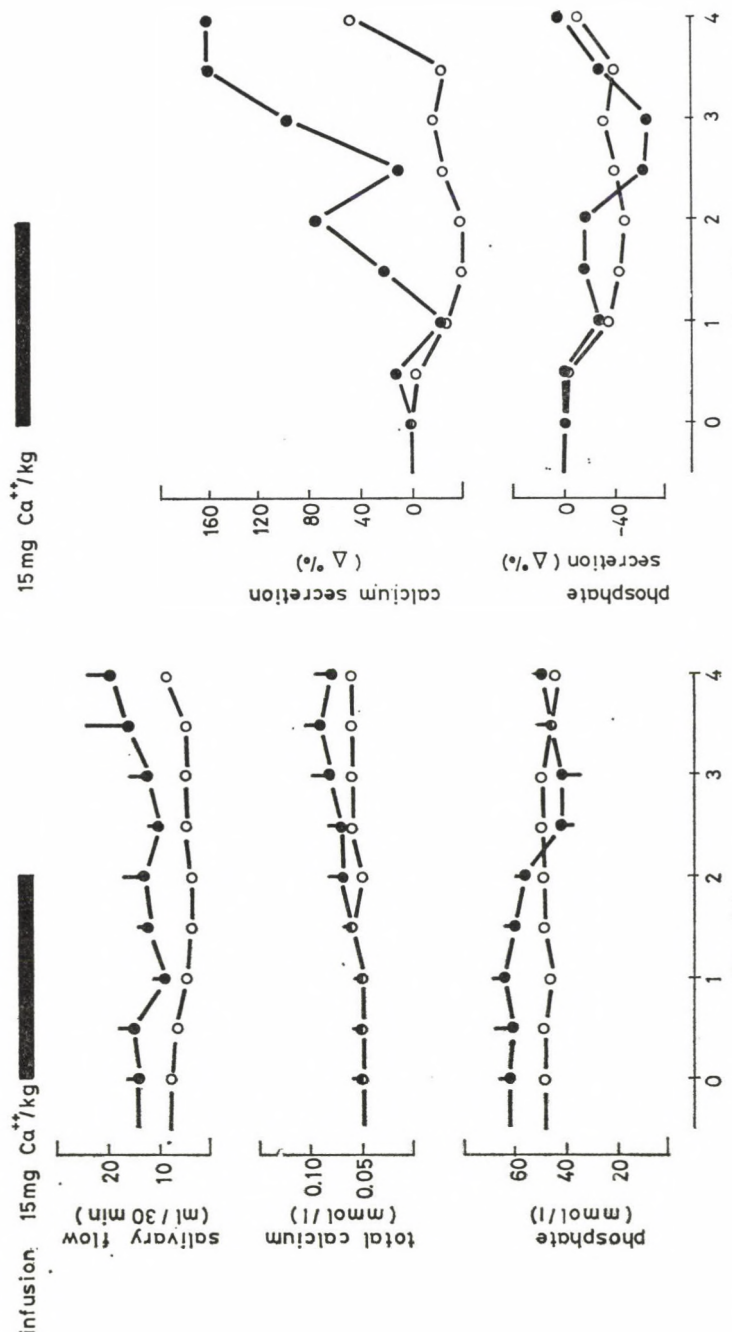

Fig. 4. Effects of calcium and propanolol on the salivary flow rate and the concentrations of calcium and inorganic phosphate in normal (●——●) and t.x.p.t.x. (○——○) goat. The mean values are expressed ± S.E.M.

alone and with propranolol all failed to influence calcium levels in saliva throughout the infusion in either intact or t.x.p.t.x. goats (Figs 1, 4). In contrast, after 1 h of calcium gluconate infusion a small but steady rise appeared in salivary calcium concentration (Fig. 3). This was considered a logical observation as the continuous infusion provoked a significant rise ($p < 0.01$) in calcemia (Table 1) which in turn may have led to an increase in ion filtration at the level of the acini, thus raising calcium levels in the saliva. This effect was not observed in animals lacking thyroid and parathyroid glands (Fig. 3), owing most likely to the fact that the calcium gluconate infusion led to lower levels of calcemia in such animals. Given that salivary calcium concentration is dependent on reabsorption processes within the ductal system (13), the smaller rise in plasma calcium levels in operated animals would be insufficient to cause a clear increase in salivary cation levels.

The noteworthy phenomena which should be mentioned with regard to salivary calcium secretion: 1) The changes noted in this parameters following the infusion of PTH in intact or t.x.p.t.x. goats followed the same pattern as salivary secretion rates, and 2) calcium gluconate led to salivary calcium levels which reflected the changes in concentration described above in both groups of animals.

Considering the relative insignificance of salivary calcium secretion in ruminants both in quantitative terms and in light of its role in digestion, it is not surprising that hardly any hormonal effect on cation movement through the various glandular components should have been detected (14, 23).

The rise in salivary phosphate concentration following the administration of PTH (Fig. 1) in t.x.p.t.x. animals, could be attributed to the concomitant hormone-induced fall in salivary flow, as previous studies (9) have described a negative correlation between salivary secretion and phosphate levels in the same. When salivary phosphate levels are expressed as the percentage of total saliva in the form of phosphate, the effects of PTH become obvious: phosphate levels rise in intact goats through the entire period of infusion returning to basal values when administration ceases. Hence exogenous PTH clearly influences salivary phosphate concentration, in agreement with previous findings in conscious (4) and anesthetized sheep (23, 28). The present results also support the hypothesis developed by Wright et al. (29) according to which the PTH-induced rise of salivary phosphate levels is thought to be a consequence of a higher degree of phosphate secretion by the acini, as under normal conditions the parotid ducts in the sheep gland are known not to reabsorb phosphates (29). Moreover the previously described effects on salivary flow are necessary conditions for the PTH-induced rise in salivary phosphate concentration.

The effects of PTH on salivary phosphate levels in normal animals were not seen in t.x.p.t.x. animals. Several different factors may serve to account for this finding: 1) Under the present experimental condition the t.x.p.t.x. animals

received supplementary thyroxine but not calcitonin, and as Clark [4] and Matsui et al. [15] have pointed out, the latter hormone may play a key role in phosphate secretion by the ruminant salivary gland. Alternatively, 2) as it was mentioned earlier with regard to the changing levels of salivary flow, it cannot be ruled out that the lack of PTH effects on salivary phosphate in t.x.p.t.x. animals might be due to excess levels of circulating PTH.

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CAERULEIN-INDUCED DESENSITIZATION OF ENZYME SECRETION FAILS IN NEONATAL RAT PANCREAS[1]

M. PAPP, G. VARGA, I. DOBRONYI

Institute of Experimental Medicine, Hungarian Academy of Sciences,
Budapest, Hungary

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Pancreatic segments of 1-, 3-, 5-, 10-day-old and adult female OFA (Sprague-Dowley strain) rats were superfused with graded concentrations of caerulein (10^{-12}—10^{-7} M) to establish concentration-response relation of amylase release. Furthermore, pancreatic segments of 3-, 5-, 10-day-old and adult rats were superfused with 10^{-10} or 10^{-8} M caerulein and then superfusion was repeated with 10^{-10} M concentration of caerulein to show whether the phenomenon of desensitization of amylase release can be induced in the postnatal period. The 1-day-old pancreas was found practically insensitive to caerulein. The 3- and 5-day-old gland was by one order of magnitude less sensitive ($ED_{max} = 10^{-8}$ M) than the adult pancreas ($ED_{max} = 10^{-9}$ M). Repeated superfusion of the 3- and 5-day-old pancreas with 10^{-10} M caerulein after the first 10^{-8} M caerulein superfusion failed to cause desensitization, while the same (10^{-10} M) repeated superfusion of the 10-day-old adult pancreatic segments after the first 10^{-8} M caerulein superfusion evoked desensitization of enzyme release.

The authors suggest that the failure of desensitization of enzyme secretion for caerulein may be due to the maturation process of newborn rat pancreatic acinar cells at receptorial and postreceptorial level.

Keywords: caerulein — desensitization — enzyme release — neonatal pancreas — rat

Incubation of intact pancreatic acini prepared from mouse, rat [12] or guinea pig pancreas [1, 2] with supramaximal concentration of carbachol [12] or CCK (cholecystokinin) and its analogues [1, 2, 12] results in a submaximal amylase release. Prior incubation of acini with supramaximal concentration of carbachol [12] or CCK-OP [1, 2] abolished the subsequent stimulation of amylase discharge induced by other secretagogues, the stimulatory action of which is mediated by mobilization of intracellular calcium (CCK, caerulein, carbachol e.t.c.). The phenomenon is termed as "desensitization", [12] or "restricted stimulation" [2].

Correspondence should be addressed to
Miklós PAPP
Institute of Experimental Medicine,
Hungarian Academy of Sciences,
H-1450 Budapest, P.O.Box 67, Szigony u. 43, Hungary

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In previous studies it was found that the newborn rat pancreas was less sensitive to caerulein *in vivo* [9] and *in vitro* [4, 8, 11] than the pancreas of adult rats. Starting from this point we examined whether desensitization of enzyme release can be induced in the newborn rat pancreas *in vitro* subsequent upon such a dose of caerulein which is supramaximal in adult rats.

Materials and methods

Studies were performed on isolated segments from 1—10 days old suckling rats and adult rats (180—220 g) [10]. The day of birth was called Day 1. Female OFA (Sprague-Dowley strain) rats were killed by a blow to the head and the pancreas was quickly removed and placed in a modified Krebs—Henseleit solution of the following composition: NaCl 103 mM, KCl 4.7 mM, $CaCl_2$ 2.56 mM, $MgCl_2$ 1.13 mM, $NaHCO_3$ 25 mM, NaH_2PO_4 1.15 mM, D-glucose 2.8 mM, Na pyruvate 4.9 mM, Na fumarate 2.7 mM, Na glutamate 4.9 mM, pH 7.4. The solution was maintained at 37 °C and gassed with 5% CO_2 in O_2.

The pancreas was cut into small segments (3—5 mg). A total weight of about 150 mg was placed in a tissue flow chamber of 1 ml capacity, and superfused at a steady rate of 1.4 ml/min with Krebs—Henseleit solution. The first 30 min effluent was discarded. Subsequently, 3-min fractions were examined.

The amylase concentration in the effluent fractions was measured by a spectrophotometric method [3]. The stimulus-evoked amylase release (i.e. the above basal amylase output) was routinely expressed in terms of the peak response in U/min/100 mg tissue. Caerulein (Takus^R, Batch TF/23326, Farmitalia, Carlo Erba, Milano) was added directly to the superfusion solution in known concentrations. Each concentration was given for 6 min.

In the first study pancreatic slices of 1-, 3-, 5-, and 10-day-old suckling and adult rats were used to construct *concentration-response curves for caerulein* in a dose range of 10^{-12}—10^{-7} M. Amylase release was expressed in percentage of the maximal peak response, except in the study with newborn rats younger than 24 hrs. In that case changes in peak amylase output were given in per cent of basal secretion prior to stimulation.

In the second study *caerulein-induced desensitization* was investigated. Pancreatic segments from 3-, 5-, and 10-day-old suckling rats and from adult rats were used. The slices were first superfused either with 10^{-10} or with 10^{-8} M caerulein for 6 min. Then the segments were washed with Krebs—Henseleit solution for 21 min. Thereafter, they were superfused by 10^{-10} M caerulein. Peak amylase response was measured after the first and the second stimulation period, and expressed in U/min/100 mg pancreas (mean$\pm$S.E.M.). Statistical differences were calculated, after analysis of variance using Dunnett contrasts [6].

