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VASCULAR BIOMECHANICAL FACTORS IN REGULATION OF ARTERIAL HEMODYNAMICS: COMPUTER MODELS

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We have the honor to dedicate this publication to the memory of Prof. Dr. László Kalmár, member of the Hungarian Academy of Sciences, founder of the Neumann Colloquiums in Szeged. He personally participated in the first six Colloquiums ... Dr. Kalmár would be 85 years old this year. Forty-five years ago he became a University Professor and 15 years ago – a year before his death – he retired.

By that time Dr. Kalmár had been awarded a Kossuth Prize and a 1st grade governmental decoration. As a prominent mathematician, professor Kalmár tried to comprehend the unknown laws of the Univers. He was internationally recognized for his achievements in mathematical logic, theory of sets and computer science. Prof. Kalmár founded the Laboratory of Cybernetics and the Department of Computer Sciences at the József Attila University, Szeged .... and still a bicycle remained his favorite means of transport. We will always keep his encouragements at the time of the very first Neumann Colloquium in our memory.

*

This work, based on the results of experimental biomechanical studies was designed to present two – partially hypothetical – models of cardiovascular regulation realized with digital computer. The first model simulates the classic pressure-induced myogenic vascular response, while the other shows the regulation of the mean arterial blood pressure using an optimization principle.

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*Dr. László Kalmár Memorial Lecture. MEDICOMP' 90 Colloquium, Szeged, 3 Dec., 1990

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1. Linear viscoelastic model of stretch-induced and spontaneous intrinsic vasoconstriction

In most parts of the arterial system pressure-induced contraction (Bayliss-phenomenon) as a rate-sensitive adaptive vascular reaction is a basic mechanism of autoregulation [5, 12, 20, 21]. Recent data indicate the existence of similar intrinsic mechanisms in veins which might autoregulate the capacity of these vessels [27, 28]. The efficiency of such response can be markedly modified under normal and pathologic conditions, both in veins [30] and in arteries [36]. Aside from that, spontaneous oscillating contractions also occur in most vessels (for example vasomotion of precapillary sphincters) [1] probably maintaining adequate local blood supply of the tissues. As far as the vessel caliber is concerned, the rate and efficiency of these primary changes of the smooth-muscle tone are mainly determined by biomechanical properties and relationships of the passive and active components of the vascular wall.

It is recognized that the non-linear, anisotropic viscoelastic properties of the vascular wall are due to the heterogeneous biomechanical properties of its passive and active elements and special characteristics of their orientation. Apart from these basic properties, intrinsic biophysical and biochemical changes induced by physiological stimuli contribute to the viscoelastic behaviour of the vascular wall [7, 8, 15, 26, 33]. The mathematical modelling of these physiological properties is one of the basic requirements for quantitative evaluation of viscoelastic parameters. Without simulation experiments based on such models, the complex relations between the passive and active structural elements of the vascular wall, as well as their biomechanical behaviour can not be adequately assessed, since the possibilities of direct measurements are strongly limited. Without comprehension of these relationships, neither short- and long-term normal adaptation processes, nor pathological alterations can be understood.

Analogue mechanical models of various combinations of elastic elements storing potential energy (spring) and viscous friction elements absorbing energy (damping), which can be mathematically evaluated are widely accepted in literature for the assessment of the viscoelastic behaviour of living soft tissues [22]. Maxwell's, Voigt's and Kelvin's (or St. Venant's standard linear solid state model) are the three basic types of these models. Maxwell's model primarily simulates stress-relaxation, Voigt's model predicts creep, while the solid state model simulates both phenomena (Fig. 1). By increasing the number of viscous and elastic elements and combining them, the model of passive viscoelastic behaviour can be improved and even expanded by introducing an inertial component storing kinetic energy (mass). In ideal elastic elements (springs), the stretch-induced counterforce (mechanical stress, σ_e) is time-independent (proportional to stretch – ε, and elastic modulus – E). In

viscous and inertial elements stretch-induced stresses (σ_v, σ_i) are proportional to the first and second time derivatives of stretch, respectively:

$$\sigma_e = E \cdot \varepsilon \text{ (elastic element)}$$
$$\sigma_v = \eta \ d\varepsilon/dt \text{ (viscous element)}$$
$$\sigma_i = \rho \ d^2\varepsilon/dt^2 \text{ (inertial element)}$$

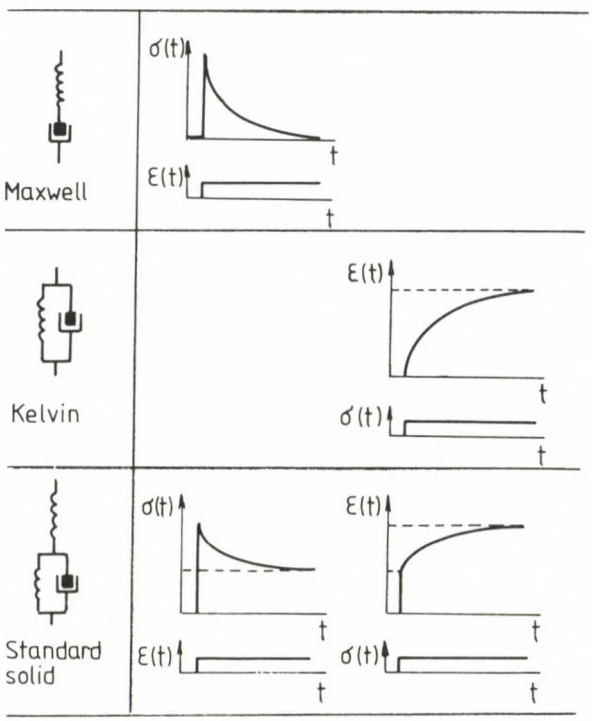

Fig. 1. Analogue mechanical models for viscoelastic behaviour of the soft tissues. σ: mechanical stress; ε: elongation (strain); t: time. The Maxwell-model describes stress-relaxation to unit step input stretching; the Voigt–Kelvin-model describes creep to a unit-step mechanical stress; the standard solid-state (St. Venant) model simulates both phenomena in a simple way

However, due to their dissipative character, these models are only suitable for the assessment of the passive mechanical behaviour of the vascular wall, and can not be used for the modelling of active contraction.

A new mechanical component previously introduced by us [18] provided an approach to the mathematical evaluation of contractions induced by stretch (e.g. intraluminal pressure) and of diameter oscillations of the vessels. The equation defining this new element is a logical continuation of equations of the above-

mentioned passive mechanical elements. This element "actively" produces mechanical stress (neither storing, nor absorbing) elicited by constant stretching:

$$\sigma_a = \alpha \int \varepsilon dt \text{ (active element).}$$

It is noteworthy that while capacity, ohmic resistance and inductivity are the electrical analogues for elastic, viscous and inertial elements, no such analog exists for the new component.

We developed a relatively simple analogue model structure simulating the properties of the vascular wall by introducing this active element into the St. Venant's model (Fig. 2). The differential equation of the model derived from the mathematical definition of the constituting elements is presented in Fig. 3. Simulation studies can be conducted in two different ways depending on the character of the input signal, which can be either stretch (stress-relaxation model), or mechanical stress (creep model). A model structure for both versions has been developed for an IBM-PC XT/AT computer [42, 45]. Due to the continuous character of the active analogue

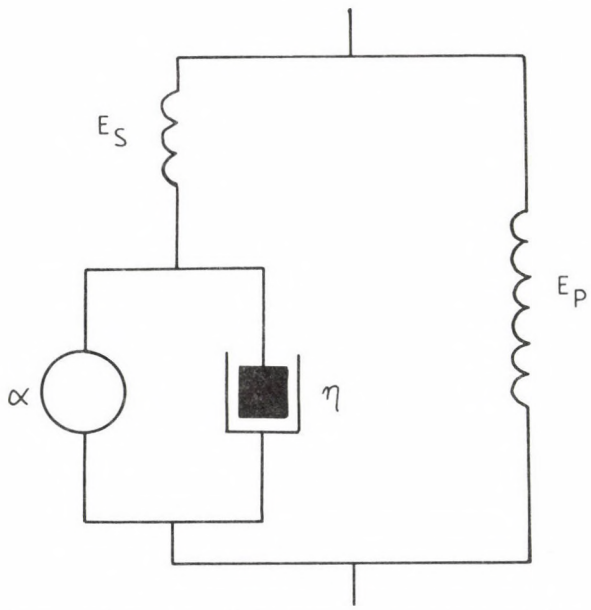

Fig. 2. A four-element analogue viscoelastic active model for the simulation of vascular wall mechanics. E_s and E_p – elastic moduli of the series and parallel elastic elements. α – rate constant of stress generation, η – viscosity coefficient of the damping element; to constant strain the active element responds with a constant rate stress generation

model, its differential equation can not be directly solved with a digital computer without sampling discrete data at certain time periods. Therefore a Z-transformation

(discrete Laplace-transformation) was performed. The solution of the equation for stretching (stress-relaxation model) as input is shown in Fig. 3. To determine the periodic spontaneous ("pacemaker") activity of the smooth muscle cells, a small sinusoidal modulation of the α parameter of the active component was introduced. Hence, the diameter-oscillation phenomenon can be simulated even at constant input values.

$$n\sigma'' + E_s\sigma' + \alpha\sigma = n(E_s + E_p)\varepsilon'' + E_s E_p \varepsilon' + \alpha(E_s + E_p)\varepsilon \qquad [1]$$

$$A_2\sigma_{k-2} + A_1\sigma_{k-1} + A_0\sigma_k = B_2\varepsilon_{k-2} + B_1\varepsilon_{k-1} + \varepsilon_k \qquad [2]$$

$$\varepsilon_k = A_2\sigma_{k-2} + A_1\sigma_{k-1} + A_0\sigma_k - B_2\varepsilon_{k-2} - B_1\varepsilon_{k-1} \qquad [3]$$

Fig. 3. Differential equation of the model shown in Fig. 2. (1): differential equation based on the definition of the component elements; (2): recursive algorithm obtained on the basis of the k-2 and k-1 sampled values of the Z-transformed form of equation (1). (3): response of equation (2) to strain elongation. By introducing the A, B (and other) constants the differential equation can be easily comprehended. $A = E_s/n$; $B = E_sE_p/n$. For further details see Figs 1 and 2

The program was written in Turbo Pascal language (version 5.0). Our purpose was to make an easy tool for research and demonstrations. The colored pull-down menu system provides an easy overview and interactive user interface. With the menu tree structure different functions (Fig. 4) or submenu items (Fig. 5) can be selected. The functions can either indicate the tasks defined by the program or adjust parameters. Within the menu tree structure the submenus of the same level are of the same color to facilitate their identification by the user. The selection of menu items requires only four keys to handle. The items of the main menu are as follows:

1. Current values
2. Parameters
3. Time
4. Simulation
5. Oscillation
6. Information
7. Print
8. Quit.

The legend to Figs 4 and 5 explains the 1st, 2nd and 6th menu items. By selecting submenus from the 3rd "Time" menu the "Sampling time" and the observation time of simulation experiments ("Max time") can be adjusted. The 4th and the 5th items of the main menu graphically display the results of four simulation experiments (simultaneously displaying four curves with different parameter combinations). "Simulation" starts a model without α-parameter oscillation (Fig. 6),

while "Oscillation" initiates an oscillation experiment with various parameters. One can exit the program by selecting the 8th "Quit" item.

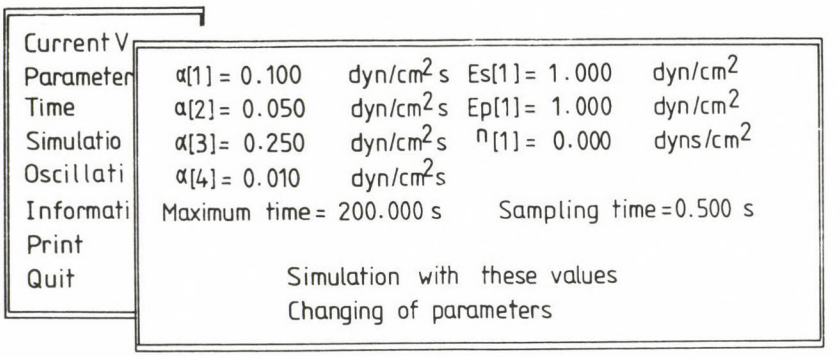

Fig. 4. Menu system for the active vascular biomechanical model program written for a digital computer. For example, by selecting the "Current values" from the main menu, the actual parameters of a simulation experiment and the physical dimensions used can be visualized

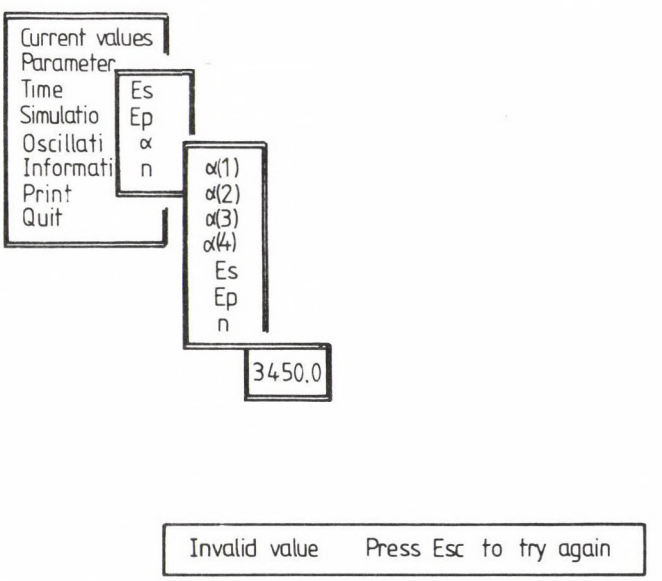

Fig. 5. Menu system of the digital active vessel model. The submenu of the main menu item "Parameters" shows the parameters (Es, Ep, α or n) which determine the difference between the four curves visible on the monitor; they can be selected by the user in the course of a simulation experiment. In the submenu of the following level of the tree structure the parameters can be adjusted. If input data error occurs, an error message appears on the screen (the ranges of different parameters can be verified by selecting "Help" of the "Information" menu item)

Simulation results are graphically displayed with automatic scaling on a high resolution monitor presenting 4 different parameter combinations. The graphic display of data can be stopped at any time and coupled by deleting or storing.