Results

Concentration-response relation to caerulein

No maximum of amylase release was found for caerulein in newborn (1-day-old) rat pancreas. Therefore, at this age group, the peak amylase response was expressed in per cent of basal amylase release. No stimulation was observed at 10^{-12}—10^{-9} M caerulein. Peak amylase response increased dose-dependently for 10^{-8} M, 10^{-7} M, and 10^{-6} M concentrations of the peptide (9.7$\pm$5.9%, 15.9$\pm$11.5%, 33.4$\pm$8.1%, respectively) (Fig. 1). The maximum of amylase secretion was found at 10^{-8} M caerulein concentration at the postnatal 3rd and 5th days, while it was measured between 10^{-8} and 10^{-9} M concentrations in the 10-day-old rat pancreas. In adult rat pancreas the maximum of amylase release was observed at 10^{-9} M caerulein (Fig. 2).

Caerulein-induced desensitization

The first superfusion with either 10^{-10} M or 10^{-8} M caerulein induced a substantial increase in amylase output from pancreatic segments both in suckling and adult rats. The caerulein-induced amylase response was about five times higher in the adult pancreas than in the glands of 3—10 day-old suckling rats corresponding to the higher amylase content of the adult pancreas [4]. The

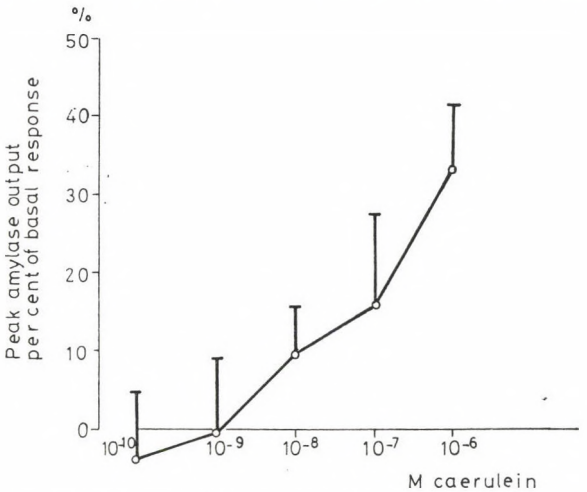

Fig. 1. Concentration-response curve (mean±S.E.M. of per cent of basal release) of amylase elease from pancreatic segments to graded concentrations of caerulein in newborn (1-day-old) rats (each point represents 5 experiments)

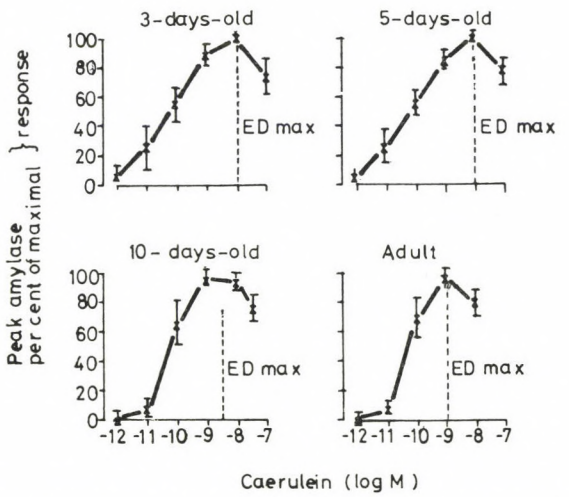

Fig. 2. Age-dete rmined dose-response curves (mean±S.E.M. of per cent of maximal response) of amylase relea se from pancreatic segments to graded concentrations of caerulein in suckling and adult rats (each point represents 4—6 experiments)

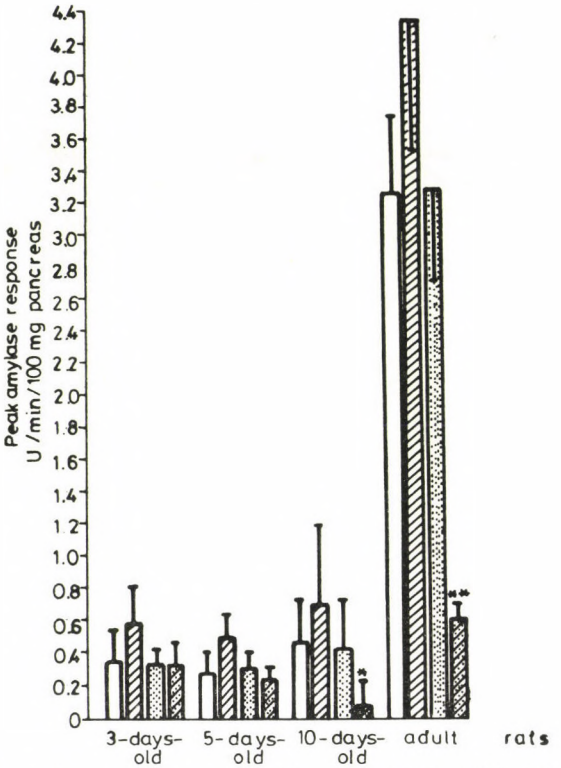

Fig. 3. Amylase release of pancreatic segments (mean±S.E.M. of U/min/100 mg pancreas) to the first superfusion with 10^{-10} (1) or with 10^{-8} (2) M caerulein and release to the repeated superfusion with 10^{-10} M caerulein after the first 10^{-10} (3) or 10^{-8} (4) M caerulein (each column represents 4—6 experiments). * $p < 0.05$ vs third column of the same age group (i.e. significant difference in amylase response for 10^{-10} M caerulein depending on the previous submaximal or supramaximal stimulation) (numbers in brackets mean those of columns)

second superfusion with the 10^{-10} caerulein concentration did not reduce the amylase release in any investigated age group if the pancreas was superfused prior by 10^{-10} M caerulein. In contrast, when the pancreas was first superfused by 10^{-8} M caerulein, the second superfusion with the 10^{-10} M dose reduced the amylase release (caused desensitization) in the pancreas of the 10-days-old and adult rats, while it did not cause desensitization in the pancreas of 3- and 5-day-old rats (Fig. 3).

Discussion

In the present study we have confirmed the earlier observations that the pancreas is insensitive to caerulein at age younger than 24 h [4, 11], and that the gland is less sensitive *in vivo* [9] and *in vitro* [4, 8, 11] to the peptide in the

early postnatal period than the pancreas of adult rats. After a previous supramaximal caerulein stimulation of enzyme release we elicited desensitization of enzyme discharge with subsequent submaximal stimulation of the adult rat pancreas with a CCK analogue as it was published earlier [1, 2].

The new point of our study is that pancreatic segments of 3- and 5-days-old suckling rats previously superfused with a dose of caerulein which is supramaximal in adults do not respond with desensitization — a decreased discharge of amylase — to the subsequent submaximal concentration of caerulein.

In adult rats combination of submaximal concentrations of agonist stimulants (e.g. carbachol+caerulein) acting through intraacinar Ca^{2+} mobilization has an additive effect on amylase release, combination of their supramaximal or maximal and submaximal concentrations has no additive effect on amylase discharge [12]. The previous application of a supramaximal caerulein concentration inhibits the effect of a subsequent agonist (carbachol) or the response to Ca^{2+} ionophore. Thus, the inhibition is due to a postreceptor phenomenon distal to the receptor [12].

In contrast to the adult pancreas, the neonatal pancreas at age younger than 24 hrs is practically insensitive to CCK-OP *in vitro* but gives secretory response to Ca^{2+} ionophore [7, 11]. The newborn pancreas at age older than 24 hrs is still less sensitive to the CCK analogue caerulein *in vivo* and *in vitro* than the adult rat pancreas [4, 8, 9, 11]. The amylase release and the specific binding of labelled CCK-OP to acinar cell receptors increase with age from 2-day-old pups to 14-day-old rats. The latter reaches a plateau between 3 and 14 postnatal days succeeded by a higher plateau in weanling and adult rats [11]. The responsiveness to cholinomimetics is also acquired by the newborn pancreas during a maturational process postnatally [5].

In the present study we observed that 10^{-8} M caerulein dose induced maximal amylase discharge in 3- and 5-days-old rat pancreas, while this caerulein concentration was supramaximal in 10-day-old or in adult rats. Consequently, this 10^{-8} M caerulein induced desensitization in the pancreas of rats older than 10 days, but not in the 3- and 5-day-old rat pancreas, showing that acinar cell desensitization is a direct consequence of supramaximal caerulein stimulation. We suggest that the failing desensitization in the early postnatal period may be due to the maturation process of acinar cells at receptorial and postreceptorial (e.g. maturation of intraacinar transduction events) [4] levels. The low sensitivity of the newborn pancreas to excessive stimuli might be regarded as a "self-defense" mechanism against an accidental nutritional supramaximal stimulus in the early postnatal period.

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LIPID LATERAL DIFFUSION MEASUREMENTS IN MODEL MEMBRANES*

T. PÁLI, L. I. HORVÁTH

Institute of Biophysics, Biological Research Center, Szeged, Hungary

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The rate of lipid lateral diffusion has been investigated by computer simulation of electron spin resonance (ESR) spectra of spin-labelled dimyristoyl phosphatidylcholine (DMPC) vesicles. An optimization method has been developed to fit the experimental spectra to the theoretical ones calculated from the modified Bloch-equations in order to determine frequencies of probe-probe collisions and the lipid lateral diffusion coefficients. The main results of this study are: (i) Due to the sensitivity of our method to the extent of the overlapping of hyperfine spectral lines it is possible to determine the spin exchange contribution to linebroadening. (ii) It is obvious from these computer analyses that over a wide range of temperatures well above the phase transition both static dipolar interaction and dynamic spin exchange make significant contributions to the linebroadening. (iii) Lipid lateral diffusion coefficient in DMPC bilayers at 36 °C was $(2.3 \pm 0.2) \times 10^{-11}$ $m^2 s^{-1}$.

Keywords: lipid lateral diffusion, spin-labelling, ESR

Due to its unique time scale, spin-label ESR spectroscopy has optimal sensitivity for studying lipid dynamics in model and biological membranes [4]. Bulk lipids and their spin-labelled analogues undergo rapid acyl chain isomerization and lateral diffusion in fluid bilayers [8]. Their motionally averaged ESR spectra can readily be resolved from the powder pattern of solvation lipids immobilized by integral membrane proteins [3]. Interaction of lipids with peripherial membrane proteins is more difficult to investigate because the short intramembrane polypeptide loops result in less effective acyl chain immobilization, particularly close to the methyl terminals [5]. Electrostatic adsorption of peripherial membrane proteins on membrane surfaces should, however, result in a reduced rate of lipid lateral diffusion. As a first step to prove this hypothesis we have generalized the Sackmann-Trauble algorithm [7] to include motionally averaged anisotropic lineshapes in order to study lipid lateral diffusion. Here we report our first results in conjunction with an optimization method of computer-assisted lineshape analyses of ESR spectra of spin-labelled DMPC vesicles.

Correspondence should be addressed to
L. I. HORVÁTH
Institute of Biophysics, Biological Research Center
H-6701 Szeged, POB 521, Hungary

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Materials and methods

Materials. 1,2-dimyristoyl-sn-glycero-3-phosphorylcholine (DMPC) was obtained from Fluka (Buchs, Switzerland). The lipid gave single spot by thin layer chromatography (solvent system: $C_6H_{14}/CH_3OH/33\%$ NH_3, 65/30/3, and molybdenium staining followed by sulfuric acid charring) and was used without further purification. C-16 positional isomer of spin-labelled stearic acid (16-SASL) was obtained from Aldrich Chemical Co. (Beerse, Belgium).