Our simulation results show good correlation with physiological observations suggesting new interesting aspects of intrinsic vascular control. For example, if the stress generating parameter α exceeds a critical value, the stress response exhibits damped oscillation (Fig. 6). On the other hand, the sinusoidal modulation of this parameter at appropriate frequency elicits a "parametric resonance", suggesting a new theory of spontaneous smooth muscle oscillation. The parameter value of the series elastic element (modulus of elasticity) determines the amplitude of the transient, as well as the steady-state response and the time required to reach this level.

The complete model in function had been first presented at the MEDICOMP' 90 Colloquium.

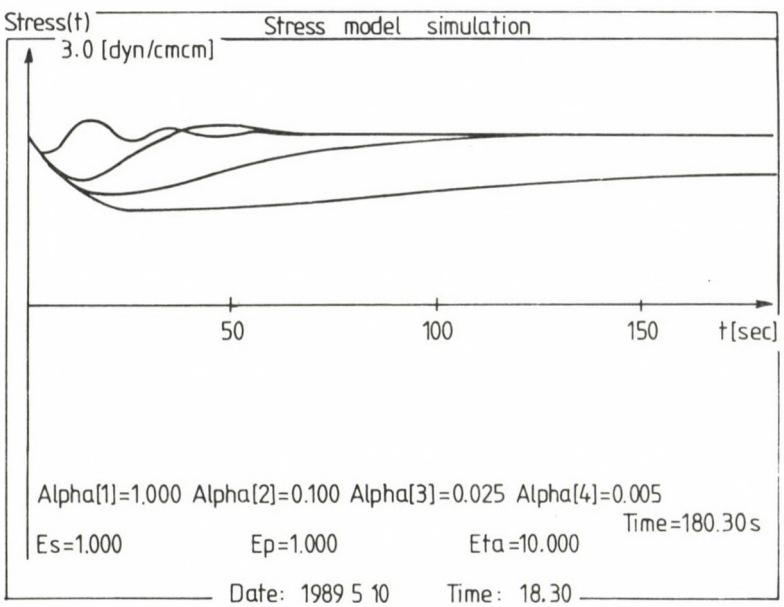

Fig. 6. Graphic interpretation of a simulation experiment performed with the active vascular model at 4 different rate constants of stress generation (α). The elastic moduli of the series and parallel elastic elements (Es and Ep), as well as the coefficient of the viscous element were the same in all 4 cases. At a chosen parameter combination, active stress response to unit-step elongation compensating passive stress-relaxation reaches the steady-state, following periodic damped oscillations. Hence the interaction between the active and passive elements of the vascular wall can elicit smooth muscle oscillations even without periodic induction (parametric resonance) of the α-parameter simulating metabolic energy production

2. Computer model of the forced-oscillation extremal control of the blood pressure

Most physiological regulatory mechanisms including cardiovascular control, are based on complex parallel and hierarchic relationships. To more deeply understand their function, experiments should be performed at different organizational levels involving both local and systemic mechanisms [23]. The aim of the present study is to evaluate the results of recent experiments in vascular biomechanics and circulatory dynamics focusing on possible optimization mechanisms of the blood pressure control. Several attempts have been made to assess blood pressure regulation in terms of system analysis and computer models. Hence, along with the classic set-point regulation [10, 11, 34], optimization principles (least energy regulation, or self-tuning control) have also been introduced [9, 38]. In our model, based on available physiological data and structural-functional analogies between physiological and technical regulatory systems, the minimalization of the flow impedance in major arteries was considered as an optimization criterion [39, 41, 43]. In vitro studies in isolated arteries have demonstrated that the characteristic impedance of large and middle sized arteries as a function of transmural pressure exhibits a parabolic shape (Fig. 7) with the minimum value in the physiological mean pressure range [2, 4, 24, 25]. These results were supported by in vivo experiments [37]. Physiological changes in the smooth muscle tone shift the minimum impedance along the pressure axis without significant alterations of its value, while the slope of the ascending loops of the curve can be markedly modified (Fig. 8). It is noteworthy that while the intrathoracic arteries (in the vicinity of the heart) exhibit parabolic pressure vs. characteristic impedance relationship even being dilated, in most of the extrathoracic arteries impedance is a monotonously increasing function of pressure exhibiting a U-shape only following the activation of the vascular smooth muscles [31]. Thus, assuming constant flow pulses in the arteries the minimum pressure-wave amplitudes are expected to be in the physiological mean arterial pressure range at rest. These results suggest that the optimal biomechanical properties adjusting the hydraulic functions of the heart, the large arteries and the microcirculation are genetically predetermined and are due to the specific structure and function of the vascular wall. We propose that the Wind-kessel arteries playing a major role in the damping and shaping of the 1st-order (pulsatile) waves should be called impedance vessels, since smooth-muscle induced modulations suggest that impedance is a controlled variable of cardiovascular regulatory mechanisms.

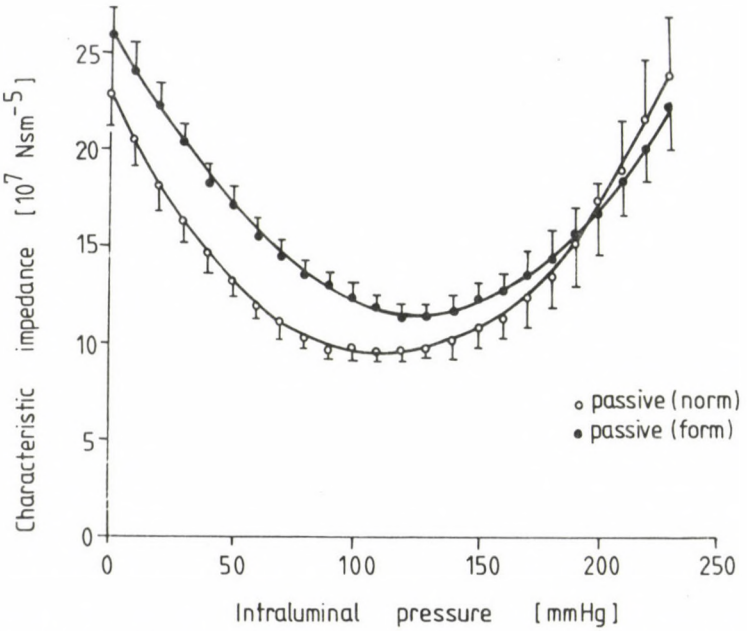

Fig. 7. Characteristic impedance vs. intraluminal pressure curve of normal bovine internal thoracic artery with relaxed smooth muscles and that treated with 0.1% formaldehyde for several days. Even after formaldehyde treatment the curve maintains its parabolic shape (F. Juhász, L. Nagy, E. Monos, unpublished data). Method: in vitro quasi-static large-deformation mechanical test [3, 16, 29]

The above biomechanical properties of the arteries closely correlate with our previous results related to spectral properties of the short-term pressure waves [32, 40]. These results show that the 1st-order pressure waves, in addition to frequency and amplitude, can also be characterized by the power density of their stochastic signal components. Some 1st-order pressure wave components, such as the variance of the stochastic components (Fig. 9) or the modulus of 1st harmonic of the pulse waves (Fig. 10) show a parabolic relationship with mean arterial blood pressure along the physiological range. In addition, the power density and the dominant frequency of slow, 3rd-order pressure waves closely follow the steady changes in mean arterial blood pressure (Fig. 11). The relative value of the stochastic signal components of these waves is markedly higher than that of 1st-order waves. Close dynamic correlation exists between the 3rd-order waves and the fluctuations of some 3rd-order components of the 1st-order waves [44].

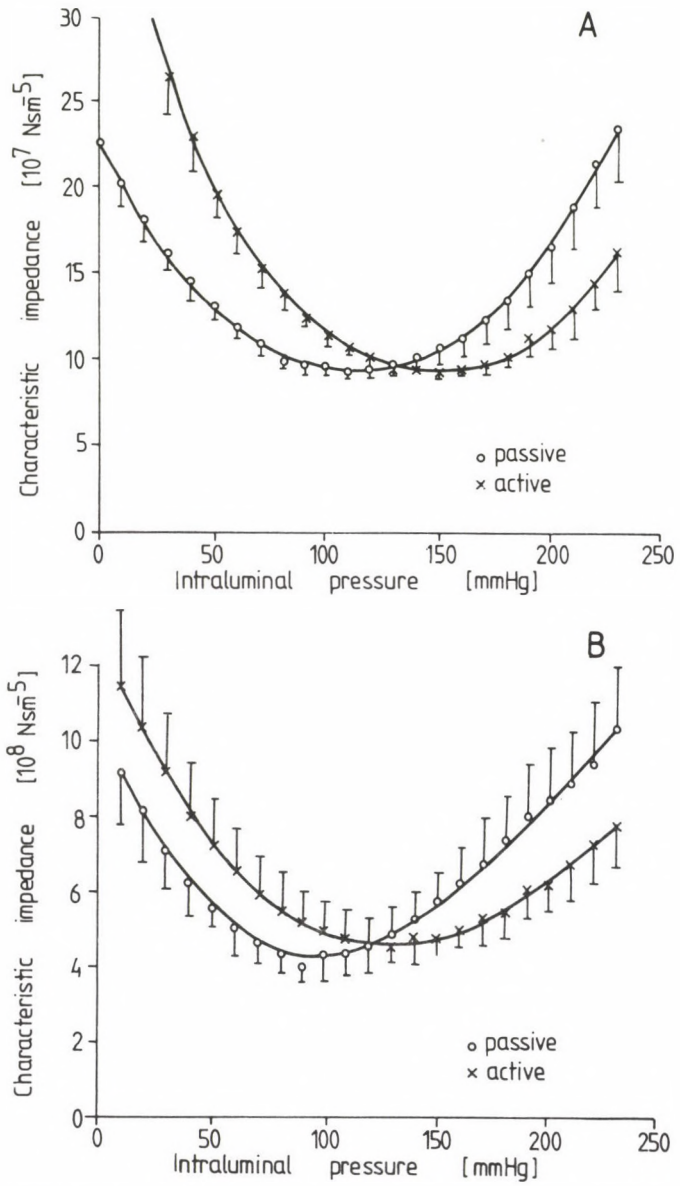

Fig. 8. Characteristic impedance ($\bar{X} \pm$ SEM) of isolated bovine internal thoracic artery (A) and the thoracic part of canine vertebral artery (B) as a function of intraluminal pressure before (passive) and after activation with a dose (0.5 μg/ml) of noradrenaline. An increasing smooth muscle tone shifts the minimum of the U-shaped curve to the right, that is towards higher values of mean arterial blood pressure. Method: same as in Fig. 7

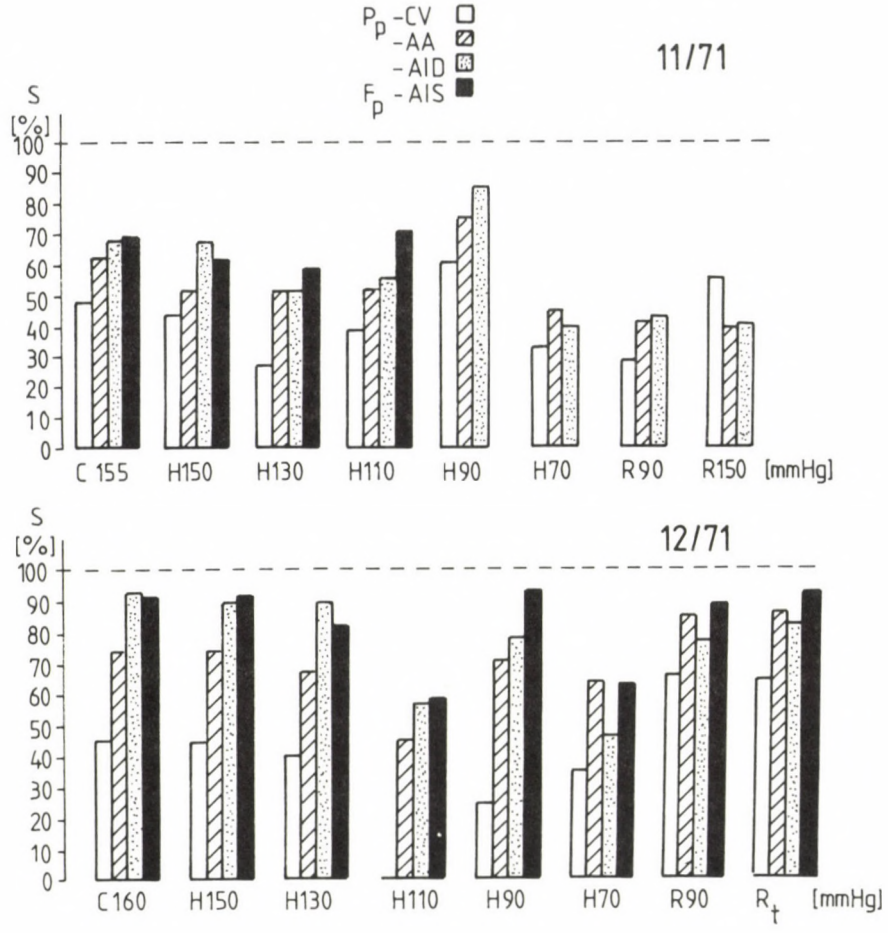

Fig. 9. Changes in pulse-synchronous stochastic circulatory wave components (S) as a function of mean arterial pressure (anesthetized dog). CV – central venous pressure; AA – ascending aorta; AID, AIS – right and left iliac arteries; Pp – pulse-synchronous pressure waves; Fp – pulse-synchronous flow waves; C – control mean pressure; H and R – values of mean arterial blood pressure set by bleeding and retransfusions respectively. Exp. No. 11/71 and No. 12/71 [32]

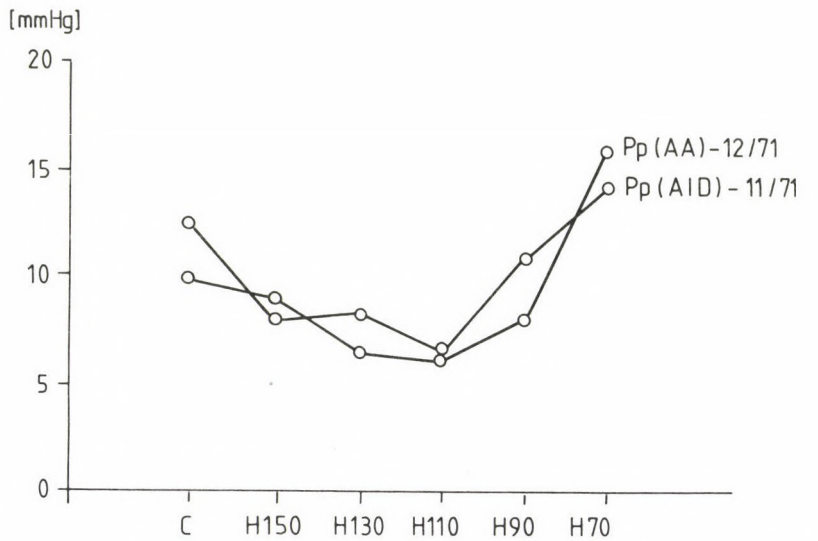

Fig. 10. The moduli of the 1st Fourier harmonics (pulse-synchronous pressure) as a function of mean arterial pressure (for further details see Fig. 9)

Analyzing these biomechanical and hemodynamic results we have developed a hypothesis of a complex regulatory mechanism optimizing arterial blood pressure. The optimization criterion is that instantaneous value of the mean arterial pressure and the minimum of arterial impedance coincide. That is, the optimal operating point of this pressure controller is defined by the extreme (minimum) of the arterial impedance characteristics [41]. This hypothesis is realized in a forced oscillation extremal control model (Fig. 12). Besides some system characteristics with an extreme value (such as a parabolic impedance curve), two interacting processes are also essential for this type of control: a searching process and a considerably slower basic process [6]. In our model either the pulsatile waves synchronous with cardiac activity (1st-order waves) or the respiratory waves synchronous with respiration (2nd-order waves) are considered as a searching process, while the 3rd-order waves (Traube–Hering–Mayer vasomotor waves) as the basic one. If external effects or internal changes disturb the system shifting its operation point, i.e. the momentary value of the mean arterial pressure, from the minimum impedance value, the searching process would initiate a control signal, the magnitude and sign of which depend on the position of the operation point on the parabolic (extremal) impedance curve. The control signal modifying the basic process in accordance with the time constant of the transfer characteristics of the system components forces the operating point back to the minimum of the impedance curve.

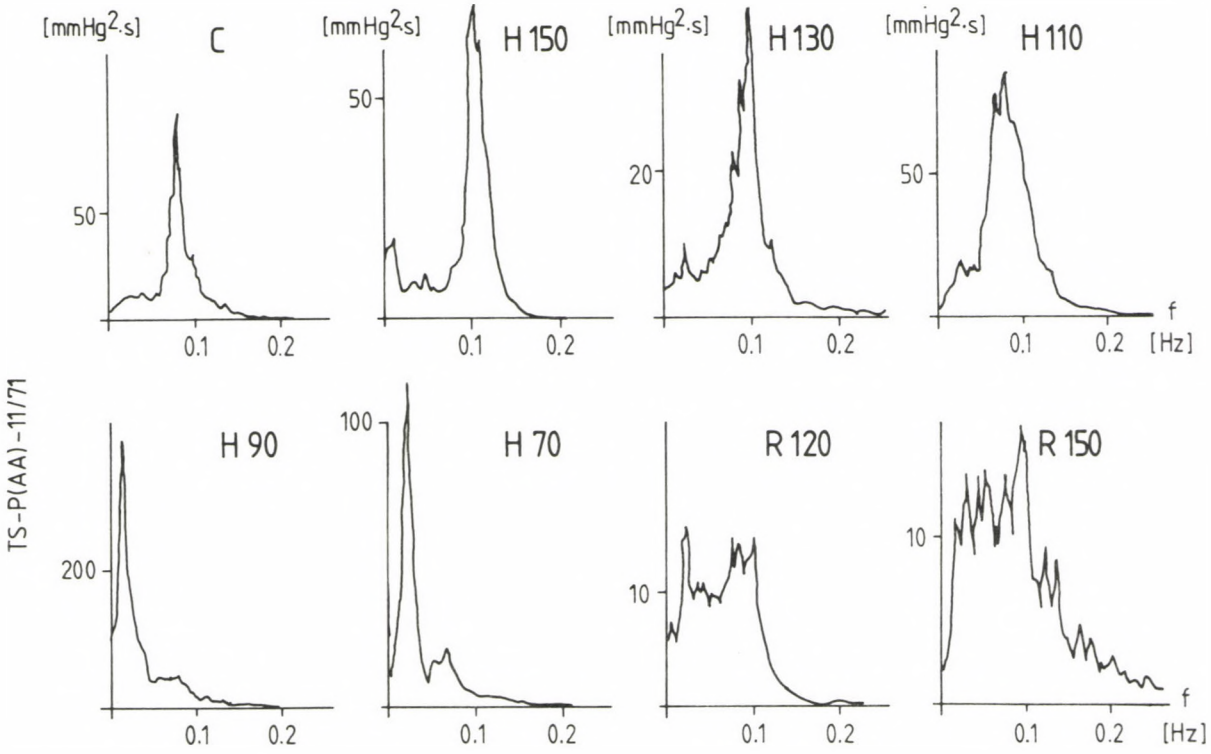

Fig. 11. Power-density spectra (TS) of 3rd-order slow (below 0.2 Hz) pressure waves as a function of mean arterial pressure. Lowering of the mean arterial pressure (bleeding) shifts the dominant waves towards lower frequencies, while their power increases. The expansion of stochastic waves following the retransfusion of the shed blood indicates terminal state of the experimental animal (for further legend see Fig. 9)

Most of the components of such type of control are verifiable to exist in the cardiovascular system. Thus, some circulatory parameters such as arterial impedance and other parameters characterizing the mechanical properties of the arteries exhibit extremal (parabolic) changes [26]. Hemodynamic processes with different frequencies, such as the blood pressure wave-components of different order can be clearly identified in the circulatory system. Close correlations found between the 1st- and 3rd-order wave components give evidence of their interaction. The slope of the U-shaped impedance-pressure curves, as well as their localization on the pressure axis differ in individual arteries and also depends on smooth muscle tone, providing the adaptation ability of the control system. Other system components required for the control functions might exist in the nervous system, where they can function as converters, band-pass filters, multipliers (or phase discriminators) and integrators (memory), however, at present their existence is merely theoretical and they are not yet identified in physiological terms. This optimizing control mechanism does not require set-point generation, thus a traditional problem of barostat hypothesis, that is to identify such a generator in the organism, is eliminated. This mechanism explains the physiological role of the rate-sensitive baroreceptors of the aortic arch and carotid sinus: assumingly they transduce impedance-dependent wave-dynamics information, thus playing a crucial role in the activation of the reflexes adjusting the instantaneous biomechanical state of the large arteries. The forced oscillation optimum-searching control hypothesis provides a uniform outlook on the physiological role of fast pulse-synchronous hemodynamic processes and the slower waves occurring in the circulatory system. Aside from the "short-term" blood pressure control such mechanisms also may play an important part in long-term regulation, since the basic histological structure primarily determining the parabolic shape of the arterial impedance curve is probably genetically determined, thus its modification or reorganization takes a substantial time.

The mathematical description and stability examination of this model have been successfully performed [13, 14, 35]. A module-structured computer program which can be easily further developed was written in Pascal and 8086 Assembly languages for IBM PC XT/AT configuration. The program consists of 4 parts: numerical algorithms, graphic procedures, a menu, and the main program. It provides possibility for the analysis of the 10 signals of our model by adjusting 17 different parameters at will. To display all the parameters independent subprograms assess the functions of all the components of the model. The options of the menu systems presented on Fig. 13 facilitate this task. Each menu item runs the applications or open a new submenu.

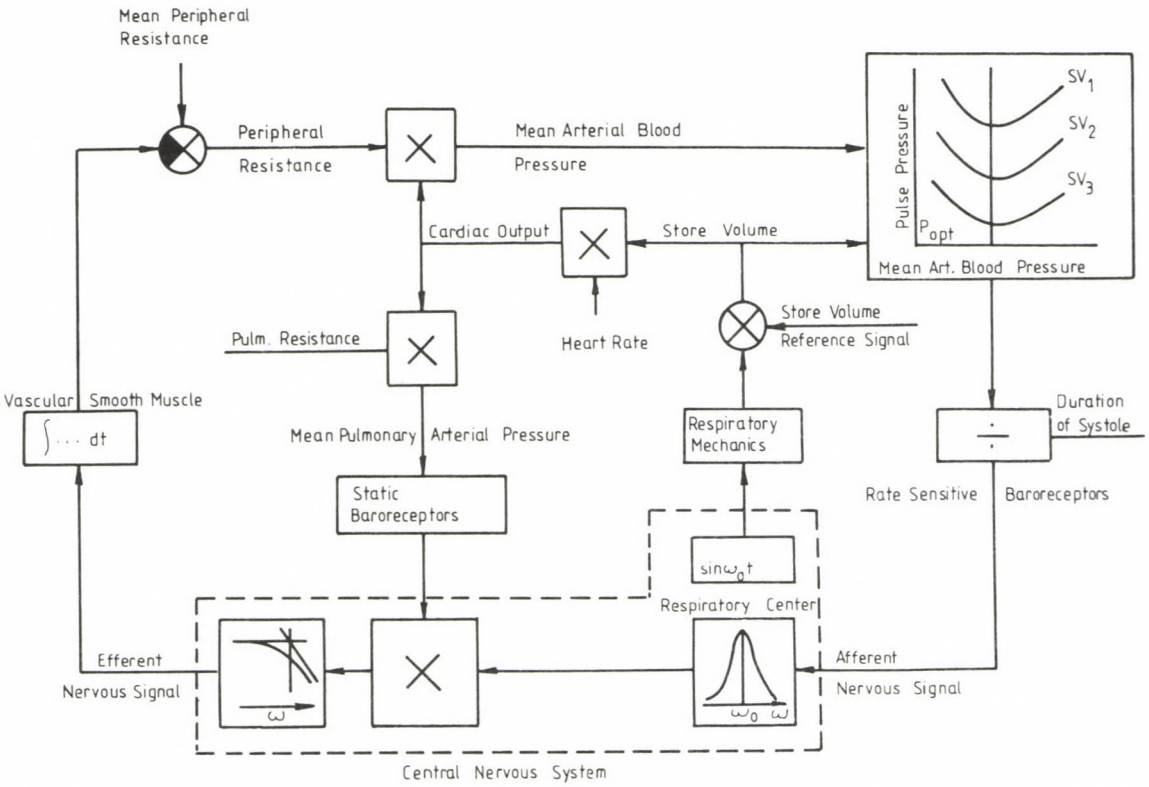

Fig. 12. **Model of a forced oscillation control mechanism optimizing arterial blood pressure. P_{opt} – optimal operating point set by the minimum value of the characteristic impedance of the large arteries. In this model the respiration-synchronous blood pressure waves are considered as a searching process (19)**

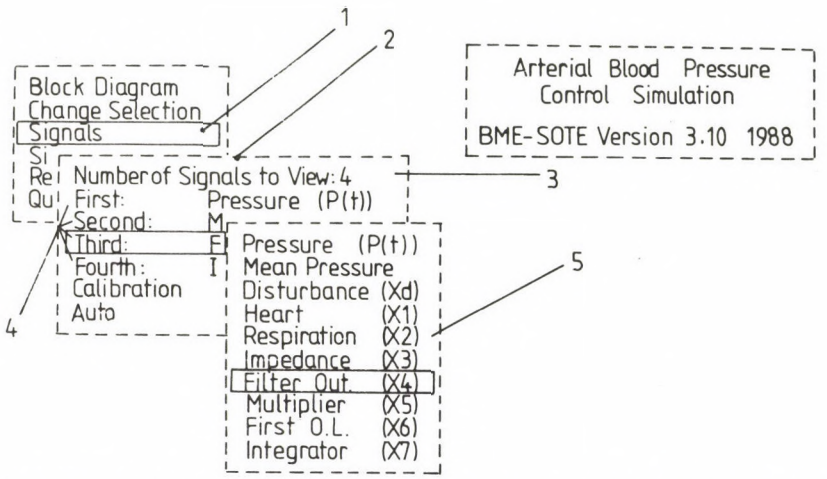

Fig. 13. Menu system of the digital computer model of the control mechanism optimizing blood pressure The Figure shows selection options displayed on the computer monitor in the course of a simulation experiment. Selection of main menu item "Signals" (1) opens a submenu window (2). Selection of its first item (3) gives the number of signals (1, 2 or 4) to be displayed simultaneously. Individual signals of the model are selected by (4). The selection of any item displays a list of signals of the control model (5) which can be used for selection in the customary way

In simulation experiments two different transient processes can be analyzed in the model: the effect of unit-step-like disturbance signal (Fig. 14) and the response to a shift of the minimum value of the parabolic curve at different system parameters. Both processes are closely related to physiological changes. The simulation results indicate that this control model is capable of effective regulation in case of disturbances and shifts the operating point in accordance with physiological observations. The resetting of the controlled variable (arterial pressure) to the preset control value can occur either aperiodically, or with damped oscillations, depending on the feedback gain of the system and the slope of the impedance-pressure curve. If these parameters exceed a critical value which is also a function of other parameters and disturbing signals, the control process becomes instable. This example indicates some possibilities of the simulation experiments in the analysis of normal and pathological processes.

Though both the above models can be already successfully used for special-purpose simulation experiments, they need further improvement. Development of our active vascular model is a basic requirement for the introduction of cylindrical vessel geometry and branching into larger complex vascular network models with distributed parameters. The optimizing control mechanism of blood pressure can also be further developed. A new version should display the distributed parameter structure of the cardiovascular effectors (heart and blood vessels) better resembling the in vivo conditions and should closely correlate with anatomic structure and the physiological data.