Preparation of lipid vesicles. Lipid and spin probe at different ratios were mixed in chloroform/methanol, evaporated to dryness by N_2 gas stream, and evacuated for several hours to remove solvent traces. Lipid vesicles were prepared from these dry lipid films by dispersion in 2 mM Hepes, 1 mM EDTA (pH 7.4) buffer solution at a final concentration of 10 mg/ml.

ESR spectroscopy. ESR spectra were recorded with a JEOL X-band ESR spectrometer (JES-PE-1X, Japan) using 100 kHz modulation technique. Typical instrumental setting: microwave power, 8 mW; scan range, 10 mT; modulation amplitude, 1.6 G_{p-p}; gain, 10^3. The ± 100 mV analog output of the spectrometer was digitized by a $12+1$ bit A/D converter and stored as 1 kword datafiles in a PDP 11/40 computer (Central Research Institute of Physics, Budapest) using softwares written by Dr. J. Czégé (this Institute). Spectra recorded at low label/lipid ratios were smoothed according to a polynomial algorithm; baseline drifts were corrected by adjusting vanishing first integrals and overlapping aqueous peaks were digitally subtracted using our ESR software package written in Fortran.

Theoretical lineshape analyses. At low label/lipid ratios (0.1—2 mol%) the observed linewidth is the sum of the intrinsic linewidth and the exchange effects of the unresolved proton and nitrogen hyperfine structures. Sachse et al. [6] explored several methods for the measurement of the translational diffusion based on different terms; molecular collision rates were determined from exchange narrowing of the unresolved proton hyperfine structure and from exchange/dipol broadening of the nitrogen hyperfine structure. The two contributions, spin exchange and dipole-dipole interaction, were separated by their different temperature dependences. In the range of intermediate label/lipid ratios (2—8 mol%) the contribution of the exchange averaged proton hyperfine structure to linewidth can be neglected [6]. Sackmann and Träuble [7] derived a lineshape formula from the steady state Bloch-equations [1]:

$$(T_{2,\mathrm{eff}}^{-1} + 3\nu_{\mathrm{ex}})u_m - \nu_{\mathrm{ex}} \Sigma u_{m'} + (\omega_m - \omega)v_m = 0 \tag{1}$$

$$(T_{2,\mathrm{eff}}^{-1} + 3\nu_{\mathrm{ex}})v_m - \nu_{\mathrm{ex}} \Sigma v_{m'} + (\omega_m - \omega)u_m = -\gamma H_1 M_m \tag{2}$$

where the subscripts m and m′ refer to the nitrogen nuclear quantum number ($m_I = +1, 0, -1$), u and v are the x and y components of the transverse magnetization, $T_{2,\mathrm{eff}}$ is the effective spin-spin relaxation time, and ν_{ex} is the spin exchange frequency. (Further notations: ω_m is the angular resonance frequency, γ is the giromagnetic ratio, H_1 is the microwave magnetic field, and M_m is the value of the steady-state magnetization in the direction of the applied magnetic field.) It should be noted that no explicit reference is made to dipole-dipole interaction in eqs (1) and (2); its line-broadening effect is absorbed in $T_{2,\mathrm{eff}}^{-1}$

$$T_{2,\mathrm{eff}}^{-1} = T_2^{-1} + T_{2,\mathrm{dd}}^{-1} \tag{3}$$

The imaginary part of the magnetization, $v = \Sigma v_m$, is proportional to the intensity of the ESR absorption.

Best fitting simulated lineshapes were adjusted by least square optimization; the variance σ defined as

$$\sigma = \Sigma(Y_{\mathrm{exp}} - Y_{\mathrm{sim}})^2 / \Sigma Y_{\mathrm{exp}}^2 \tag{4}$$

was minimized by fitting T_2 to lineshapes recorded at low label/lipid ratio and then varying $T_{2,\mathrm{dd}}$ and ν_{ex}. (Y_{exp} are Y_{sim} are the experimental and simulated spectra.)

Results and discussion

According to Eqs (1) and (2) the exchange contribution to the linewidth can be determined as

$$3\nu_{\mathrm{ex}}/(T_{2,\mathrm{eff}}^{-1} + 3\nu_{\mathrm{ex}}). \tag{5}$$

The fitting error (σ) between theoretical and experimental spectra was found to be strongly dependent on the exchange contribution even at constant total line-broadening ($T_{2,eff}^{-1} + 3\nu_{ex} = \text{const.}$). This was observed at magnetically nondilute spin-label mole fractions (2—8 mol%) as the nitroxide hyperfine structure began to merge into a broad singlet due to spin exhange (exhange narrowing). As illustrated in Fig. 1 a unique minimum point could be found for the relative contributions of ν_{ex} and $T_{2,dd}^{-1}$ to the observed line-broadening and lineshape changes. The spin-label collision frequency can be determined from ν_{ex}, found at the optimal fitting error, using the simple relation [7] $\nu_{coll} = 3\,\nu_{ex}$. Our least square optimization is, therefore, a rapid way to follow changes in the rate of lipid lateral diffusion with no need for collection a set of spectra as a function of temperature.

ESR spectra of 16-SASL in DMPC bilayers using various label/lipid mole fractions are shown in Fig. 2. These spectra were recorded at 36 °C in the liquid crystalline phase of DMPC and so motionally averaged 3-line spectra were observed. On increasing the label/lipid mole fraction a Lorentzian line-broadening was first detected (spectra A and B) and then the spectral effects of spin exchange were dominating as expected for intermediate label/lipid mole fractions (spectra C-E). The experimental spectra are shown together with best fitting simulated spectra in order to illustrate the quality of the simulations. Best fitting simulations for intermediate label/lipid mole fractions were obtained with approximately equal contributions from the spin exchange and the dipole-dipole terms to the line-broadening as the nitrogene hyperfine structure began to collapse into an exchange averaged lineshape. Monomolecular lipid lateral diffusion coefficient (D_{diff}) of $(2.3 \pm 0.2) \times 10^{-11}$ m^2 s^{-1} at 36 °C was calculated using a hexagonal lattice model of two dimensional diffusion from

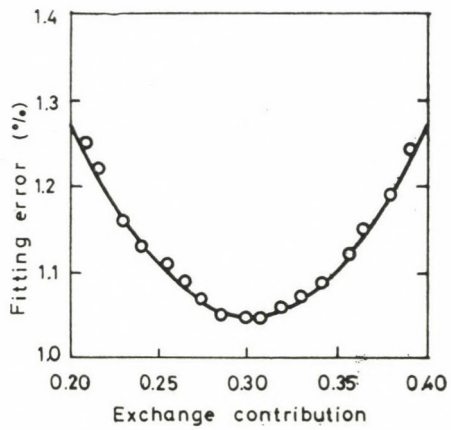

Fig. 1. Relative fitting error defined by Eqn. (4) as a function of spin exchange contribution defined by Eqn. (5). Spectral simulations of the experimental spectrum of Fig. 2D were done at constant total line broadening

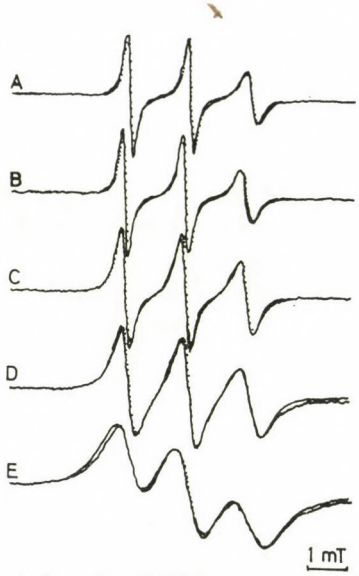

Fig. 2. Simulation of the spin-spin broadened ESR spectra of 16-SASL spin label in dimyristoyl phosphatidylglycerol (DMPC) bilayers at various label concentrations. Dotted lines are experimental spectra and full lines are the simulations according to Eqs. (1) and (2). Spectrum A is for 0.020 mole fraction and $\nu_{ex} = 4.8\ 10^6\ s^{-1}$; spectra B, C, D and E are for mole fractions of 0.038, 0.074, 0.107 and 0.137 and exchange rates of $8.0\ 10^6\ s^{-1}$, $14.0\ 10^6\ s^{-1}$, $22.7\ 10^6\ s^{-1}$, and $28.8\ 10^6 s^{-1}$, respectively

the dependence of label-label collision frequency on mole fraction of spin-label (C) with the Einstein-formula [2]:

$$D_{diff} = d^2\, \nu_{coll}/(12C), \tag{6}$$

where d is the lipid-lipid spacing in the hexagonal lattice, which was choosen to be 0.93 nm [9].

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QUANTITY OF DNA IN THE ORGANS OF ADULT SWISS MALE MICE AFTER AET AND MEA TREATMENT

Jadwiga MANOWSKA, Lidia MAZUR

Department of Animal Physiology, Institute of Zoology, Jagiellonian University, Cracow, Poland

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Adult Swiss male mice were injected intraperitoneally with 2-aminoethylisothiouronium bromide hydrobromide (AET) or cysteamine hydrochloride (MEA) in a dose of 400 mg/kg body weight. In the thirtieth, sixtieth or ninetieth minute after the injection, the animals were killed and the deoxyribonuclein acid content in 100 mg of fresh tissue of testes, spleen and liver, was measured. DNA was extracted from the organs by means of Burton's method, which is based on the estimation of deoxiribose content in the colour reaction with diphenylamine.

The injection of AET and MEA did not distinctly influence the DNA content in the organs of mice. Statistically significant differences among the groups of mice were not observed compared to the controls, in mice treated with the compound, a decreasing tendency in the quantity of the DNA in the organs was found only.

It is now well accepted that DNA is the most important biomolecular site regarding the effects of ionizing radiation in cells or in living organisms [25, 37, 41]. Thus major part of the literature is focused on two principal aspects: the estimation of radiation damage in physical and chemical ways [2—4, 7, 10—16, 18, 22, 23, 27, 30—32, 35, 39, 40] and the research of strong radioprotectors which are able to reduce the harmful effects of ionizing radiation [6, 9, 17, 19, 20, 24, 26, 28, 38]. The administration of a radioprotector to the animals leads to radiosensitive reactions connected with the radiation biochemistry of nucleic acids. The protectors somehow normalize DNA metabolism after irradiation [6, 17, 26].

It has been demonstrated that DNA synthesis was inhibited in tissues when rats were injected with an efficient radioprotector 5-hydroxytryptamine (5-HT) (36). For purposes of comparison the radioprotectors AET and MEA were used. The aim of the present study was to determine DNA content in the organs of adult mice after AET or MEA treatment.

Correspondence should be addressed to
Jadwiga MANOWSKA
Jagiellonian University, Institute of Zoology
Kraków, Mieczyslawa Karasia 6., Poland

Materials and methods

Experiments were carried out on adult Swiss male mice. All were kept under constant conditions, fed on standard granulated chow and given drinking water ad libitum.

The males were injected intraperitoneally with AET (2-aminoethylisothiouronium bromide hydrobromide, Sigma, USA), or MEA (cysteamine hydrochloride, International Enzymes Limited, England), in a dose of 400 mg/kg body weight, in 0.2 ml aqua pro injectione (Polfa, Poland). The control mice were treated intraperitoneally with 0.2 ml of aqua pro injectione alone.