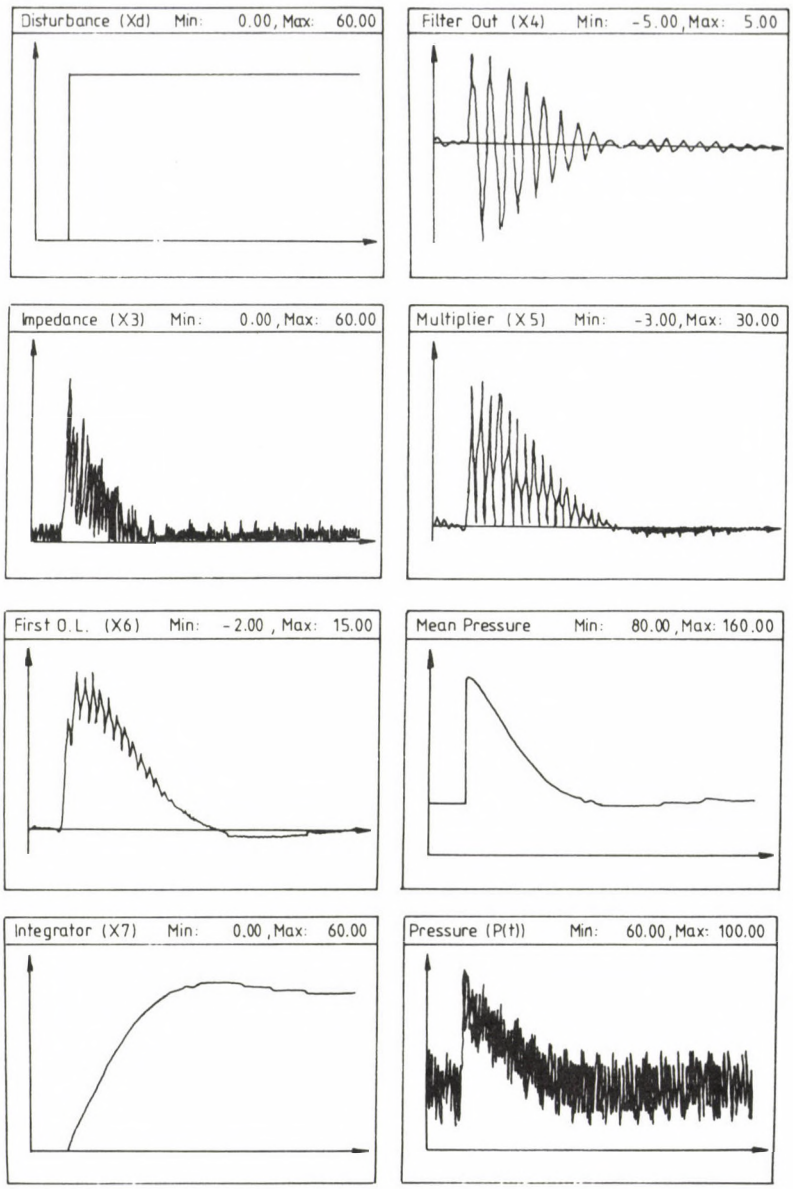

Fig. 14. Signals (2x4) of the optimizing model as function of time displayed on the monitor if a unit-step 50 mmHg disturbing signal (Xd) is selected. Arterial blood pressure {P(t)} resumes its initial values (in this case aperiodically) even if the disturbing signal is maintained. The blocks of the diagram display identifying marks and calibration signals of the Y axis

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EFFECT OF FLUMECINOL (ZIXORYN) ON THE CYTOCHROME P_{450} AND CYTOCHROME P_{448} DEPENDENT HEPATIC MICROSOMAL MONOOXYGENASE ACTIVITIES IN MALE RATS

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The effect of three-day oral administration of 50 mg/kg bw. and 100 mg/kg bw. flumecinol (Zixoryn, Gedeon Richter Chemical Works Ltd., Budapest, Hungary) and intraperitoneal administration of 50 mg/kg bw. phenobarbital as well as the single intraperitoneal administration of 20 mg/kg bw. 3-methylcholanthrene on various cytochrome P450 and P448 dependent hepatic microsomal enzyme activities was studied in male albino Wistar rats.

50 mg/kg bw. flumecinol had no significant effect. 100 mg/kg bw. flumecinol had an inducing effect comparable to the one of phenobarbital. The activity of the cytochrome P448 dependent 7-ethoxyresorufin O-deethylase was enhanced by all three substances, but flumecinol's effect was by far behind that of 3-methylcholanthrene, so the carcinogenic promoter effect of flumecinol can be questioned.

Keywords: flumecinol, microsomal enzyme activities, rats

The metabolism of xenobiotics may lead to the generation of toxic, potentially carcinogenic intermediers [4]. The cytochrome P450 dependent hepatic microsomal polysubstrate monooxygenase system plays thus a key role not only in the detoxification of various agents but also in the generation of toxic metabolites.

Cytochrome P448 activity is closely linked to the generation of carcinogenic metabolites [11]. The cytochrome P448 content of tumor tissues is high and the increase of it is an early indicator of malignant transformations [14]. All xenobiotics with inducing effect on cytochrome P448 content and/or activity are considered

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potential carcinogens. This activity can be characterized by the determination of 7-ethoxyresorufin 0-deethylase activity [15].

The promoting effect of the potent microsomal cytochrome P450 inducer phenobarbital is well documented [2, 5]. Clinical investigations clearly demonstrate the coincidence of various cancers and prolonged phenobarbital treatment [14].

Zixoryn (flumecinol, 3-trifluoromethyl-alpha-ethyl-benzhydrol; Gedeon Richter Chemical Works Ltd., Budapest, Hungary) is a potent phenobarbital type microsomal enzyme inducer [3, 17, 18, 19] and is metabolized by the hepatic microsomal monooxygenase system [8]. We studied the effect of flumecinol on various cytochrome P450 and P448 dependent hepatic microsomal monooxygenase activities in male rats in order to get data on potential promoter or non-promoter role of the agent.

Materials and methods

Male albino Wistar rats weighing 150 to 250 grams were used. The rats were housed in plastic cages and the room was kept at constant temperature with fixed illumination rhythm. The rats were fed with LATI food pellets. Before decapitation all animals were kept fasting for 16 hours.

The rats were treated with 50 and 100 mg/kg bw. daily oral dose of Zixoryn through gastric tube for three consecutive days. Zixoryn was dissolved in distilled water containing 10% Tween 80. Controls received physiological saline.

In order to control the accuracy of our methods further two, smaller animal groups were treated with phenobarbital (50 mg/kg bw. intraperitoneally for 3 days) and the cytochrome P448 inducer 3-methylcholanthrene (20 mg/kg bw. intraperitoneally as a single dose). The rats were killed 24 and 48 h after the last treatment, respectively.

After decapitation the liver was removed, put into ice cold isolating solution (1.15% KCl in 50 mM Tris buffer, pH: 7.4) and homogenized followed by centrifugation at 7500 *g* for 10 min and ultracentrifugation of the supernatant at 105 000 g for 90 minutes in order to obtain microsomal fractions. The aliquot containing the microsomes was suspensed in 0.1 M Tris-HCl buffer and stored at -20°C until analysis. Liver weight was previously measured and registered.

The cytochrome P450 and b_5 content [13], the NADPH cytochrome c reductase (modified Jansson and Schenkman method, [7]), the aniline hydroxylase [1], the 7-ethoxy-coumarin 0-deethylase [6] the aminopyrine N-demethylase [10] and the p-nitroanizole 0-demethylase activities [12] were measured in order the determine the effect of the agents on cytochrome P450 dependent hepatic microsomal monooxygenase activities. The activity of cytochrome P448 was measured by the 7-ethoxyresorufin 0-deethylase [16] activity.

The microsomal protein content was determined according to the method of Lowry et al. [9].

A Specord M-40 spectrophotometer (Carl Zeiss, Jena, Germany) and Hitachi 650 10S fluorimeter (Japan) were used. For statistical analysis the Student's *t*-test for two means was performed.

Table I

Effect of flumecinol, phenobarbital and 3-methylcholanthrene on rat microsomal enzyme activities

	liver weight (gr)	cytochrome P-450 (nmol/ mg protein)	cytochrome b5 (nmol/mg protein)	cytochrome-c reductase (nmol/min/ mg protein)	aminopyrine N-demethylase (nmol HCHO/ min/mg protein)	aniline hydroxylase (nmol p-aminophenol/ min/mg protein)	p-nitroanizole 0-demethylase (nmol p-nitrophenol min/mg protein)	7-ethoxycoumarin 0-demethylase (pmol/min/ mg protein)	7-ethoxyresorufin 0-demethylase (pmol resorufin/ min/mg protein)
Control									
n	11	11	10	11	11	11	11	11	11
$\bar{x}$	8.89	0.59	0.9	93.21	11.13	0.87	1.93	1537	53.73
±SEM	0.25	0.03	0.05	7.55	0.53	0.06	0.11	136	3.16
Flumecinol									
50 mg/kg(n)	11	11	11	11	11	11	11	11	11
$\bar{x}$	10.07*	0.62	0.80	109.09	10.40	0.92	2.06	1849	53.40
±SEM	0.59	0.03	0.20	8.03	0.50	0.02	0.19	174	3.49
Flumecinol									
100 mg/kg(n)	11	11	11	11	11	11	11	11	11
$\bar{x}$	10.25***	0.83**	0.96	106.86	15.97*	0.99	2.74*	2438***	98.64***
±SEM	0.24	0.07	0.06	6.92	1.21	0.06	0.19	190	7.01
Phenobarbital									
n	3	3	3	3	3	3	3	3	3
$\bar{x}$	11.00***	1.21***	1.06	147.99**	17.98***	1.33***	3.85***	3291***	122.30***
±SEM	0.3	0.09	0.15	21.00	2.51	0.06	0.34	529	1.10
3-MC									
n	5	5	5	5	5	5	5	5	5
$\bar{x}$	11.31	0.86	0.87	67.73	7.13	0.80	3.45**	1857	4133***
±SEM	0.58	0.08	0.04	11.65	0.65	0.13	0.63	185	1016

* $p<0.05$ – ** $p<0.01$ – *** $p<0.01$
3-MC: 3 methylcholanthrene
n: number of cases

Results

There was a significant rise in liver weight in rats treated with 50 mg/kg bw Zixoryn. The administration of 100 mg/kg bw Zixoryn resulted in significant increases in liver weight, cytochrome P450 content, aminopyrine N-demethylase, p-nitroanizole 0-demethylase, 7-ethoxycoumarin 0-deethylase and 7-ethoxyresorufin 0-deethylase activities (Table I).

Phenobarbital significantly induced all activities measured except cytochrome b_5 content. Methylcholanthrene significantly enhanced the p-nitroanizole 0-demethylase and 7-ethoxyresorufin 0-deethylase activities.

Discussion

The potent phenobarbital type microsomal polysubstrate monooxygenase inducer flumecinol is rapidly absorbed from the gastrointestinal mucosa and undergoes a strong "first pass" effect [8]. Only 4.5% of the applied dose reaches the circulation. It is metabolized by the hepatic microsomal monooxygenase system to three main metabolite forms (para hydroxylation of the phenyl ring, hydroxylation of the aliphatic side-chain and the combination of the two). The main metabolic route in man is the secunder site hydroxylation of the aliphatic side-chain [8]. The primer structure and the trifluoromethyl group on the aromatic ring remain unchanged. The metabolites are excreted as glucoronides and sulphate conjugates with the urine and feces. Only 1.2% of the dose administered is excreted unchanged.

Flumecinol is a fairly strong phenobarbital type microsomal enzyme inducing agent without any other known pharmacological effect [3, 17, 18, 19].

Our results strengthen the inducing potency of flumecinol. We emphasize, however, that the rise of the 7-ethoxyresorufin 0-deethylase activity reflecting cytochrome P448 activity (promoter effect) was only a tiny fragment of the activity obtained after the administration of 3-methylcholanthrene. Phenobarbital's inducing effect was slightly above of that of Zixoryn in this respect.

We conclude that flumecinol's inducing effect on cytochrome P448 dependent microsomal enzyme system is very weak and hence its promoter effect can be questioned.

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POSSIBLE ROLE OF BILE DEFICIENCY IN THE DEVELOPMENT OF INTESTINAL SYNDROME OF ACUTE RADIATION DISEASE

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Experimentally induced bile deficiency significantly accelerates the development of acute gastrointestinal syndrome in the irradiated rat. The usual 11 days of survival is reduced to 5 days. In view of the experimental results it seems justifiable to conclude that endotoxins play an important role in the development of this syndrome.

Keywords: bile, endotoxin, acute radiation sickness, gastrointestinal syndrome

Nuclear establishments keep spreading all over the world. World-wide energy supply is almost inconceivable without them. Ionizing radiation gains also in popularity in industry, agriculture and medicine. In the wide scope application of atomic energy, however, radiation and nuclear accidents with severe consequences (see Chernobyl) may happen despite strict safety regulations.

The most radiosensitive tissue of the organism the bone marrow responds with impaired haemopoiesis to irradiation. Higher radiation doses induce gastrointestinal syndrome. Extremely high irradiation doses, may be followed by damages to the central nervous system [11].

Gastrointestinal syndrome was selected as our object of study because according to the sad statistics of radiation accidents (nuclear accidents) this disease is most likely lethal. Damages of the central nervous system, being likewise incurable, occur less frequently.

The development of the gastrointestinal syndrome is attributable to:

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– severe damage to the gastric and intestinal mucosa manifesting itself in denudation of villi and exulceration [11];

– a decreased bile production after irradiation [6];

– release of endotoxins from the cellular wall of Gram-negative bacteria in the small intestine.

Experiments on endotoxin hypersensitivity induced by lead acetate have shown that in gastrointestinal syndrome endotoxins enter blood circulation [4, 7] causing enteroendotoxaemia that may account for the lethal outcome of the disease.

It is a well known that bile acids are able to detoxicate endotoxins [2, 3]. In view of these facts, it is supposed that decreased bile production after irradiation with appropriate dose is not capable to inactivate the endotoxins released. Thus, through the damaged intestinal barrier they may enter blood circulation eliciting endotoxin shock.

In this work we have intended to prove that experimentally induced bile deficiency accelerates the development of gastrointestinal irradiation syndrome with fatal outcome. In preliminary experiments rats were rendered bile deficient for a relatively long period of time (30 days) by bile cannulation. Technically this was achieved by leading a plastic cannula from the bile duct to the external surface of the abdomen. In the present work similarly operated rats were irradiated on day 11 after surgical procedure with a dose sufficient to induce the gastrointestinal syndrome.

Material and methods

Experiments were performed on 60 female Wistar-R (LATI, Gödöllő) rats of 160-180 g body weight maintained on granulated LATI rat chow and tap water given ad libitum. The animals were anaesthetized with 3 mg/100 g Nembutal intraperitoneally. A plastic cannula of 1 mm diameter was introduced into the common bile duct. Its delivery end was led through the closed abdomen and skin to the external surface of the abdomen. The cannula was fixed by a two-component adhesive "UVERAPID 5" (UVESZ, Budapest) to the tissue between musculature and skin of the abdomen. On the external surface of the abdomen the position of the cannula was also fixed to this tissue with compatible adhesive. A small amount of it was piled up around the plastic tube to protect it against any external mechanical damage (otherwise the animal could have pulled it out).

The bile fistula animals were then irradiated from a THX-250 device (Parameters: 200 kV, 20 mA, target to midline distance 60 cm, 0.5 mm (Cu filter, dose rate: 0.516 Gy/min, total dose: 8 Gy).

Results

Radiation dose inducing intestinal syndrome was determined in a preliminary study, performed with 40 rats. This dose was found to be 8 Gy. The death occurred during 7 to 11 days. Diarrhoea was observed from the 4th postirradiation day onwards. Post mortem examinations verified that the animals had died of intestinal syndrome, indeed. Characteristic haemorrhages were detected in all the stomach,

small and large intestines. In each case, the stomach was filled and dilatated. In the majority of the cases the coecum was also enlarged. Bowels frequently appeared as swollen. Intestinal lymph nodes were enlarged and hyperaemic. Adrenals got nearly doubled in size. The thymus, however, diminished or sometimes wholly disappeared.

After these preliminary experiments we started our recent study. Altogether four experimental groups were established (see Table I). The table shows the results of the experiment.

Table I

Effect of bile deficiency on the spontaneous death of rats after whole body irradiation

Group	No. of animals	Treatment	Time of death following irradiation (days)
1	5	bile fistula	**
2	5	Rtg (8 Gy)	9, 9, 12, 12 12
3	5	(sham operated + Rtg (8 Gy)	9, 9, 11, 12, 13
4	5	bile fistula + Rtg (8 Gy)	2, 4, 6, 6, 8

** these animals were still alive 2 weeks after the completion of the experiment

Rats belonging to Groups 2, 3 and 4 revealed the same characteristic pathological alternations at autopsy as those in the preliminary experiments. Consequently, it seems right to suppose that they died of gastrointestinal syndrome. All the bile fistula and irradiated rats (Group 4) died earlier than any of the other groups. Difference in the time of death was statistically significant.

These observations support our idea that in animals with long-term bile deficiency, irradiation with 8 Gy induces gastrointestinal syndrome much earlier than in the non-operated but irradiated counterparts.

Discussion

Results obtained in this study indicate that bile deficiency developing after whole body irradiation at a sufficiently high dose level plays a considerable role both in the induction and course of subsequent gastrointestinal syndrome. Our experiments evidence that rats rendered bile deficient prior to irradiation develop the gastrointestinal syndrome within a shorter time. Contrary to other authors who deny the pathogenic role of endotoxins [8, 9, 10] we believe that circulating endotoxins represent a major contribution to the rise of subsequent shock [2, 3]. Earlier

experiments with the lead acetate hypersensitizing method support this hypothesis [4, 5, 7, 12].

Naturally, further investigations on the effects of isolated abdominal irradiation, antibiotic pretreatment and induction of endotoxin tolerance are required to illuminate the exact role of endotoxins in this process.

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NEWLY IDENTIFIED STEADY-STATE POTASSIUM CHANNELS IN RAT HIPPOCAMPAL NEURONS

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We report two new types of potassium channels in cultured hippocampal neurons of rat. Both channels occurred in the soma membrane of these cells at very low density. They were active in steady-state conditions, within a wide voltage range that included the resting membrane potential. Their open probability was enhanced by membrane depolarization, but not influenced by Ca ions. In symmetrical 150 mM KCl the channels showed a slope conductance of ca. 40 and 80 pS, respectively. Current-voltage relations of both K channels show a negative slope at high positive voltages.

Keywords: potassium channel, hippocampus, membrane potential, depolarization, calcium ion, current-voltage relations

Potassium channels form the most diversified and widespread class of membrane ion channels. They have been found in all preparations, and more than twenty distinguishable types of potassium channels have been described. Some are ubiquitous and well characterized (i.e., delayed rectifiers, inward rectifiers, A channels, calcium-activated K channels; for a review see [11]), others are restricted to specific cell types, organisms or tissues (i.e., M-channels described in bullfrog sympathetic neurons [1]; inverted A channel-like from *Ascaris* esophageal muscle [5]; pacemaker, I_f, channels from cardiac node cells [6]).

Although a large number of potassium channels have already been described, new ones are continuously being discovered. In this paper we report the identification and preliminary biophysical characterization of two new K channels found in cultured hippocampal neurons of rat. The channels occur in the soma membrane at very low density, and are active in steady-state conditions within a wide voltage range which

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includes the cell resting membrane potential. The functional role of the background activity of these K channels and their contribution to the stabilization of the resting potential will be discussed.

Methods

Experiments were carried out on hippocampal neurons dissected from 18-day-old rat embryos, and plated on collagen/polylysine-coated culture dishes containing basic tissue culture medium (N5) supplemented with a fraction of horse serum. Neurons were kept in culture for 2-4 weeks before the experiment.

The patch clamp method [10] was used to record single-channel currents in the inside-out configuration. The cell membrane was voltage-clamped and current was recorded with a List EPC-7 patch clamp amplifier. The pClamp-5 (Axon-Instrument, USA) package of hardware and software was routinely used during experiments for stimulating, recording the currents, and analyzing the data. Single channel currents were filtered at 1.0 kHz (-3 dB) and digitized at 2.5 kHz. Single-channel records were taken in steady-state conditions attained by stepping the membrane voltage to the desidered level, and maintaining that value for tens of seconds to minutes. Recording began several seconds after the step potential, to allow relaxation processes subsequent to the voltage step to reach completion. Current amplitude histograms were constructed from 20-120 s stretches of current records, and used to measure single-channel current as interpeak distance.

Intracellular and extracellular solutions contained 150 mM of either NaCl or KCl as the bulk salt, 2 mM morpholinopropanesulphonic acid (MOPS), and 1 mM EGTA. The solutions were adjusted to pH 7.3 by addition of the hydroxide of the bulk cation. These solutions will be referred to by specifying the concentration (mM) and species of the bulk salt, e.g. 150 KCl//150 NaCl for intracellular//extracellular. For experiments investigating channel sensitivity to calcium, 0.96 mM $CaCl_2$ was added to the 150 KCl solution to give free Ca^{2+} of 4μM. Effective change of test solutions at the cytoplasmic surface of the patch in the inside-out configuration was obtained with a perfusion system similar to that described in [7]. Experiments were carried out at 22-25 °C.

Results and discussion

Of the over 200 successfully excised inside-out patches, only seven contained either of the K channels illustrated in Figs 1 and 2. These excised patches displayed no channel activity until they were depolarized for several seconds to voltages greater than +60 mV.

Figure 1 shows single channel recordings from one of the two types of K channels we report here. The identification of this channel as a K channel was based on the following observations: (a) When the patch was perfused with 150 KCl//150 NaCl (upper 5 records), the extrapolated reversal potential was near the electrochemical potential for K, which indicates that in these conditions K, but not Na or Cl is the major carrier of the current. (b) When KCl solution perfusing the cytoplasmic side of the membrane was replaced with a 150 NaCl solution (bottom record) elementary currents were no longer observed, further showing that K is the permeant ion through the channel. Current-voltage relation in symmetrical 150 KCl (not shown) gave single channel conductance of 80 pS.

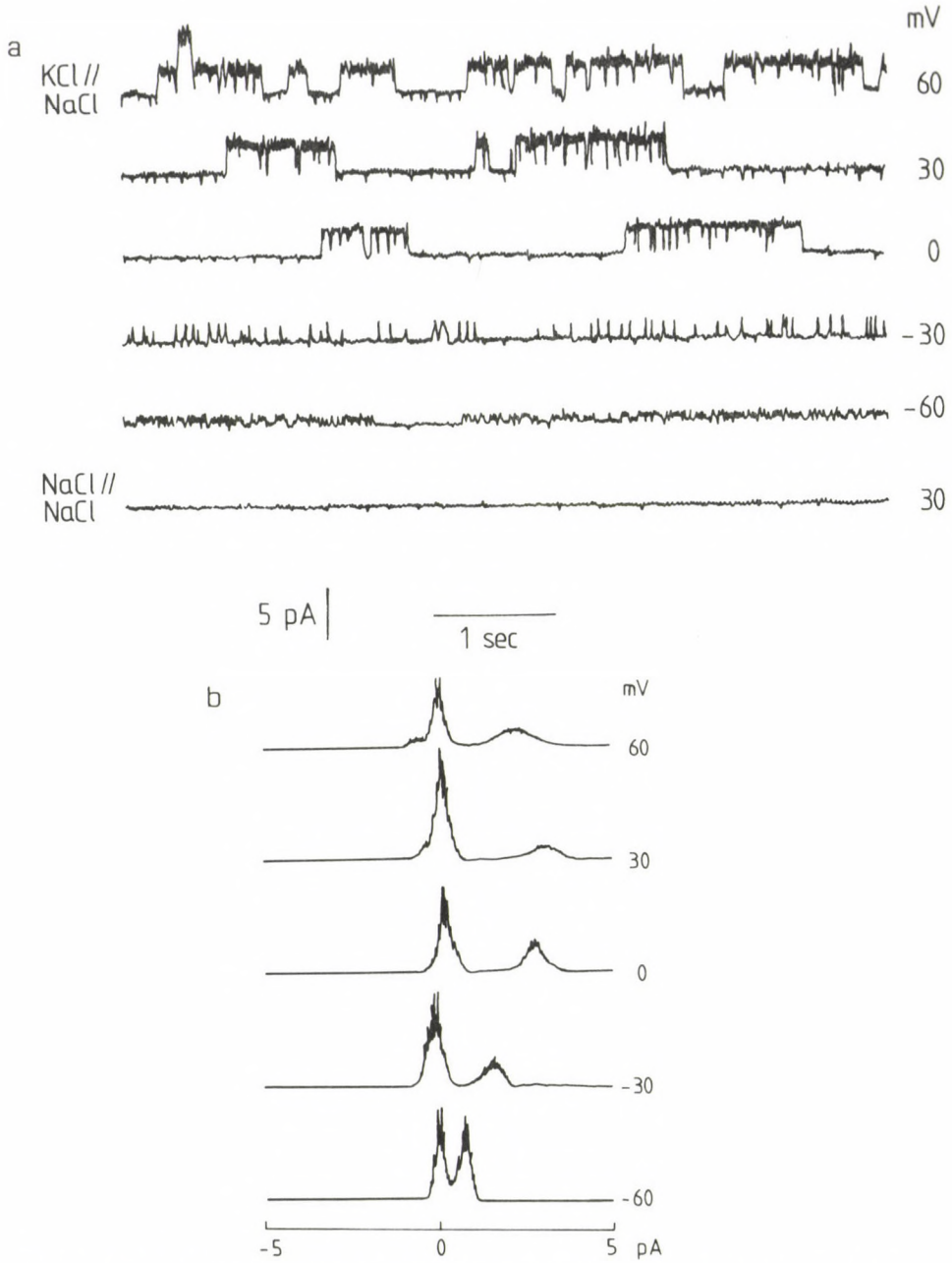

Fig. 1. (a) Single-channel recordings of the 80 pS (symmetrical 150 K) K channel at various voltages (indicated), from an excised inside-out patch of hippocampal neuron of rat. Perfusion conditions were 150 KCl//150 NaCl (top five traces), and symmetrical 150 NaCl (bottom trace). Records were filtered at 1 kHz; calibration as indicated. Outward currents are upwards. (b) Current amplitude histograms constructed from 6–36 s stretches of single-channel recordings at different voltages. The corresponding segments in (a) contributed to the histograms. The measured currents, sampled every 400 μs, were binned at 0.05 pA resolution, and the number of observations in each bin plotted against the current range for each bin