The animals were killed by cervical dislocation in the thirtieth, sixtieth, or ninetieth minute after the injection. The body weight and the fresh weight of testes, spleen and liver, was measured, the deoxyribonucleic acid content in 100 mg of fresh tissue was determined.

DNA content in the organs of males was determined according to Burton's method [5], modified by Giles and Myers [8], Abraham et al. [1] and Setaro and Morley [33, 34]. This method is based on the estimation of deoxyribose content in the colour reaction with diphenylamine. Pure deoxyribonucleic acid from calf thymus (Department of Biochemistry, University of Lódz, Poland) was used as a standard. The determinations were made with a Zeiss Specol photocolorimeter at 595 nm.

Statistical evaluations were made by the Student's t-test.

Results

The results of the present study are given in Table I. Among the groups of mice, statistically significant differences in the values analysed were not observed.

Discussion

Administration of chemical radioprotectors into animal organism causes biochemical and structural alterations, and affects the cellular metabolism [21, 29].

The results of the present study indicate that after AET and MEA treatment of male mice with a radioprotective dose of 400 mg/kg body weight, a decreasing tendency in the quantity of DNA in the testes, spleen and liver exists. Compared with the controls, and among all the groups of mice, however, statistically significant differences in the values were not found.

After the injection of 5-HT alone, without irradiation, a short period of depressed DNA synthesis was observed. However, one hour later the rate of DNA synthesis was normal again [36].

Cellular toxicity and the decrease in DNA synthesis in mammals appears to be reponsible for the protective action. So the radioprotectors inhibit DNA synthesis without irradiation, and this effect is certainly connected with the protection of the recovery processes. Perhaps the delay in cell division and the prolongation of the cell cycle gives the cell more time to repair the radiation damage before next division [21, 36]. But the mechanism of both the toxic effect of sulphydryl compounds to DNA and their radioprotection of DNA is not well known [19, 24].

Table I

Body weight of males, weight of their organs and DNA content in 100 mg of fresh tissue in the particular groups of Swiss mice

Group	Time of killing after injection	Organ	Number of males	Weight of body (g) mean±SD	Weight of organs positive (mg) mean±SD	Weight of organs relative	DNA content (μg) in 100 mg of tissue mean±SD
control	30	T	9	22.50±2.59	154.22±13.15	0.0068	652.79±25.79
		S	9	22.67±2.41	105.61±15.44	0.0046	3075.87±259.62
		L	11	22.45±2.41	1184.30±167.50	0.0527	392.48±45.87
	60	T	7	23.93±2.74	161.57±9.45	0.0067	651.12±54.27
		S	10	24.40±3.48	118.80±27.11	0.0049	2956.42±191.27
		L	10	23.70±3.60	1278.71±212.97	0.0544	390.37±31.79
	90	T	8	24.00±3.24	153.06±25.41	0.0064	646.36±28.74
		S	9	24.22±3.95	120.89±14.75	0.0050	3005.68±198.39
		L	12	24.00±3.66	1270.65±220.07	0.0529	400.45±33.36
treated with: AET	30	T	9	22.78±2.26	160.22±15.67	0.0070	632.02±44.93
		S	11	22.59±2.09	106.14±23.86	0.0047	3058.11±241.16
		L	12	22.83±2.07	1292.00±152.70	0.0566	387.67±47.16
	60	T	10	24.40±2.14	156.85±9.75	0.0064	636.37±42.53
		S	10	24.45±2.15	114.25±10.72	0.0047	2953.57±221.09
		L	12	24.33±1.99	1349.57±224.80	0.0555	383.76±34.88
	90	T	9	23.67±2.38	155.39±9.64	0.0066	650.69±27.76
		S	13	23.61±2.48	117.27±21.53	0.0050	3045.48±221.47
		L	10	23.90±2.48	1326.23±172.60	0.0555	406.87±42.31
MEA	30	T	9	24.44±2.26	158.44±19.98	0.0065	635.48±21.35
		S	12	23.67±2.47	117.71±35.04	0.0050	3038.86±294.65
		L	13	23.58±2.39	1268.06±192.00	0.0538	387.79±31.69
	60	T	11	24.68±2.62	158.04±17.55	0.0064	637.50±37.44
		S	10	24.05±2.84	112.25±22.36	0.0047	2981.49±249.16
		L	12	24.37±2.71	1276.60±198.10	0.0524	396.27±41.42
	90	T	11	23.73±1.82	160.91±12.75	0.0068	642.10±41.29
		S	13	23.15±2.17	114.31±19.03	0.0049	3015.77±192.61
		L	14	23.21±2.10	1240.28±135.69	0.0534	395.68±27.89

T-testes, S-spleen, L-liver

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A PREVENTIVE APPROACH OF THE POSTMENOPAUSAL BONE LOSS

(REVIEW)

L. J. Benedek JASZMANN

Regional Protestant Hospital, Out-patient's Clinic for Climacterics, Bennekom, Netherlands

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In countries where osteoporosis in the ageing process tends to be a major problem, this phenomenon is of particular interest. The consequences of the osteoporosis in an ageing population, especially in the coming decades could create serious problems for the health authorities. It would be very interesting to see that in an early stage i.e. at menopause it could be influenced by prophylactic treatments

In a few countries the prophylactic approach of the menopausal osteoporosis has been already introduced. But it will take a long time before prophylaxis of postmenopausal osteoporosis could be considered as a standard and routine procedure.

Keywords: postmenopausal osteoporosis, prophylactic approach, estrogen-progestagen treatment.

Bone loss with ageing is largely a female problem resulting from a loss of gonadal function during the climacteric period. There is a rapid escalation of this process few years after the menopause. However, Albright [2] drew already our attention to the postmenopausal osteoporosis as long ago as 1941. It is only in the last decade that this growing problem attracted the proper interest of many investigators [6, 8, 9, 12, 13, 14]. As a gynaecologist my aim is to draw attention to the prevention of the postmenopausal osteoporosis.

We have to make difference between the osteoporosis after the menopause and the osteoporosis in ageing process. There is however, a third group, called "risk group" which is not related to age. Osteoporosis is characterised by decreased bone mass and resulting increased susceptibility to fractures. The process usually starts in women earlier than in men. The exact cause of the demineralisation of the bones is not yet clear but hormones especially the sex hormones play here a major role [5, 10].

Osteoporosis has two main aspects: a. material and b. non-material one. In the Netherlands we carried out an epidemiological survey of this problem [9]. The consequences of osteoporosis could be seen at the age of 65 in women and this phenomenon shows a significant increase in the last 10 years.

Correspondence should be addressed to
L. J. Benedek JASZMANN
Regional Protestant Hospital Out-patient's Clinic for Climacterics
6720 GA Bennekom, The Netherlands

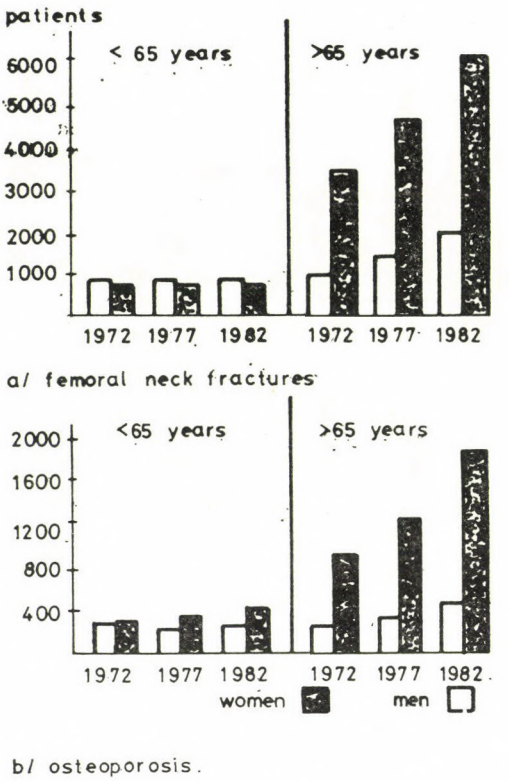

Fig. 1. a) Femoral neck fractures admitted in Dutch hospitals. b) diagnosis osteoporosis in Dutch hospitals according to the Medical Registration Institute (S.M.R.)

In 1984 there were 8100 femoral neck fractures admitted to the Dutch hospitals and 2200 fractures of the vertebral column. The medical costs of these two fractures came nearly to 200 million guilders; on revalidation and other medical costs the Health Authorities spent more than 250 million guilders. If this tendency will be continued in the following 25 years, the costs will be raised three-fold and the material aspects of osteoporosis will amount to more than 800 million guilders.

The non-material aspects in many cases cause no complaints. Very often, however, the patients are suffering from acute and chronic bone and muscle pain. A reflective muscle pain already occurs by small spontaneous fractures of the vertebral column. The age-related problems of osteoporosis are mostly symptomatic. 27% of the patients with fracture of the femoral neck will die in the first year of the complications. The average mortality around the 72nd year is otherwise still at 9 per cent. Only 30 per cent of these patients will, after a long revalidation reach their full health and activity which they had before the accident.

Especially in the countries where the ageing process is a serious problem to the general health, the osteoporosis and its risks require more attention, our interest is not only a theoretical one in the prevention of osteoporosis.

If we are convinced that osteoporosis is a major public health problem, as well it rises some questions. Firstly, what is the present attitude of medical profession and, secondly, what is the susceptibility of peri- and postmenopausal women to this problem. Table 1 shows [21] the primary care physicians' attitude in the U.S.A. according to Wallach.

Strategies have to be developed to change the negative attitudes of doctors well as a women. In the Netherlands 57 years was the average age in the first two decades of this century, and today the life expectancy of women has risen to 80 years. This "third period" of life which starts at menopause, needs more attention than before [4].

Table II shows the risk factors for osteoporosis and table III the candidates for osteoporosis.

In view of strategy for diagnosing of osteopenia/osteoporosis Table IV details the medical evaluation to be accomplished on osteoporosis candidates as they present for routine periodic medical care. It is anticipated that the baseline evaluation will be preceded by carefully collected medical history, physical examination and laboratory studies in order to exclude other pathology.

Table I

Primary care physicians' attitudes concerning osteoporosis (Wallach, 21)

1. 'Bad backs' are common and are almost always due to functional problems
2. It is normal for old people to lose skeletal mass, become kyphotic, lose height, and break bones.
3. It is difficult, expensive and unrewarding to diagnose and treat metabolic bone diseases.
4. The scientists take too much time trying to explain their area of expertise and are then obscure and indecisive in their recommendations.
5. No two experts agree on a practical approach to the problem and the advocated procedures and treatments must therefore have questionable effectiveness.
6. This subject is too complicated for primary care physicians who must treat scores of patients each day to survive.

Table II

Risk factors for osteopenia/osteoporosis

1. Postmenopausal status, regardless of age.
2. Advanced age (over age 60).
3. History of prolonged immobilization.
4. Medical conditions predisposing to osteopenia; hypogonadism, hyperthyroidism, hyperparathyroidism, Cushing's disease and syndrome, malabsorption, idiopathic hypercalciuria, chronic renal failure, chronic hepatic insufficiency, rheumatoid arthritis.
5. Lack of physical conditioning.
6. Dietary avoidance of daily products.
7. Alcohol abuse.
8. Corticosteroid or heparin therapy, regardless of dose.

Table III

Candidates for osteopenia/osteoporosis
(listed by Wallach, 21)

1. Postmenopausal women, regardless of age.
2. Men age 60 and above.
3. Patients with one or more additional risk factors (see Table II.).
4. Patients with a prolonged history of intermittent or chronic back pain and disability; a history of hip or Colles fractures.