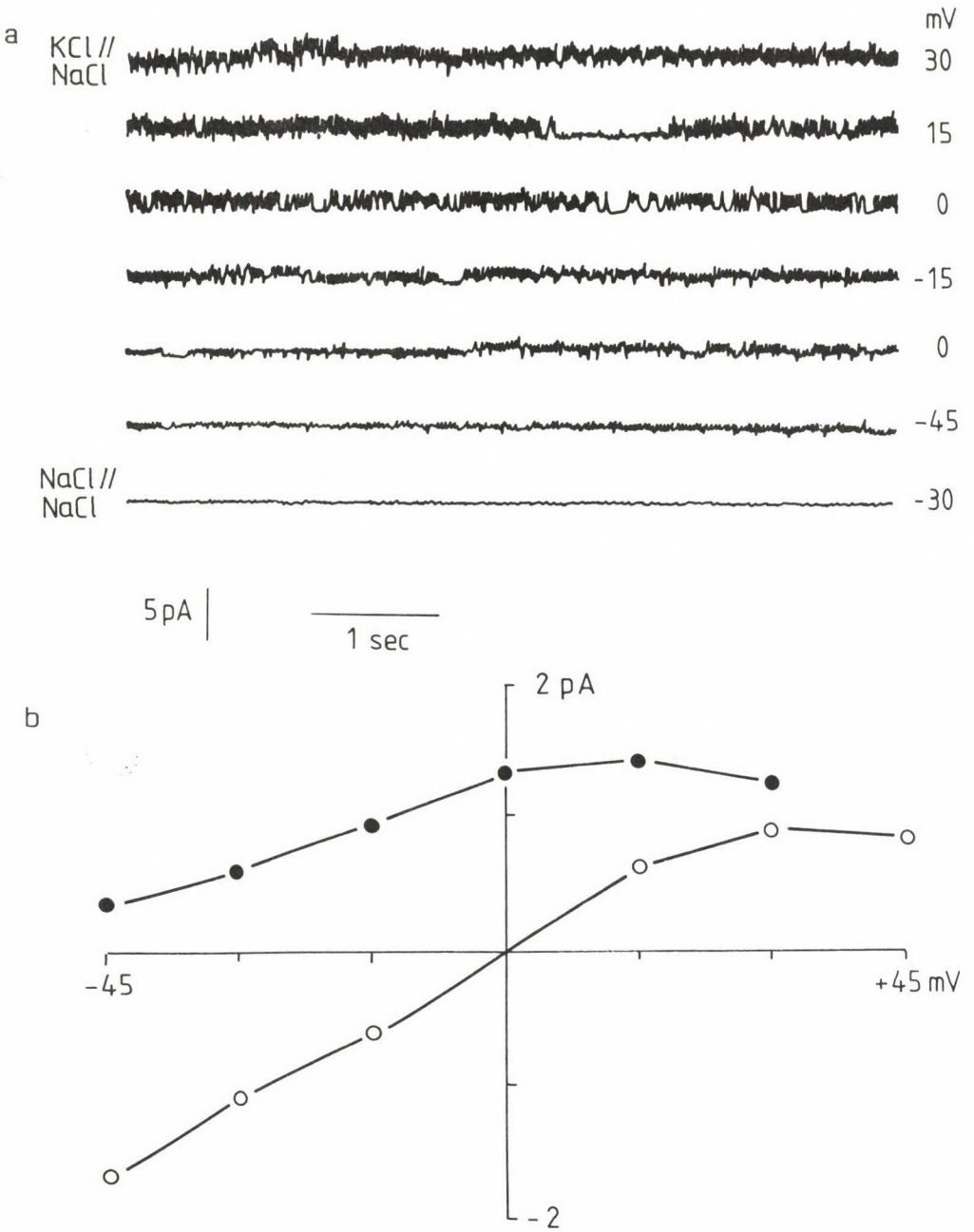

Fig. 2. (a) Single-channel recordings of the 40 pS (symmetrical 150 K) K channel at various voltages, obtained from an excised inside-out patch of hippocampal neuron of rat. Perfusion conditions were 150 KCl//150 NaCl (top six traces), and symmetrical 150 NaCl (bottom trace). Filter setting was 1 kHz; calibration as indicated. (b) Filled symbols show the current-voltage relation in 150 KCl//150 NaCl, obtained from the data shown in (a). Empty symbols are from the same patch, and show the i/V relation in symmetrical 150 KCl. Single-channel currents were measured as interpeak distance from amplitude histograms (not shown)

The channel is modulated by a gating process with complex kinetics. This is illustrated in Fig. 1a showing that the channel can enter phases with markedly different kinetics. The standard kinetics of the channel, that is, the mode in which the channel spends most (>95%) of the time, is illustrated by the upper three traces. It is characterized by the presence of long openings and closures, and no bursting episodes. Alternative modes can be seen from the recording at −60 mV where openings and closures are very short and densely grouped to generate a bursting behavior, and from the recording at −45 mV featuring instead needle-like openings separated by very long closures. (In the latter mode the open probability is markedly lower than in the other modes.)

Another characteristic of this channel, already noticeable from the single-channel records, is more clearly illustrated in Fig. 1b which plots the amplitude histograms of long stretches of single channel recordings at various voltages. The histograms show that single channel current increases with voltage until a certain point; at voltages above this point the current does not just level off, as expected, instead it decreases. Ionic current always saturates as the concentration of the permeant ion or the electromotive force is increased. This occurs because channel binding sites, like enzymes active sites, take a finite time to translocate an ion from one side of the membrane to the other, with the result that a maximum ion flux rate is reached when the binding site, due to high ion concentration, becomes permanently busy.

Although this phenomenon readily explains how a channel's conductance may saturate, it does not give insight into the decrease of single-channel current at positive voltages, as shown in Fig. 1b. Saturation and eventual decrease of macroscopic potassium conductance has been shown to occur in squid axon following elevation of ion (external K) concentration [16]. Decrease in reaction velocity and non compliance with the Michaelis equation is reported to occur also in enzyme kinetics, and usually attributed to inhibition by high substrate concentration. We planned to test this hypothesis by increasing the concentration of permeant K ions to see whether in this condition the i-V relation would begin levelling off at lower voltages as expected if ions crowding the channel's binding site were causing the inhibition of the ion flux. Because of the rare occurrence of this channel, we were not able to carry out these experiments.

Representative single channel recordings from the other K channel that we found in hippocampal neurons are shown in Fig. 2a. The identification of the channel as a K channel was based on the same observations as before, that is, under perfusing conditions of 150 KCl//150 NaCl, the extrapolated reversal potential was near the electrochemical potential for K, and in symmetrical 150 NaCl channel current was completely eliminated.

The kinetics of this channel is markedly different from the standard kinetics of the K channel just described. Channel activity occurs in bursts that last for hundreds of milliseconds, and are separated by long closures. Within the bursts, openings and closures are very short and not fully resolved under our recording conditions.

As before, the current-voltage relation obtained in 150 KCl//150 NaCl shows a negative slope at high positive voltages. The fast kinetics of this channel that prevents full resolution of a considerable number of open and closed events very likely causes this result. At more positive voltages, openings and closures become shorter, thus more severely chopped by filtering. Therefore, the reduction in the channel current amplitude is probably due to the increasing number of unresolved discrete channel openings and closures. Negative slope in the i-V relation is also obtained in symmetrical 150 KCl (not shown). In these ionic conditions, slope conductance is 40 pS (Fig. 2b).

Conclusions

This paper reports two new types of potassium channels in cultured hippocampal neurons of rat. Because of their rare occurrence in excised membrane patches, a preliminary description of some of their features was possible. Therefore, rather than summarizing the scant biophysical information on the channels already discussed in the previous Section, here we will address more broad topics that the data presented above inspire: (a) The functional role of steady state K channels. (b) The significance of the high number of different types of K channels.

Potassium channels are thought to have two main functions. (a) They repolarize a depolarized cell after an action potential. (b) They stabilize the membrane potential by moving it towards the potassium equilibrium potential and further away from the firing threshold. Different types of K channels account for these functions. The repolarization of the membrane is primarily accounted for by two types of K channels which are activated [11] by membrane depolarization (delayed rectifiers), and [1] by an increase in intracellular calcium (Ca-activated K channels).

A stabilizing function for K channel would instead involve any K channel with a significant probability of being open in steady-state conditions at voltages near the resting membrane potential. Virtually all Na channels are closed at rest. Therefore open K channels will increase potassium permeability to values much greater than sodium permeability. The membrane voltage approaches the Nernst potential for K. For a long time the best example of K channel serving this function has been the inward rectifier. Recently, however, several K channels capable of undertaking this task, at least in principle, have been reported, and this paper provides two further examples [4, 8, 9, 12–14].

The major question then to be addressed is why there are so many K channels with the same basic characteristics and presumably the same function. This dilemma could be partly resolved by the possibility that differences among these K channels may arise by unsuspected contributions from experimental methodology, as suggested by Bretag [3] for Cl channels. If this were the case, the steady-state K channels could be much more similar to each other than experimental results indicate: in other words, there would be fewer K channel types than presently thought. This possibility appears plausible due to the high and ever increasing number of different types of K channels being described. So, it is very difficult to satisfactorily characterize each of them, to assess their individual contribution to the total membrane current, and to understand their specific function. This creates a vicious circle: the more channel types, the poorer their characterization and the greater the chance of experimental errors. So, more types of channels are being described than actually exist.

Alternative splicing, which is known to be an ubiquitous mechanism for the generation of multiple protein isoforms [2], could also be considered a possible cause of the high number of steady-state K channels described. Indeed, this mechanism was shown to produce two distinct K channel types responsible for the two different A current components [15]. However, until the organization of the genes coding for the steady-state K channels and their transcription mechanisms are clarified, alternative splicing cannot be considered the mechanism which explains the diversity of these K channels.

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IMPACT OF SINGLE NEONATAL ALLYLESTRENOL TREATMENT ON THE ESTRUS CYCLE OF RATS TREATED WITH FSH + LH OR TSH

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Neonatal allylestrenol treatment administered to female rats significantly increases the duration of estrus phase in the sexual cycle. Treatment with follicle stimulating hormone (FSH) + luteinizing hormone (LH) in adulthood prolongs the duration of estrus even on its own; the effect, however, is more pronounced in those animals who were treated (imprinted) with allylestrenol neonatally. When administered to the control animals, the chemically related thyreotrop hormone (TSH) is either indifferent or it even decreases the estrus index. In animals having received neonatal allylestrenol treatment, however, TSH administration increases significantly the duration of the estrus phase. Either with or without FSH + LH treatment, the ratio of estrogenic to gestagenic phase increases following neonatal allylestrenol treatment. The experiments call attention to the potential functional risks inherent in neonatal allylestrenol treatment. The actual risks, however, seem to be smaller than the effects seen at the receptor level.

Keywords: perinatal imprinting, steroid treatment, FSH + LH, TSH, estrus cycle

The quality of the hormone receptors is programmed at the gene level. Nevertheless, both the permanent structure and the maximal number of receptors evokable in adulthood evolve in parallel with the maturation of the cell – or, in the case of membrane receptors, in parallel with the maturation of the cell membrane – [3]. In mammals the relation between the hormone and its receptor is immature at birth, and the maturation of the receptor is completed in the presence of the hormone characteristic in its quality and quantity to the given individual [4]. The first interaction of the hormone and the receptor takes place in the perinatal period, usually after birth, and is known as hormonal imprinting. As an effect of this

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imprinting, the receptor completes its maturation and reaches the binding capacity characteristic to adulthood, i.e. secures the characteristic responsiveness of the cell [5].

The neonatal period represents critical phase for the maturation of the receptor. This period is of special importance, since if during this period molecules are present which, however, differ from the proper hormone but are able to bind to the receptor, faulty imprinting may occur and it may evoke life-long alterations of the binding capacity of the receptor and, thus, of the response capability of the cell [6]. A single highdose gonadotropin treatment was sufficient to induce life-long decrease of the binding of the related TSH hormone to the receptors of the thyroid gland [7]. Similarly, a single dose of oxytocin administered in the critical period resulted in life-long modification of the response to vasopressin [8]. The phenomenon of imprinting occurs not only in the case of membrane receptors but in the case of cytosol receptors as well; a single neonatal dexamethasone treatment was able to exert permanent effect on the binding capacity of thymic glucocorticoid receptors [13].

In the critical phase of the maturation of the receptor hormonal overlaps may occur. Or to put it in another way, a given steroid hormone may influence not only its own receptor but the similar receptors in other organs as well [14].

In order to protect the endangered pregnancy, numerous pregnant women have received steroid treatment. Previously diethylstilbestrol (DES) treatment was used which – after the realization of its cancerogenic effect on the descendants [2, 11, 12] – has been replaced by allylestrenol therapy. Under experimental circumstances, however, even allylestrenol treatment did not seem to be harmless. Single neonatal allylestrenol treatment decreased the binding capacity of the uterine estradiol receptors in adults to its half [9]; moreover, the glucocorticoid receptors of the thymus also changed in a similar, i.e. overlapping manner [14]. Due to postreceptor mechanisms and compensatory reactions, the actual values characteristic to the binding and influence are not always the same. Therefore it seemed to be necessary to investigate just how far neonatal allylestrenol treatment can influence the estrus cycle of adult rats. In order to clarify also the quality of the effect, the changes of estrus cycle were determined both under the influence of gonadotropins and the overlapping thyreotropic hormone.

Materials and methods

Newborn Wistar rats were treated within 24 hours after birth with a single subcutaneous dose of 1.75×10^{-2} mg allylestrenol (Turinal, Richter, Budapest) dissolved in 0.1 ml sunflower seed oil. The control rats did not receive any treatment. At the age of 5 months, 3x12 female animals were selected both from those having received neonatal treatment and from the controls. One group was left untreated (control), the animals of the other group received FSH+LH (Pergonal, HUMAN, Budapest) treatment

in a dose of 5 IU/animal for 12 days, whereas the animals of the third group were treated with TSH (Ambinon, Organon, Oss) in a dose of 2 IU/animal administered also for 12 days.

Twelve days before, during and 6 days after the treatment administered in adulthood vaginal smears were obtained from the rats. The smears were washed into water drops, stained with toluidine blue and evaluated every day. All smears were classified according to the phases of the rat estrus cycle. The smears were obtained always at the same times of the day (9 a.m.) [10]. Chronic treatment began on the 12th day, i.e. already being aware of the results of the preliminary investigations on the cycle, and was performed in the form of subcutaneous injections. The total duration of the experiment was 30 days.

For numerical evaluation of the results, the phases of the cycle were characterized by numbers. Estrus phase of one day duration was marked with 1, that of two days duration with 2, etc. The sum of these numbers gave the total duration spent in estrus during the period investigated. The average duration of estrus (further: duration of estrus) was calculated by dividing the total duration of estrus with the frequency numbers. The average frequency rate was calculated by dividing the number of the frequency of estrus with the animals' number multiplied with the duration of the experiment in days. The ratio of estrogenic to gestagenic phase (on the basis of the literature [10] the phase characterized by the preponderance of estrogens was regarded as estrogenic, while the phase characterized by the preponderance of gestagens as gestagenic phase) is represented by the quotient of the two values. In the case of the duration of estrus, the numerical data were compared with the help of the two-tailed "t" test.

Results

Duration of estrus. During the first 12 days, the duration of the estrus of the animals untreated in the neonatal period was 1.27 day, on the average. During the second 12 days and the 6 days after that, further increase could be detected. During the first 12 days, the duration of estrus was significantly longer (1.44 day) in the animals treated neonatally with allylestrenol than in the untreated controls. Later on, however, no further significant increase could be observed (Fig. 1).

In the animals treated with FSH+LH (but left untreated in the neonatal period) the duration of estrus increased significantly during the treatment, while after the treatment it decreased – albeit not significantly – belows the control level. In the animals having received neonatal allylestrenol treatment, FSH+LH treatment resulted in markedly pronounced increase of the duration of the estrus, both when compared to its own control and to those animals who did not receive neonatal treatment but were treated with FSH+LH in adulthood. The increase, however, was not significant when compared to the latter.

TSH treatment did not influence the duration of estrus in the group untreated in the neonatal period, meanwhile during the treatment the duration of estrus increased significantly in the animals having received neonatal allylestrenol treatment.

Frequency of estrus. As far as the frequency of estrus was concerned, no difference of considerable extent could be observed between the animals treated or not treated with allylestrenol in the neonatal period. Some differences were observed in the groups treated with FSH+LH, depending on the fact whether the animals received neonatal treatment or not (Fig. 2).

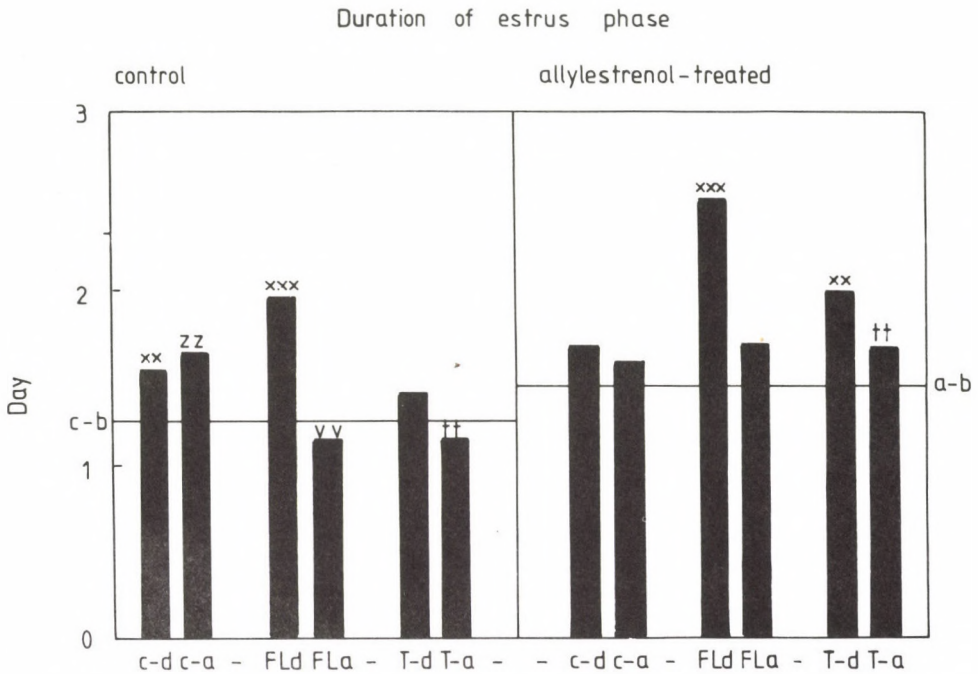

Fig. 1. Duration of estrus in the controls (c) and in the rats treated with FSH+LH (FL) or TSH (T), untreated neonatally or imprinted with allylestrenol, during (-d) and after (-a) the treatment in adulthood. c-b = untreated neonatally, before the treatment in adulthood, a-b = neonatally treated with allylestrenol, before the treatment in adulthood. Significance: c-b → a-b = $p < 0.01$; x = significance between the results obtained before and during the treatment in adulthood, v = significance between the results obtained during and after the treatment in adulthood, z = significance between the results obtained before and after the treatment in adulthood, t = significance between treated and untreated with allylestrenol neonatally. The increasing number of the letters indicates the degree of significance in the order of 0.05, 0.01 and 0.001

Ratio of estrogenic to gestagenic phase. During the first 12 days, i. e. before the initiation of treatment, differences could be observed between the animals treated or not with allylestrenol in the neonatal period. The ratio shifted in the neonatally treated animals towards the estrogenic phase. Similarly, the duration of the estrogenic phase increased in the animals having received neonatal treatment, both during FSH+LH treatment and after TSH therapy (Fig. 3). Estrogenization was more marked after FSH+LH treatment in the imprinted group than in the controls; the difference, however, occurred somewhat later during the treatment (on the 5th day instead of the 2nd).

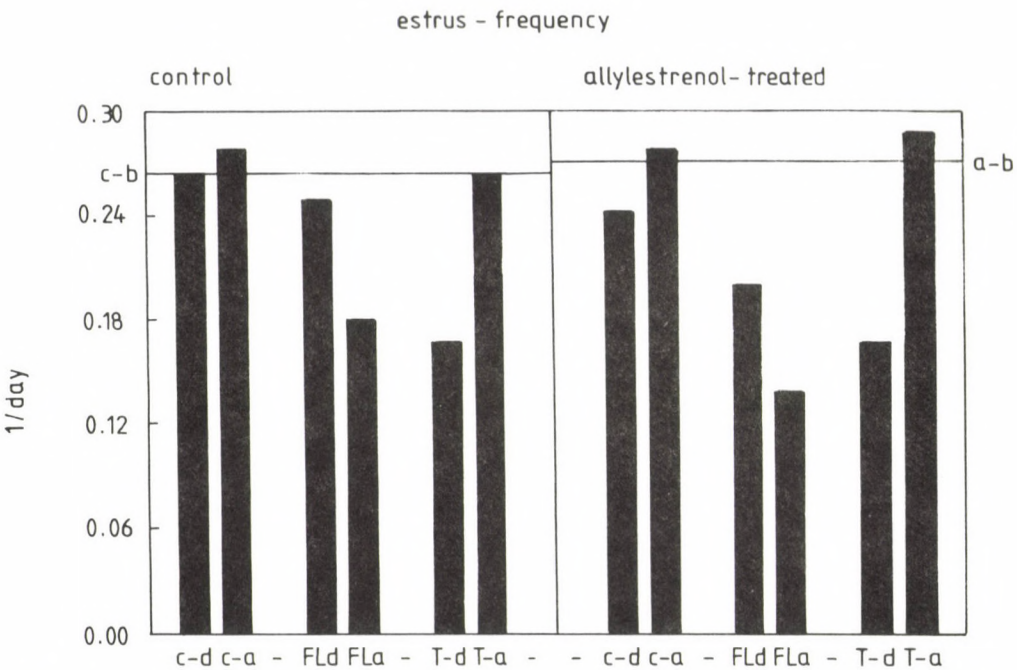

Fig. 2. Frequency of estrus in the controls and in the rats treated with FSH+LH or TSH, untreated neonatally or imprinted with allylestrenol, during and after the treatment in adulthood. For abbreviations see Fig. 1

Discussion

The experiments have rendered possible several aspects to be simultaneously investigated. On the one hand, the effect of neonatal allylestrenol treatment on the functions in adulthood could be investigated and compared to the results of previous investigations on the binding of receptors. On the other hand, it was also studied just how far the neonatal treatment could influence the effect of the trophormone in adulthood, namely, the effect of FSH+LH acting specifically on the ovarium as well as the effect of the chemically related and – in the critical period – overlapping TSH.

On the basis of the experiments it can be drawn the conclusion that neonatal allylestrenol treatment increases significantly the duration of estrus even on its own; i.e. even without further treatments. However, the increase is mathematically significant, from the biological point of view it does not seem to be relevant. Treatment with FSH+LH, which prolongs the duration of estrus even on its own, results in even more pronounced increase in the animals having received neonatal allylestrenol treatment. Though the increase is mathematically insignificant when

compared to the animals left untreated neonatally; biologically it may be significant, partly because it could be detected both during and after the treatment and partly because it could be observed also with respect to the frequency of estrus and the estrogenic/gestagenic ratio.

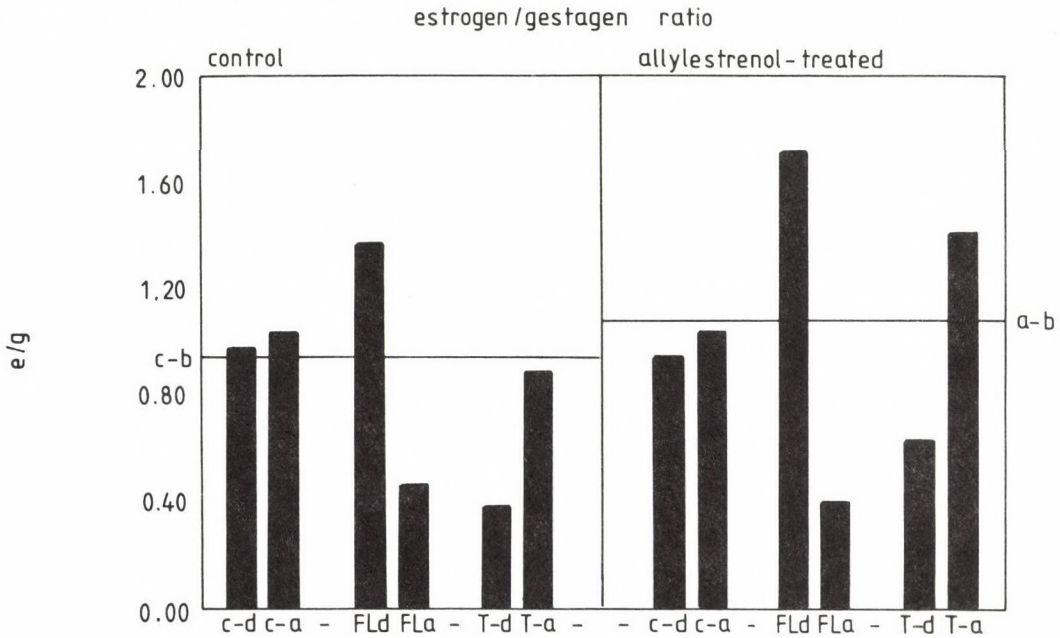

Fig. 3. Ratio of estrogenic to gestagenic phase in the controls and in the rats treated with FSH + LH or TSH, untreated neonatally or imprinted with allylestrenol, during and after the treatment in adulthood. For abbreviations see Fig. 1

In our previous experiments it has been demonstrated [9] that neonatal allylestrenol treatment decreased the adult number of uterine estradiol receptors by 50%. When this previous observation is compared to the present results, the conclusion may be drawn that the changes of binding are more pronounced than those detectable functionally, i.e. on the basis of the cycle. This difference is presumably due to compensatory mechanisms possessed either by the organism or the receptor-bearing cell itself. These compensatory mechanisms could have evolved during the 5 months (or even earlier) following the neonatal hormone treatment. On the other hand, when it is also taken into account that the functional alteration developed as the consequence of but one neonatal treatment, the difference is definitely not to be underrated.

As far as perinatal imprinting is concerned, the chemically related pituitary hormones, i.e. FSH + LH and TSH exert overlapping effect. The alfa subunit of these

hormones is identical and it is the slight difference in the beta subunit which is responsible for the different action [1, 15]. The previous investigations have indicated [7] that these hormones are able to disturb the effect of one another on the immature receptor. The present investigations, however, seem to show that neonatal allylestrenol treatment modifies not only the effect of FSH + LH but makes the receptor more sensitive to the effect of TSH as well. The problem is of special interest, since the effect of FSH + LH is manifested in the modification of the steroid synthesis in the ovarium, and it is the newly synthesized hormone which acts on the uterine steroid receptors having suffered damages as a consequence of neonatal allylestrenol treatment. Considering that the effect of TSH on the uterus is indirect, it can be assumed that neonatal allylestrenol treatment exerted some effect on the ovarium, too, and it was this effect which made the ovarian steroid-producing cells more sensitive to TSH.

Allylestrenol (Gestanon-Organon; Turinal-Richter) has been widely used for the protection of endangered human pregnancies. Certainly, it is always difficult to draw conclusions from animal experiments to humans. It is especially so in the present case, since the placentae are highly different; nevertheless, steroid hormones are known to pass the placenta both in humans and in rats. It should be emphasized that beside the alterations at the membrane level and in the behaviour, also functional changes in the cycle could be detected as an effect of allylestrenol treatment administered in the critical neonatal period. It seems to be worthwile even for the clinical praxis to carry out a follow-up study lasting to adulthood concerning/monitoring reactions of those who received allylestrenol treatment *in utero*.

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HEMODYNAMIC OBSERVATIONS FOLLOWING ORTHOTOPIC CARDIAC TRANSPLANTATION: HEMODYNAMIC RESPONSES TO UPRIGHT EXERCISE AT 1 YEAR*

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Central hemodynamic responses during upright exercise were studied at 1 year in 40 orthotopic cardiac transplant recipients. Hemodynamic responses were characterized by slow rise in heart rate and blunted peak exercise heart rate response, a significant early increase in stroke index followed by a plateau phase, and a steady increase in ventricular filling pressures and pulmonary artery pressure. In spite of exclusive utilization of the Frank-Starling mechanism to augment cardiac output during early exercise, the pressure responses were comparable to those reported in normal subjects. Our observations also indicate that similarly to normal subjects, the heart rate response plays an important role in the cardiac output achieved at maximum exercise.

Although patients with younger donor hearts achieved a more favorable maximum heart rate, the other hemodynamic parameters showed no correlation with the donor heart age. Thus, no hemodynamic disadvantage of older donor hearts could be demonstrated. These data provide further enlightenment regarding the mechanisms of the well-preserved functional capacity noted in these patients.

Keywords: cardiac transplantation, hemodynamics

The unique characteristics of resting central hemodynamics and the hemodynamic responses during supine exercise have been exhaustively studied after cardiac transplantation [2, 3, 6, 8–11, 14]. There is evidence that exercise in the

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upright posture, in the setting of an initially low pre-load, is hemodynamically more favourable in this group of patients [12]. In order to further characterize the hemodynamic responses to upright exercise, 40 patients were studied at 1 year after cardiac transplantation.

Methods

Patient demographics

The patient group consisted of forty 1 year survivors of orthotopic cardiac transplantation (36 male and 4 female). The mean age at the time of transplantation was 51 ± 10 years (range 23 to 61) and the mean donor heart age was 32 ± 12 years (range 14 to 53). Twelve patients (30%) were treated by a calcium antagonist (diltiazem or nifedipine) at 1 year and 10 patients (25%) were treated for hypertension with an angiotensin converting enzyme inhibitor. No patient was being treated with a beta blocker, Digoxin, or Verapamil at the time of the study. Medications were not routinely withheld at the time of hemodynamic assessments.