Table IV

Evaluation schedule for osteopenia or postmenopausal osteoporosis and follow-up during treatment

1. Baseline evaluation
   a) carefully collected medical history;
   b) general physical examination including vertebral column
   c) laboratory tests: hormonal evaluation for menopausal status; thyroid function test; renal- and liver function tests; calcium and phosphor serum levels; albumin and globulin serum evaluation; serum lipoproteins; routine blood tests as sedimentation rate, hemoglobine etc. Urine tests: 24 hours excretion of calcium or fasting two hours morning urine for calcium- and hydroxyproline/creatinine ratio;
   d) for bones: X-ray examinations and measurement of bone mass with non-invasive techniques.
2. Annual follow-up evaluations
   a) a, b, c, as above annually or sooner if indicated
   b) annual measurements of non-invasive techniques.

Besides the physical examination we use laboratory tests and biochemical parameters in the detection of osteopenia/osteoporosis. We determine hormones as: FSH, LH, E_2, E_1 and androstenedione; we perform thyroid function test as T_4, T_3 uptake and FTI; biochemical tests: as serum creatinine and ureum, calcium and phosphor, albumin and globulin; liver tests: as gammaGT, alkaline phosphatase, ALAT, ASAT; serum lipoproteins: HDL, LDH total and fractions as well as HDL-cholesterol, tryglicerides. Tests for further routine blood tests: are sedimentation rate, haemoglobine, haematocrit, red- and white blood cell analysis, serum glucose. Tests for urine analysis are: albumin, reduction, sedimentation; as specific tests: fasting two hours urine for calcium/creatinine and hydroxyproline/creatinine ratio. Tests for bones are: X-ray examination of the vertebral column, especially for anatomical purposes.

To measure bone density: dual photon absorption method was applied. There are different kinds of methods, the so-called "non-invasive" techniques for the measurement of bone mass and bone loss.

a) The radiogrammetry is a simple measurement of the thickness of bone cortex as measured on X-ray film that has been exposed according to the prescribed standards; the classical approach has been to measure metacarpal or radial cortical thickness. However, this study can be done in any department of radiology, but it requires the use of a fine-grain film and a carefully standard-

ized tube-to-film distance in order to avoid problems with variable enlargement; the film should be examined with a 10-power loupe. This method does not give a picture of what is going on in the trabecular bones. It must be remembered, though, that trabecular bone loss may constitute the major part of bone loss, particularly in the years immediately after menopause. Also ordinary X-ray have been used for many years to make the diagnosis, but they are not very accurate in helping to recognize when an individual has less bone than it is expected for her age.

b) The single photon absorptiometry with the Norland-Cameron device belongs to the non-invasive techniques. It is more accurate than the radiogrammetry. In this technique the photon beam originates, usually from iodine^{125}. One can detect osteopenia (decreased bone mass), up to a large degree, it determines cortical bone, because using distal forearm measurements, it is possible to get about 20% input from the trabecular bone too.

c) In our research we use the dual photon approach, where single isotope, commonly Gadolinium^{153} is used, that emits at two different energy levels and two different gamma rays. This method has the advantage of a very radiation exposure and we can study the trabecular structures measuring L-1 and L-2. Trabecular bone appears to be highly responsive to changes at the time of menopause. The rate of loss of trabecular bone is several time higher than that of cortical bone, perhaps in the order of 8% per year, while overall bone loss is in order of 3% per year. Except the vertebral body we always measure both sides of the femoral neck, and the mineral content of the femur. Physicians as well as women must apply measures that retard or halt the progress of osteoporosis before irreversible structural defects occur. Todays attitude to postmenopausal osteoporosis must be changed and prophylactic measurements as well as follow-up must be accepted for many years. We are disposing only motivated women. There are three basic rules: a) control of diet; b) stimulation for doing exercises; c) estrogen-progestagen replacement therapy

ad a) all women attending our out-patient clinic have to consult the dietist for a food analysis. The necessary calcium intake must be 1400 mg daily with a ratio 1:1 to phosphorus. If the calcium intake is too low, the woman takes extra calcium with vitamine D_3. In cases of milk product intolerance and malabsorption, supplement is very important [17].

ad b) Inactivity leads to bone loss. There are recent studies suggesting that weight-bearing exercises reduce bone loss [17, 18]. Our advice is body exercise in women's clubs with a trained supervisor. In many cases physiotherapy proves to be successful especially in women who already have some vertebral problems.

ad c) Estrogen replacement therapy is highly effective for preventing osteoporosis in women. Estrogens reduce bone resorption and retard postmenopausal bone loss. In the literature one can find many case control studies with benefi-

cial effects showing a substantial reduction in hip and wrist fractures in women whose estrogen replacement therapy was begun within the first years of menopause [6, 7, 8]. Studies also suggest that estrogens reduce the rate of vertebral fractures. In a prospective study of 5 years Gambrell [12] showed the advantage of combined estrogen-progesterone treatment. In the epidemiologic part of his study he found an incidence of endometrial cancer in postmenopausal women of 158:100 000 women per year. The lowest incidence of endometrial cancer, 70·8:100 000 woman, was observed in the largest group, the estrogen-progestagen users. The highest incidence was found in women using estrogens alone (434:100 000). The difference in the incidences between the estrogen-progestagen users and those who use only estrogenes a statistically highly significant ($p < 0.0001$). The second highest incidence of endometrial adenocarcinoma was observed in the untreated group, an incidence of 242:100 000 women per year. The estrogen-progestogen users not only had a significantly lower incidence of endometrial cancer than those using estrogens only, but the incidence was also significantly lower than that of those who use no hormones ($p < 0.05$). Concerning the breast cancer in this study of 5563 postmenopausal women followed for 24.599 patient-years were found to have a breast malignancy, which means an overall incidence of 174:100 000 women per years. The expected incidence for this age group, according to the Third National Cancer Survey for ages 55 to 59, is 188:100 000 women, since the mean age in this study was 57.5 years. The difference in incidence between both the estrogen users and the estrogen-progestogen group was significantly lower ($p < 0.01$) than that observed in the untreated women; again an incidence of 95.6:100 000 in the estrogen-progestagen group versus 500:100 000 incidence in the untreated group. Gambrell [12] clearly showed the advantage of the combined estrogen-progestagen replacement therapy in the postmenopausal women. Also he paid attention to the beneficial effects of estrogen in postmenopausal osteoporosis, the influence on cardiovascular disease and the lipid metabolism. Still there are women at whom hormon substitution forms a contraindication of replacement therapy.

One problem of the cyclical estrogen-progestagen treatment has not yet been mentioned. A 55 year old postmenopausal women probably can not accept the consequences of regular or irregular monthly blood loos for another 6 to 8 years. The motivation and continuation of a hormon replacement therapy, notwithstanding the benefits, is losing its goal. The future research has to concentrate on hormon replacement therapy, without uterine blood loss. During the latest few years an estrogen-progestagen combination without causing blood loss was being developed which inducing an atrophic endometrium prevents not only the climacteric extragenital complaints, but also the postmenopausal bone loss. At the Fourth International Congress on the Menopause a paper was presented [3] on the "Long-term placebo controlled efficacy and safety study of Org. OD 14", now registrated in the U.K. as Tibolone^R tablets

of 2.5 mg [1]. Tibolone^R is a synthetic steroid; which has been shown in animals to have a combination of weak estrogenic, progestogenic and androgenic (anabole) activities. In patient trial no androgenic effect was found. Since lipoprotein levels have been directly related to the incidence of coronary heart diseases, as it has been shown in a 5 year study [11] OD 14 does not cause significant changes in lipoprotein profiles and may be beneficial regarding coronary heart disease risk. The beneficial effect on the postmenopausal bone loss also has been shown [15]. This compound showed a significantly better effect than placebo on hot flushes and sweting. A similar beneficial effect was seen on other extragenital symptoms listed by the Blatt Menopausal Index, albeit in lesser extent. In this respect Tibolone^R (Org OD 14) is a compound with considering as an alternative to conventional estrogen replacement therapy causing an atrophic endometrium, even after provocating the endometrium with lynestrol 5 mg as progestagen agent.

There is another new compound Kliogest^R which consist of 17-beta-estradiol, estriol and as progestagen norethisteronacetate [20] with the same effects as Tibolone^R.

Another combination with Premarin^R and as progestan, norethisteron registrated as Prempak^R [16] has been used for treatment of postmenopausal complaints; one succeded in to get amenorrhea and endometrial atrophy, but the investigators have to get more experience to confirm their findings.

Future hormone replacement therapy in the postmenopause requires a treatment with high estrogen in combination with a low progestagen effect to avoid osteoporosis with no growth effect upon the endometrium and with a favourable lipoproteine profile.

Research on this subject is still going on.

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ENDOCRINE FACTORS IN THE NEONATAL ADAPTATION

(REVIEW)

E. SULYOK

Country Children's Hospital, Pécs, Hungary

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In this review an attempt has been made to summarize our current knowledge on the involvement of various endocrine systems in the adaptation of the neonate to extrauterine life.

Clinical and experimental evidence has been provided to indicate the role of catecholamines, glucocorticoids, prostaglandins, vasopressin, endorphines and the renin-angiotensin-aldosterone system in the cardiovascular and respiratory adaptation.

Furthermore, the contribution of prolactin, vasopressin and the renin-angiotensin system to the redistribution of the body fluid compartments after birth and to the regulation of neonatal salt and water metabolism has been demonstrated.

It has been concluded that the endocrine reactions induced by birth process and perinatal pathological events are important regulators of successful transition.

Keywords: neonatal adaptation, endocrine system, catecholamines, glucocorticoids, prostaglandins, vasopressin, endorphines, renin-angiotensin-aldosterone system

Transition to extrauterine life is a complex process encompassing changes in the function of organ systems and reorganization of metabolic processes to achieve postnatal homeostasis. Simultaneously, pronounced endocrine reactions occur which are not only accompanying the process of adaptation, but have a definite regulatory role. The perinatal endocrine reactions, therefore, should be regarded as an important prerequisite of the adaptation to extrauterine life. When pathological events or therapeutic interventions do not allow to develop these reactions the success of adaptation is endangered and higher rate of perinatal morbidity and mortality can be anticipated.

Some of the factors influencing perinatal endocrine reaction are as follows:

1. Gestational age

With advancing gestational age increasing fetal hormone levels have been observed and the endocrine reactions to stress have been found to be attenuated in preterm when compared with full-term infants. The importance of gestational age is further substantiated by the observation that the endocrine responses in small-for-date and appropriate-for-date newborn infants with the same gestational age are quite similar.

Correspondence should be addressed to
Endre SULYOK
Country Children's Hospital
7601 Pécs, P.O.B. 76, Hungary

In this regard it is to be noted that comparing sympathoadrenal activity in preterm and full-term sheep fetuses Padbury et al. reported substantially greater increase in plasma catecholamines in preterm than in full-term fetuses, the catecholamine-related cardiovascular and metabolic responses, however, were markedly attenuated in preterm animals [54]. It is reasonable to assume, therefore, that in addition to the state of maturity, the responsiveness of the target tissues to hormones are also important determinants of fetal-neonatal endocrine reactions.

2. Stress of delivery

Cord plasma hormone levels are lower after elective caesarean section than after spontaneous delivery, in particular, after instrumental delivery and as the labour progresses significant elevation takes place in fetal hormone level [38].

3. Severity of stress imposed by perinatal asphyxia

The more severe hypoxic insult complicates the neonatal transition the more elevated hormone levels can be expected. Close correlations have been demonstrated between fetal hormone levels and Agpar score, the degree of acidosis and hypoxemia [44]. It is of importance, however, that not only the severity but the duration of asphyxia should also be considered. Prolonged asphyxia results in a gradually diminished endocrine response and depletion of hormone reserves, particularly in low birth-weight premature infants [3].

Nearly all endocrine systems studied are involved in the process of neonatal adaptation. This review, however, deals only with those which are believed to be of major clinical significance with respect to the successful transition.

Sympathoadrenal system

Clinical studies on the activity of sympathoadrenal system during the perinatal period proved to be of particular importance because the major function of this system to sustain homeostasis during perinatal stress. On the other hand, beta-mimetic tocolytic therapy is widely used in obstetric practice to prevent preterm delivery and catecholamine administration became also a powerful therapeutic measure in the neonatal intensive care.

The fetal sympathoadrenal system becomes increasingly activated during labour and delivery, in particular, during instrumental delivery and when it is complicated by perinatal asphyxia [44]. This activation may be of particular value for the neonatal transition immediately after birth for several reasons: 1) catecholamines stimulate cardiac performance through positive inotropic and chronotropic effects and redistribute blood flow towards the most vital organs that is placenta, brain, mycardium and adrenals [7]. — 2) They have been found to stimulate surfactant synthesis and release and to increase the absorption of

interstitial and alveolar fluid from the fetal lung. [47, 77]. — 3) Metabolic changes occurring after birth are also thought to be due to increased sympathoadrenal activity. Namely, glucogenolysis and lipolysis with increased blood glucose, free fatty acid and glycerol levels are assumed to be accounted for by a direct action of catecholamine surge, although suppressed insulin and enhanced glucagon secretion is also to be considered [30, 39]. The role of catecholamines as activators of non-shivering thermogenesis is also to be mentioned since they serve as the most important chemical mediators of metabolic heat production in the neonate [52]. — 4) Circulating catecholamines have been demonstrated to stimulate central nervous system function [23], to contribute to the arousal reaction of the neonate [23], to increase muscular tone [23], and to enhance the development of chemoreceptor activity [61].

The importance of the highly activated sympatoadrenal system in the immediate neonatal period is further substantiated by the observation that the failure of the neonate to activate this endocrine system was found to be associated with severe disturbances of the adaptation such as neonatal hypoglycaemia [69] and idiopathic apnoe of prematurity [40].

Dopamine, an endogenous catecholamine has been used increasingly in premature infants to treat low cardiac output and hypotension associated with hyaline membrane disease, septicaemia, perinatal asphyxia and persistent fetal circulation. Dopamine administration has also been suggested to prevent the hypotensive side effect of tolazoline given to reduce the increased pulmonary vascular resistance and to blunt the renal tubular side effects of indomethacin given to close patent ductus arteriosus [65, 66, 74].

The pharmacological effects of dopamine are dose-dependent. In low dose increased renal blood flow and improved renal function, "a dopaminergic effect" was observed and with increasing the dose myocardial performance improved ("betaadrenergic effect") and at an even higher dose "alpha-adrenergic effect" with increased peripheral resistance and reduced renal blood-flow could be seen [29].

Glucocorticoids

Much clinical and experimental evidence has accumulated to support the concept that glucocorticoids are effective in accelerating fetal lung maturation and preventing hyaline membrane disease. Exogenous steroids influence fetal lung development during the critical period of gestation and require delayed parturition for 48 hours for maximal therapeutic effectiveness [48].

Although risk associated with this type of prophylactic therapy seems to be low and possible long-term adverse side-effects remain hypothetical (immunsuppression, perinatal infection, compromised growth and development), it has been suggested that fetal adrenal function is affected by maternal glucocorti-

coid administration. With this suggestion in line decreased fetal plasma cortisol levels, delayed occurrence of the daily rhythm of fetal cortisol production and attenuated stress-reaction have been reported [26].

The incidence of clinically detectable patent ductus arteriosus complicating respiratory distress syndrome decreased significantly in low birthweight premature infants exposed to antenatal glucocorticoids [10]. This finding is consistent with the hypothesis that glucocorticoids appear to decrease the effectiveness of PG-mediated inhibition of ductus construction.

This influence of glucocorticoids may result from decreased synthesis of locally produced PG or diminished vascular PG sensitivity [76]. Glucocorticoids are known to inhibit PG synthesis by inhibiting arachidonic acid release from phospholipids [33]. On the other hand, prenatal dexamethasone treatment was found to enhance the ability of fetal lung homogenate to convert arachidonic acid to prostacyclin [74]. It seems likely, therefore, that not a decrease in vasodilatator PG production but rather the decrease in sensitivity of the ductus arteriosus to PG may account for the lower incidence of patent ductus arteriosus after prenatal exposure to glucocorticoids.

Prostaglandins

There is considerable experimental and clinical evidence to suggest a physiological and pathophysiological role for PG in the neonatal adaptation. Renewed interest for PG research in the perinatal period has emerged when it was observed that indomethacin, a PG synthesis inhibitor administered to pregnant women could be successful to prevent preterm delivery. In newborn infants born to mothers getting such treatment, however, severe disturbances of cardiopulmonary adaptation had been demonstrated. Moreover, it has been shown that indomethacin can induce complete and permanent closure of patent ductus arteriosus in premature infants providing an alternative treatment to surgical ligation.

Studies on the perinatal characteristics of PG metabolism have revealed that the fetal and neonatal tissues have high capacity to produce PG-s [50], there is a marked dominance of the dilatator PG-s in the fetal circulation [73], with advancing labour the maternal and fetal plasma PG levels increase progressively [79] and the reduced fetal pulmonary PG catabolism also accounts for the high plasma PG level [68].

The most intensive studies were performed on the involvement of PG-s in the cardiovascular and respiratory adaptation. It has been shown that the fetal patency of the ductus arteriosus is actively maintained by intramural PG-s and the oxygen-triggered constriction that occurs after birth is also mediated by PG-s. The decreased activity of vasodilatator PG-s both locally and in

the circulation and the postnatal increase of the vasoconstrictor PG synthesis may equally be responsible for the oxygen-induced ductal closure [9, 24]. In addition, a great body of evidence indicates that PG-s may contribute to the maintenance of low systemic and pulmonary vascular resistance and to the constriction of umbilical vessels [51]. PG-s are also thought to counterbalance the vascular effect of pressor hormones i.e. angiotensin II., vasopressin and catecholamines [35].

As to the role of PG-s in the respiratory adaptation it has been demonstrated that the fetal and neonatal lung produces vasodilatator PG-s, mainly prostacyclin upon ventilation and this process proved to be of prime importance in the reduction of pulmonary vascular resistance and in the respective increase in pulmonary blood flow seen after the first breath [47]. PG-s have also been claimed to enhance fetal lung maturation either acting directly on pulmonary surfactant production [11] or indirectly through increasing adrenal steroid synthesis [34]. Furthermore, with progressing labour fetal breathing movements were found to diminish or cease which has been attributed to the rise in fetal plasma PGE and to its direct action on the fetal medulla [7].

More recently, it has been suggested that pulmonary hypertension in the perinatal aspiration syndrome can be prevented by the selective inhibition of thromboxane production lending support to the view that the rise in pulmonary resistance in perinatal asphyxia may be mediated by thromboxane release [6].

The importance of PG-s in the cardiopulmonary adaptation is further stressed by the observations that maternal ingestion of drugs known to inhibit PG synthesis may cause intrauterine closure of ductus arteriosus, pulmonary hypertension and presistent fetal circulation syndrome. Our group was the first to recognize that the disturbed respiratory adaptation seen after maternal treatment with indomethacin and after elective caesarean section has a common pathomechanism, i.e. decreased PG production with subsequent persistent fetal circulation [13].

Prolactin

Highly elevated plasma prolactin (PRL) level has been demonstrated during the perinatal period. It has been found to rise with advancing gestational age (2) and the neonatal hyperprolactinaemia has been proved to be long-lasting and more pronounced in preterm than in full-term neonates [58]. The reason for the elevated plasma PRL levels during the late fetal and early neonatal period is multifactorial including high rate of PRL release due to increased sensitivity to TRH and estrogens [1, 42], the relative insensitivity of the pituitary to dopaminergic inhibition [42] and the low rate of PRL degradation by the immature kidney. It is well established that the renal PRL elimination

is proportional to the glomerular filtration rate [19] and in newborn infants the glomerular filtration rate is characteristically low.

The importance of increased estrogen stimulation is supported by the observations that in pathological conditions with impaired placental estrogen production (intrauterine growth retardation, maternal diabetes) the physiological increase in PRL secretion does not occur and significantly lower plasma PRL levels have been reported [28, 63].

The clinical significance of the perinatal hyperprolactinaemia is still ill-defined. It has been demonstrated, however, to participate in the acceleration of fetal lung maturation as shown by the depressed plasma PRL in newborn infants suffering from hyaline membrane disease [31].

Moreover, in a series of recent studies on tissue hydration of newborn rabbits *Goulter* provided pieces of evidence that the mobilization of neonatal tissue water stores is inversely proportional to the fluid intake and PRL is intimately involved in the regulation of this process. Namely, it has been observed that newborn rabbits treated with PRL or fluphenazine, a stimulant of endogenous PRL secretion retained more water while the bromocriptine-treated animals retained less water than did the untreated or saline-treated controls [12].

The involvement of PRL in the control of the volume and composition of body fluid compartments in human neonates has also been suggested by demonstrating significantly higher PRL levels in full-term newborn infants with idiopathic edema than in infants without clinically manifest edema [21.]. Furthermore, there was a clear association of sodium depletion due to renal salt wasting with high PRL level and the restoration of sodium balance with markedly reduced PRL level in low birth-weight premature infants indicating that PRL, as a salt-retaining hormone may contribute to the re-establishment of positive sodium balance [20].

PRL has also been shown to participate in the control of breast milk composition. Breast milk from mothers of preterm infants contained less PRL and more sodium than that from mothers of term infants [70] and significant positive correlation has been observed between breast milk PRL level and its total protein and lactalbumin concentration [32].

It is of interest that PRL has been reported to potentiate the ACTH-stimulated adrenal steroid secretion: aldosterone proved to be the most sensitive to PRL followed by corticosterone, androstendione and progesterone [18]. It is reasonable to assume, therefore, that the influence of PRL on fetal lung maturation and on neonatal salt- and water metabolism is the result of this indirect mechanism.

Vasopressin

Clinical studies and animal experiments have shown that the fetus and the neonate responds to specific (volume depletion, increased osmolality) and aspecific (delivery stress, hypoxia, acidosis, hypotension) stimuli with increased vasopressin release and variations in plasma vasopressin level are normally concerned with maintenance of water balance and plasma volume.

As a result of the aspecific stimulation plasma vasopressin is higher in infants born vaginally than in those delivered by elective cesarean section [55, 60] and it is further augmented when the adaptation is complicated by significant pathology [14]. Moreover, increasing number of patients presenting inappropriate antidiuretic hormone secretion with water retention and hyponatraemia has been reported among newborn infants admitted for neonatal intensive care [53].

Elevated plasma vasopressin in the immediate neonatal period may be of importance in the successful perinatal transition. Increased pressor responsiveness to exogenous vasopressin has been observed in undistressed fetal lamb [62] and vasopressin has been suggested to contribute to the maintenance of arterial blood pressure and to the redistribution of cardiac output toward myocardial and cerebral circulation in lamb fetus subjected to hypoxemia and haemorrhage [36, 41, 62]. Further studies have indicated that vasopressin in utero may have a role to increase placento-fetal sodium and transepidermal water-transport [49, 56] to promote lung liquid reabsorption [57] and to mediate the classical clinical signs of fetal distress i.e. bradycardia and meconium passage into the amniotic sac [25].

Renin-angiotensin-aldosterone system

It is well established that the activity of the renin-angiotensin-aldosterone system (RAAS) is highly elevated in the neonatal period. The reasons for the increased activity, however, are not quite clear. Perinatal stress imposed by labour and delivery, low systemic blood preassure, reduced renal perfusion, negative salt balance, end-organ unresponsiveness to angiotensin II and aldosterone as well as the low metabolic clearance rate of renin, angiotensin and aldosterone had all been implicated as possible factors [4, 16, 24].

The role of adrenergic nervous system and increased PG production in renin release during birth has also been suggested [43, 71].

The physiological and clinical significance of the increased activity of RAAS is not completely understood. However, a dependence of blood pressure on RAAS activity has been demonstrated and the correlation between blood pressure and plasma angiotensin II levels has been reported in newborn infants [4, 67]. Administration of Captopril, an angiotensin converting enzyme

inhibitor during pregnancy resulted in a markedly increased perinatal morbidity and mortality due to the reduction in placental uterine blood flow, systemic hypotension and severe oliguria/anuria. [5, 59].

Under physiological conditions cardiac output and its distribution is not influenced by RAAS. After hypoxia and hemorrhage, however, the increased activity of RAAS mediates the rise in blood pressure, heart rate, stroke volume and the overall cardiac output. At the same time it contributes to the profound alterations in organ blood flow by increasing blood flow to the brain, myocardium and adrenals at the expense of placental, umbilical, gastrointestinal and renal circulation. There is also a rise in pulmonary blood flow, presumably as a result of angiotensin induced pulmonary PG E production [37].

In addition to its role in the regulation of cardiovascular adaptation, RAAS proved to be an important regulator of neonatal electrolyte homeostasis. Our group has demonstrated that in low birth-weight premature infants the renal salt wasting, the subsequent negative sodium balance and the fall in plasma sodium concentration results in an extreme elevation of the activity of RAAS. The highly activated RAAS enhances distal tubular sodium reabsorption and contributes to the spontaneous correction of sodium depletion and the so-called late hyponatraemia [72].

Endorphins

Recent studies suggest that endorphins also participate in the adaptation of the newborn to the extrauterine life. Plasma and cerebrospinal fluid beta-endorphin level is elevated in the perinatal period [22, 78] and it was found to be further augmented when pathological events such as perinatal asphyxia, idiopathic apnoe of prematurity, severe hyaline membrane disease, hypoxia, ischaemic encephalopathy and shock supervened [45, 64].

The more severe the hypoxic depression of the central nervous system is the more pronounced increase in beta-endorphin level can be anticipated [45]. It has been assumed that the decreased respiratory response to hypxemic stress is mediated, at least in part, by endorphin accumulation. With this suggestion in line the hypoxemia-induced respiratory depression of the neoanate could be attenuated by the administration of naloxone, an opiate antagonist [8, 15].

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TREATMENTS FOR INDUCING SUPEROVULATION AND THE ENDOCRINE ASPECTS OF CYCLE DIAGNOSTICS IN CONNECTION WITH THE IN VITRO FERTILIZATION PROGRAM

(REVIEW)

Z. BOGNÁR

Department of Obstetrics and Gynecology, University Medical School, Pécs, Hungary

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On the basis of literary data and of own experience the author deals with protocols concerning hyperstimulation in the in vitro fertilization and embryonic transfer cases as well as with the endocrine monitoring of superovulatory cycles. The IVF and ET programs widen our knowledge most spectacularly in the fields of follicle maturation, cycle endocrinology, and early processes of reproduction. When discussing the theoretical and practical aspects of various superovulatory treatments, the principal differences between the natural and hyperstimulatory sexual cycles are emphasized.

Because of these differences and of the nature of the IVF-ET procedure, the importance of the traditional cycle diagnostics is necessarily limited. More important diagnostic possibilities are the daily quick hormone measurements by RIA methods, the hemagglutination tests, ELISA, and ultrasound examinations.

Keywords: in vitro fertilization, natural and superovulatory cycles, cycle monitoring, stimulation protocols.

The commencement of in vitro fertilization and embryo replacement programs resulted in a broadening of knowledge and higher accuracy in many fields of medicine. To illustrate this, the new data can be mentioned which refer to the earliest phase of the reproductive process, the early metabolic characteristics of embryonic development (pyruvate, pure albumin utilization) or to the early detectable alteration in the immune status that develops in response to the presence of the embryo already before its implantation [4, 16].

The freezing of the human embryo has brought about new knowledge on the cryoprotective substances and cryobiology (17). The experience obtained with sperm handling and with the incubation media have highly improved the results of homologous or heterologous artificial inseminations. The daily ultrasound folliculometry was a help in elaborating more refined ultrasound

Correspondence should be addressed to
Zoltán BOGNÁR
Department of Obstetrics and Gynecology,
University Medical School
H-7624 Pécs, Édesanyák u. 17, P.O.B. 89, Hungary

List of abbreviations

LH — Luteinising Hormone
FSH — Follicle Stimulating Hormone
GnRH — Gonadotropin Releasing Hormone
HCG — Human Chrionic Gonadotropin
GH — Gonadotropic Hormone
E_2 — Estradiol-17 beta
HMG — Human Menopausal Gonadotropin
SP1 — Pregnancy specific beta-1-glycoprotein
EPF — Early pregnancy factor
RIA — Radioimmunoassay
ELISA — Enzym linked immunosorbent assay
PAPPA — Pregnancy associated plasma protein A
cAMP — cyclic Adenosine Mono Phosphate
IgG — Immune globulin G
IgA — Immune globulin A
IVF — In vitro fertilization
ET — Embryo transfer

techniques. Among the most important fields of this development are still the cycle diagnostics and early gestational endocrinology and immunology mainly because by these the early reproductive processes have become practically in different moments, they could be photographed, filmed and exactly determined in time.

Aim process and methods of superovulation treatment

For natural conception or artificial insemination in cases of induced ovulation, the goal is to obtain minimally one egg (but not too many of them) and to reach ovulation. For in vitro fertilization the goal is to obtain more antral follicle in order to have three or possibly more preovulatory follicles at the time of harvesting of the eggs.

When dealing with natural or superovulatory cycles, the exact determination of the maturity of the follicle and the ovum is of utmost importance. The maturation process, prior to harvesting the ovum, can be examined only indirectly, by its endocrinological characteristics. The most important indices of the maturation process are the following:

growth of the ovum
thickening of the granulosa layer
formation of a theca cell coat
rise in the aromatase activity of granulosa cells
increase in enzyme biosyntheses in the granulosa cells
including the capacity to produce progesterone
liquification of the granulosa mass
clearing up of the antrum
development of FSH-induced LH-receptivity [7, 8, 12, 13, 14, 27].

In the natural cycle, preantral follicles are continuously produced from primary follicles during the luteal phase but, in this case, no further development takes place because of the low levels of GH (18, 28). Many follicles start developing,

hovewer, due to the increasing levels of GH (particularly FSH) in the course of luteal regression and during menstruation. The follicle being in optimal circumstances obtains a developmental advantage over the others which, in turn, atresize [3, 11, 12]. This is because the aromatase activity and estrogen production of the dominant follicle is high enough to reduce the FSH-level, and the low FSH-level, being insufficient to the less developed follicles, makes them atresize. This early follicular atresia is over by about the 5th to 6th day of the cycle. In contrast, the dominant follicle continues the maturation process described above, it becomes suitable for ovulation and luteinization. The aim of the various superovulation treatment protocols is to overcome this natural selection and by this to secure that more follicles reach the preovulatory stage. For this, there are several theoretical possibilities:

1. Preventing the decrease of the endogenous FSH-level consequent to the negative feedback effect of the estrogen

— antibodies to estrogen
— competitive antagonism
— GnRH treatment (in pulses)

2. Substituting the decreased andogenous FSH by an exogenous one

— HHG treatment
— HMG treatment
— treatment with purified FSH

3. Combination of the two methods in order to utilize the benefits of both.

Having already abandoned the manipulations with the natural cycle, the superovulation treatment protocols used nowadays in the in vitro fertilization practice, are as follows:

— Clomiphen alone (Wood-Trounson) [15, 24]
— Clomiphen plus HMG (Wood-Trounson) [25]
— HMG alone [9, 22]
— FSH alone
— GnRH (on rare occasions)

All these are accompanied by a spontaneous LH surge or they are more frequently combined with HCG-termination (i.e. induced ovulation by giving HCG). HCG induced ovulation appears to be more popular, because onset of spontaneous LH-surge is rather unpredictable and on the other hand spontaneous LH-surge may be frequently accompanied with late follicular atresia [26].

By the daily monitoring and the corresponding treatment one has to be careful to observe the following points in the course of superovulation cycles:

— Not too many follicles should start developing
— No atresia should develop (often cystic forms)
— Follicles should not be lost during late atresia
— Appropriate timing of the follicle puncture

To fullfill all these requirements and to predict the time of the expected ovulation the main role is played by the daily or even more frequent determinations of E_2 and LH levels as well as by ultrasound examinations. The most common methods used in cycle monitoring are shown in Table I. Unfortunately, the more simple conventional methods are not informative enough, they have smaller predictive value [1].

Main differences between natural and superovulatory cycles

There are basic differences in the characteristic endocrinological and morphological features of the natural and the superovulatory cycles and these differences must be taken into account in the course of monitoring the maturation of the follicles. Apart from these the superovulatory cycles also exhibit individual characteristics depending on the various protocols applied to evoke

Table I

Methods for detecting ovulation and following egg maturation

Basal body temperature measurement (Van de Velde 1904)
Cervix score (Insler)
grade of congestion (hyperaemia) in cervix
softness of cervix
opening of the cervical os
Characteristics of cervical mucus dependent on E_2
quantity↑, purity, (clarity)↑ viscosity↓, spinnbarkeit↑, presence of cellular elements↓, arborisation↑, enzyme changes (lysozyme↓, peroxidase↑, amylase↑)
protein content changes (falls in albumin, globulin, IgG, IgA)
Vaginal smear
Detection of cycle dependent changes in the temperature of certain skin areas
E_2 measurement: in serum (rapid by RIA, daily)
in urine (chemical method)
Luteal hormone: in serum (rapid RIA)
in urine (rapid haemagglutination test or ELISA)
Ultrasound: number and diameter of follicles
detection of ovulation
Laparoscopy: size, clarity and vascularization of follicles (not for diagnostic purposes)
Charateristics of follicle fluid: quantity↑, purity↑
prostaglandin content↑
E_2/androstendione↑
E_2/progesterone↓
transferrin↑
α_1-antitrypsine
PAPP-A↑
cAMP↓
Cumulus corona radiata complex microscopic study (CCCs)
size of granulosa cells
expanded state of granulosa cells
dispersity of granulosa cells
spinnbarkeit of cumulus mass
maturity of corona radiata
Microscopic examination of the egg — effective maturity
breakdown of germinal vesicle
presence or protrusion of first polar body

them [5]. Some characteristic differences are shown in Table II., Fig. 2. and 3. Besides the general characteristics the time course of each variable and their relationship to each other are also important from the point of view of practical work. Timing is difficult because a planned follicule puncture time must be decided 50—72 hours beforehand. Simultaneously with this decision HMG treatment must be stopped from that on it is necessary to wait with ovulation induction until the proper development of LH-receptivity of the granulosa tissue. On the other hand, a longer waiting increases the danger of late atresia or, alternatively, an early LH-surge may occur.

Table II

Some differences between superovulation and natural cycles

Induced ovulation or superovulation	Natural cycle
Usually *several follicles* develop	Usually *1. follicle* develops
The follicular development rate (1,6 mm/day) is different for each follicle	The follicular development rate is *linear* until —5 to 0 days (1.2)
The preovulatory follicle is slightly smaller (1—2 mm), the size of the mature follicle varies in a wide range (16—30 mm)	The increase in E_2 level is *linear* until the days —5 to 0
Preovulatory atresia is relatively frequent	Follicle maturation and growth are parallel
The E_2 level is proportional to the total volume of follicles but it is not to their diameter	The size of the mature follicle is in a small range (20—24)
The E_2 peak levels are extremely high, the rate of increase is exponential on days —5 to 0	Ultrasound alone can be informative
The diagnostic value of ultrasound is moderate, not sufficing in itself	Ultrasound and the parallel measurement of E_2 and LH are satisfactory for monitoring
Ultrasound and the measurements of E_2 and LH levels are minimally required for monitoring	The absolute values and dynamics of sexual steroids and gonadotropic hormones are different from those in superovulatory cycles

Difficulties concerning monitoring of superovulatory cycles

In the ideal case, on the day after HCG administration the E_2 values still increase or level out and the HCG application does not coincide with the early phase of the spontaneous LH-surge (6). Otherwise, the final maturation process might become unpredictable, thereby the harvesting would be less successful than in any other situation.

In the practice this difficulty is tried to be overtaken by two ways:

1. Solely on the basis of E_2 levels and folliculometry some colleagues induce ovulation by HCG according to the observation that spontaneous LH-surge rarely follows HMG-stimulation and the developing follicles are "relatively less mature". Some even discard the results of ulrasound examinations [20].

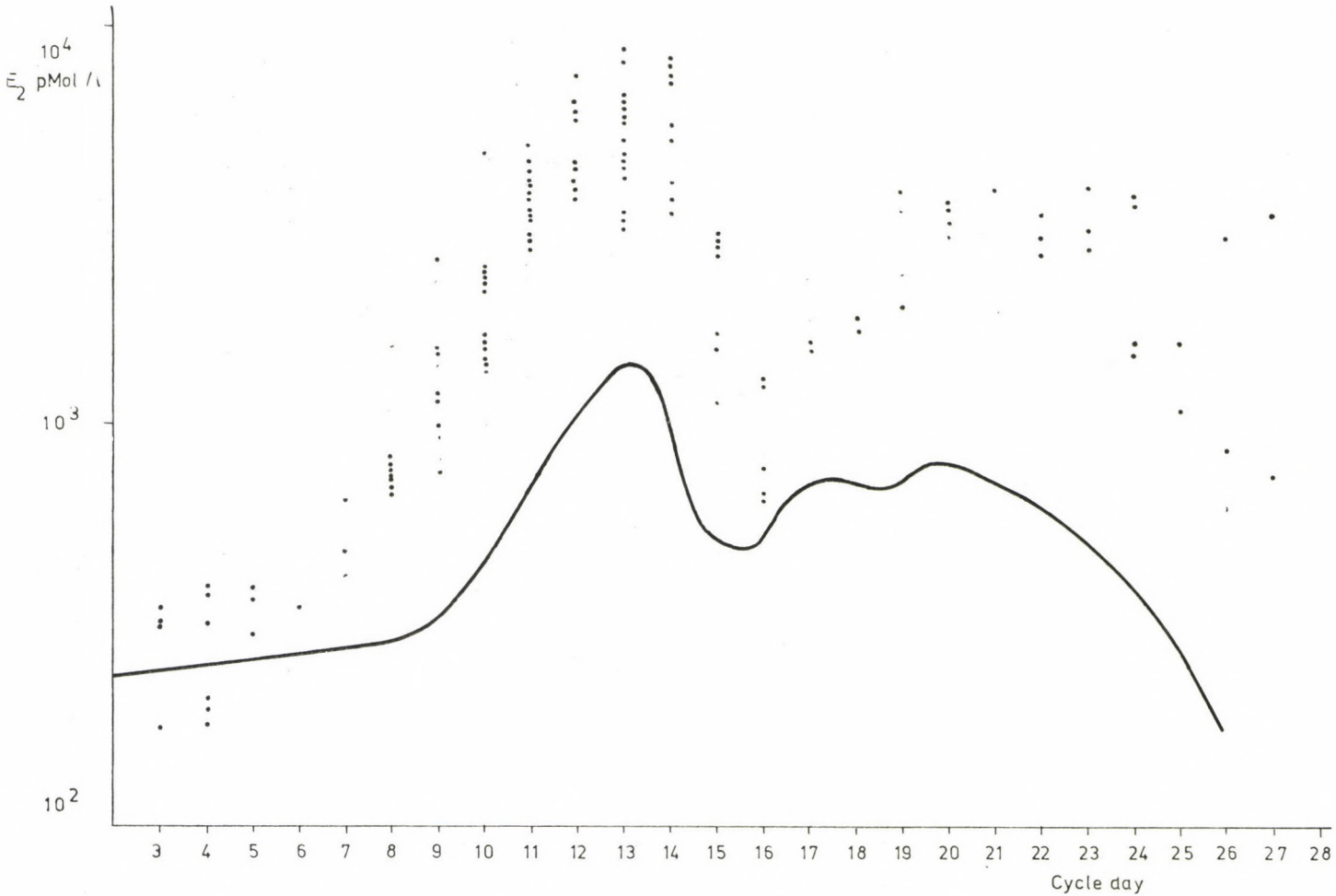

Fig. 1. E_2 values in superovulatory cycles (dots). The solid line refers to the average values of natural cycles

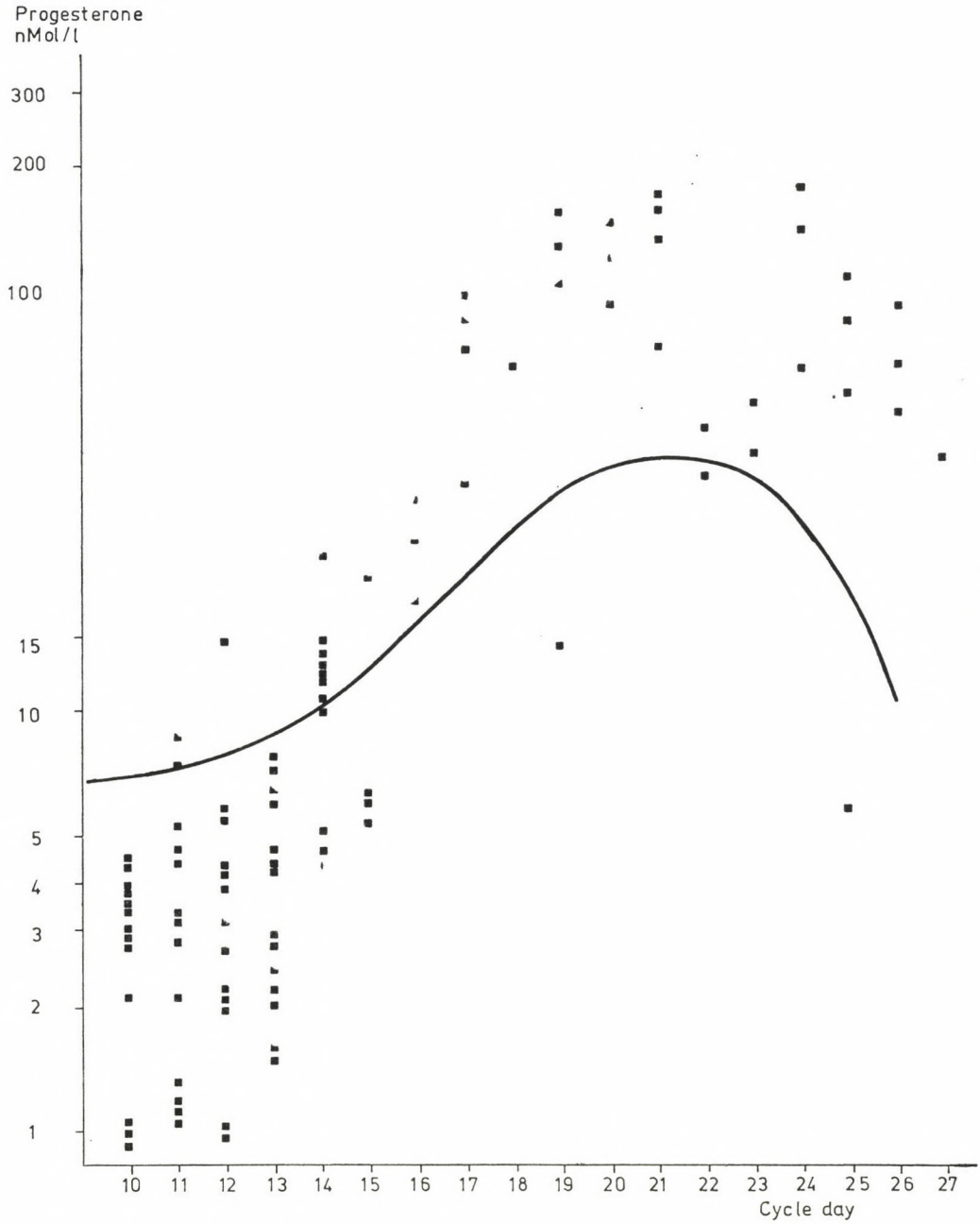

Fig. 2. Progesterone values in superovulatory cycles (dots). The solid line refers to the average values of natural cycles

2. The other approach is to apply a totally individual treatment. The time of the HCG administration is decided according to the serum or urine LH levels measured at 3 to 6 hrs intervals. This method is more complicated but it is generally accepted that the best results can be obtained in cases when the follicle puncture is performed after the occurrence of the spontaneous LH-surge [26].

Problems of controlling corpus luteum subsequent to the replacement of the embryo

No reliable difference between conceptual and non-conceptual cycles in their E_2, progesterone and GH levels appear to be, at least not until the period when early pregnancy becomes detectable by other methods. In conceptual cycles the luteinization of the punctured follicles is usually more satisfactory and the progesterone level is slightly higher than in non-conceptual cycles [10]. According to several data, the length of the luteal phase can be reasonably well predicted from the peak values of E_2 [19]. The remaining difficulty is that luteal inadequacy can be present despite progesterone levels much exceeding those seen in the natural cycle. Regarding the possibility of a HCG or natural progesterone support to the luteal phase, the opinions are not consistent.

The detection of early pregnancy or biochemical pregnancy is possible about 1 week after the replacement, on the basis of demonstrating the beta sub-unit of HCG or the pregnancy specific $beta_1$-glycoprotein (SP1). The detection of the early pregnancy factor (EPF) may be possible already about 24 hrs following the replacement, — it demonstrates the extremely rapid alteration of the immune system yet, its presence can not be regarded as a sign of real pregnancy because its appearance preceeds the implantation. Although its continuous increase seems to the vitality of the embryo, for practical reasons the continuous measurement seems to be rather difficult (rosetta inhibiting test). The immunological alterations concomittant with the early reproduction processes and the behaviour of proteins present in early pregnancy has already brought about data of great interest, their biological importance and clinical value have, however, not yet been clarified [2].

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