Hemodynamic measurements

Hemodynamic assessment followed routine endomyocardial biopsy performed through the right internal jugular vein. A flow directed thermodilution catheter was advanced into a branch of the right pulmonary artery where inflation of the balloon resulted in a satisfactory pulmonary wedge pressure tracing. Following measurement of supine resting hemodynamics, the patient was seated in an upright bicycle ergometer for repeat measurement of resting hemodynamics. The pressure transducer was positioned at the mid-axillary line supine [2, 12] and at the level of the fourth intercostal space in the sitting position [7, 12, 15]. Pressure measurements included pulmonary artery systolic, diastolic, and mean pressures, mean pulmonary capillary wedge pressure, and mean right atrial pressure. Cardiac output measurements were performed in duplicate or triplicate with a Criticon thermodilution cardiac output computer to obtain values in agreement by 10%. Both cardiac output and stroke volume were corrected for body surface area and are expressed as the indices. Pulmonary vascular resistance was calculated with the formula: pulmonary vascular resistance (Wood units) = (mean pulmonary artery pressure − mean pulmonary capillary wedge pressure)/cardiac output. The trans-pulmonary gradient was calculated with the formula: trans-pulmonary gradient = mean pulmonary artery pressure − pulmonary capillary wedge pressure. Arterial blood pressure was obtained using a Dinamap automated sphygmomanometer. The mean arterial blood pressure was calculated with the formula: mean arterial blood pressure = diastolic blood pressure + 1/3 (systolic blood pressure − diastolic blood pressure). Systemic vasculart resistance was calculated using the formula: systemic vascular resistance (Wood units) = (mean arterial blood pressure − right atrial pressure)/cardiac output.

Exercise protocol

Exercise was begun at a workload of 25 watts and increased by an additional 25 watts every 3 minutes until exercise was terminated at the point of fatigue. Patients were encouraged to complete full exercise stages. Hemodynamic measurements were obtained during the last 1 1/2 minutes of each exercise stage in the following sequence: pulmonary artery phasic and mean pressures, mean pulmonary capillary wedge pressure, mean right atrial pressure, and cardiac output. The heart rate was recorded at the time of cardiac output measurements. Hemodynamic measurements were repeated 2 to 3 minutes after completion of the exercise protocol while the patient remained in the upright sitting position.

Data analysis

Data are expressed as mean values ± 1 standard deviation. The highest exercise stage achieved was defined as maximum exercise. The hemodynamic responses to the change in posture and to exercise were assessed by a two-tailed paired *t*-testing. The effect of donor heart age on heart rate responsiveness was assessed by linear regression analysis. A p value <0.05 was considered significant.

Table I

Hemodynamic responses to postural change and exercise

	Supine	Upright	Ex 1	Ex 2	Maximum	Recovery
HR (beats/min)	93±12	100±15**	106±16¶¶	113±16¶¶	131+17¶¶	120±13++
SBP (mmHg)	149±17	147±17¶	154±17¶	161±18¶¶	169±24¶¶	153±19++
DBP (mmHg)	93±12	91±15	95±12	95±13	95±18	86±13+
MBP (mmHg)	112±11	110±14	115±12¶	117±13	120±17¶¶	108±13++
CI ((L/min/m²)	3.2±0.6	2.7±0.4**	4.0±0.5¶¶	4.8±0.6¶¶	5.9±1.1¶¶	3.5±0.7++
SI (ml/m²)	35±6	27+6*	39±8¶¶	43±9¶¶	46±10¶¶	29±6++
PAS (mmHg)	29±7	24±6**	33±8¶¶	39±10¶¶	46±11¶¶	30±10++
PAD (mmHg)	13±3	12±4	16±4¶¶	19±5¶¶	22±6¶¶	14±5++
PAM (mmHg)	20±5	18±4*	24±5¶¶	28±7¶¶	32±7¶¶	22±7++
PCWP (mmHg)	10±3	6±4**	11±5¶¶	14±56¶¶	15±6¶¶	6±4++
RA (mmHg)	5±2	2±2**	4±3¶¶	6±4¶¶	7±4¶¶	2±3++
PVR (Wood units)	1.6±0.6	2.2±0.7**	1.7±0.5¶¶	1.7±0.6¶¶	1.6±0.6¶¶	2.3±0.7++
SVR (Wood units)	18±3	22±4**	15±3¶¶	12±2¶¶	10±3¶¶	17±4++

means ± 1 SD; *$p<0.05$ and **$p<0.001$ supine vs upright, ¶$p<0.05$ and ¶¶$p<0.001$ exercise vs upright, +$p<0.05$ and ++$p<0.001$ recovery vs maximum exercise; CI = cardiac index, DBP = diastolic blood pressure, HR = heart rate, MBP = mean blood pressure, PAD = pulmonary artery diastolic pressure, PAM = pulmonary artery mean pressure, PAS = pulmonary artery systolic pressure, PCWP = pulmonary capillary wedge pressure, PVR = pulmonary vascular resistance, SBP = systolic blood pressure, SI = stroke volume index, SVR = systemic vascular resistance

Results

Rest hemodynamics

Resting hemodynamics are summarized in Table I. Systemic hypertension was common in this population. Thirty-two patients (80%) had a resting supine blood pressure > 140/90 mmHg.

Exercise duration

The mean exercise duration was 10.5±2.8 minutes (range 3–15 minutes). One patient performed 1 exercise stage, 5 patients performed 2 stages, 17 patients 3 stages, 13 patients 4 stages, and 4 patients performed 5 exercise stages.

Postural changes (Table I)

On active transition from the supine posture to sitting on the bicycle ergometer, the heart rate increased by 7±5 beats/minute within the first 60 seconds. There was a modest negative correlation between the donor heart age and the postural heart rate response ($R = -0.35$, $p < 0.05$). The change in posture also resulted in a significant decrease in cardiac index and stroke index. Both the pulmonary artery pressure and ventricular filling pressures dropped significantly and there was an associated increase in both the pulmonary vascular resistance and systemic vascular resistance.

Exercise response (Table I)

Heart rate rose slowly with exercise with a limited (32%) increase at maximum exercise. Again, there was a significant relationship between donor heart age and the maximum heart rate achieved (Fig. 1). The extent of heart rate acceleration from rest in the sitting position to maximum exercise was not related significantly to donor heart age. There was a marked and rapid increase in stroke index with exercise. Excluding the patient who achieved only 1 exercise stage, 65% of the exertional increase in stroke index occurred during the first exercise stage (Fig. 2). There was a tendency for a plateauing in stroke index with advanced stages of exercise and only 8% of the increase in stroke index occurred during the final exercise stage (Fig. 2). At maximum exercise, there was a 2.2 fold increase in cardiac index. Pulmonary artery pressure increased significantly during exercise by nearly 100%. Similarly, the pulmonary wedge pressure increased by 130% and the right atrial pressure by 230% at maximum exercise. A significant decrease in both the pulmonary and systemic vascular resistance was seen throughout the exercise.

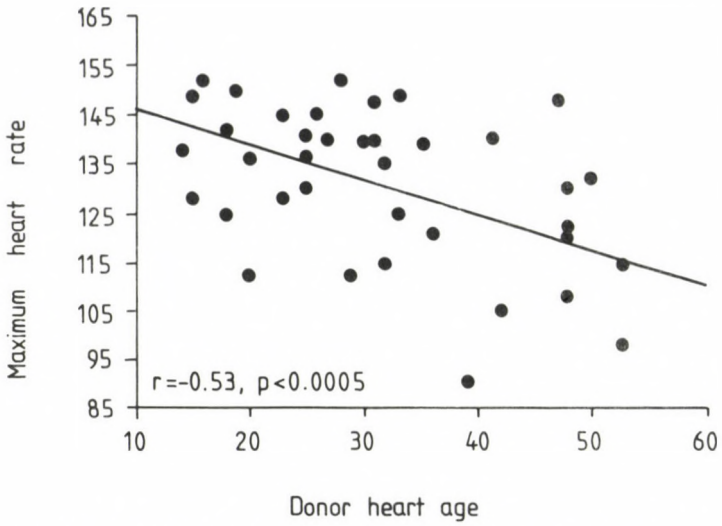

Fig. 1. Relationship of maximum exercise heart rate and donor heart age

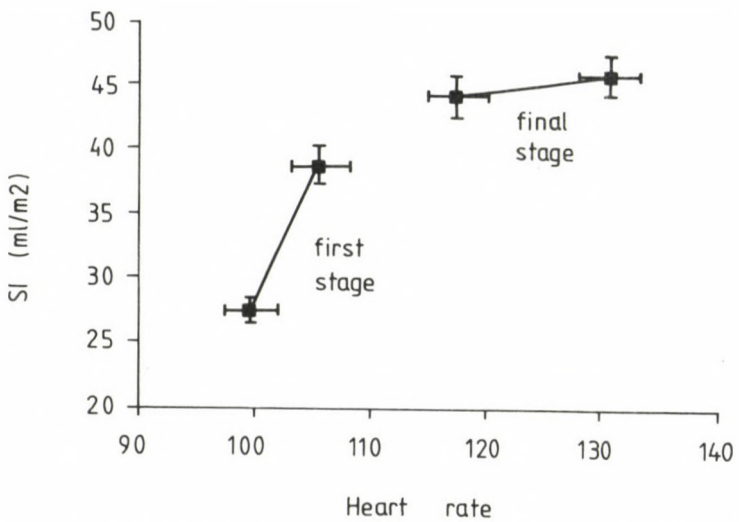

Fig. 2. Relationship of heart rate and stroke index during early and late exercise

Recovery post exercise (Table I)

The stroke index, pulmonary artery pressure, and ventricular filling pressures rapidly returned to near baseline upright values. Cardiac index and heart rate, however, both remained considerably higher than the baseline values. Heart rate deceleration after exercise (-11 ± 12 beats/minute) again showed correlation with the donor heart age ($R = -0.41$, $p < 0.001$). The deceleration was also related to the maximum heart rate and by multiple regression analysis, only the maximum heart rate retained significance.

Discussion

Role of posture in post-transplant exercise hemodynamics

It has been well documented that the transplanted heart utilizes the Frank–Starling mechanism in augmenting cardiac output during the early stages of exercise [2, 4, 5, 6, 9–12, 14]. The major determinant of this phenomenon is an abnormally blunted heart rate response [12, 14], analogous to the response of patients exercising with a fixed pacemaker rate [1]. In heart transplant recipients, both the heart rate and contractility increase as a result of increased levels of circulating catecholamines during latter stages of exercise [8, 9].

Most prior observations on exercise hemodynamics in transplant recipients have focused on supine exercise [2, 6, 8–11, 14]. In the supine resting position, venous return is significantly higher than in the upright position. Further volume augmentation with exercise, in the presence of diastolic dysfunction, results in markedly elevating filling pressure [12]. In the upright position, where pre-load is initially low, volume augmentation during exercise occurs along a flatter portion of the pressure/volume relationship. This serves to explain the more physiologic hemodynamic responses during upright exercise. The ventricular filling pressure and pulmonary artery pressures at maximum exercise in this study are comparable to those reported in normal subjects using a similar exercise protocol [7, 15]. In addition, similar to normal subjects, a plateau in stroke index during advanced exercise was documented in this study [7].

Relation of donor heart age to heart rate responsiveness

In a prior study [13] time post transplantation was a significant determinant of heart rate responsiveness. In the current study, a relationship between heart rate responsiveness to orthostatic stress and exercise was related to donor heart age. This association may reflect differential responsiveness to circulating catecholamines or a

difference of intrinsic responsiveness to the hemodynamic changes during exercise [13]. Although the donor heart age showed a negative correlation with the maximum heart rate, no such trends with regard to changes in cardiac index, stroke index, or ventricular filling pressures were found. Thus, no significant hemodynamic disadvantage of older donor hearts (up to 53 years) could be shown.

Hemodynamic responses to upright exercise, more representative of daily routine exertion than supine exercise, showed virtual normalization at 1 year after heart transplantation. These data provide further enlightenment regarding the mechanisms of the well-preserved function capacity noted in these patients.

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HEMODYNAMIC OBSERVATIONS FOLLOWING ORTHOTOPIC CARDIAC TRANSPLANTATION: EVOLUTION OF REST HEMODYNAMICS IN THE FIRST YEAR*

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The evolution of resting hemodynamics was studied at 1 week and 1 year in 40 patients following orthotopic cardiac transplantation. Abnormal hemodynamics after transplantation, characterized by elevated ventricular filling pressures (indicating diastolic dysfunction) and also by pulmonary hypertension, showed normalization by 1 year. In the absence of innervation, the resting heart rate of heart transplant recipients should theoretically be close to the intrinsic heart rate predicted by the age of the donor heart. In this study, a high incidence of relative sinus bradycardia (an indicator of sinus node dysfunction) was noted in the first post-operative week, although again there was a tendency to normalization by 1 year.

These beneficial changes help to explain the dramatic and sustained improvement in the functional capacity of these patients late after transplantation.

Keywords: cardiac transplantation, hemodynamics

With improved immunosuppressive therapy, particularly with the advent of cyclosporine, heart transplantation has become a well-established therapy for end-stage heart failure. Abnormal resting hemodynamics in the immediate perioperative period are well documented and are characterized by elevated ventricular filling pressures, elevated pulmonary artery pressure and pulmonary vascular resistance [3, 5, 6, 16, 20, 22, 24], and also by a high incidence of sinus node dysfunction [2, 12, 13, 14]. In order to determine the reversibility of these abnormalities and the time course

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of such changes, 40 patients were studied serially at 1 week and 1 year after orthotopic cardiac transplantation.

Methods

Patient demographics

The patient group consisted of forty 1 year survivors of orthotopic cardiac transplantation (36 male and 4 female). The mean age at the time of transplantation was 51±10 year (range 23 to 61) and the mean donor heart age was 32±12 years (range 14 to 53). Twelve patients (30%) were treated by a calcium antagonist (diltiazem or nifedipine) both at 1 week and at 1 year. One patient at 1 week and 10 patients (25%) at 1 year after transplantation received angiotensin converting enzyme inhibitor therapy. No patient was being treated with a beta blocker, digoxin, or verapamil at the time of the study. Medications were not routinely withheld at the time of hemodynamic assessments.

Immunosuppressive therapy

Long-term immunosuppresssive therapy consisted of cyclosporine and prednisone for all patients. The mean daily maintenance dose of prednisone was tapered from 1 mg/kg/day perioperatively to a mean of 6±2 mg/day by 1 year (range 2.5 to 12.5). The cyclosporine dose was adjusted to maintain through whole blood levels between 100 and 200 ng/ml by the whole blood monoclonal RIA method by 1 year. Seven patients at 1 week (18%) and 23 patients (58%) at 1 year also received azathioprine. None of the patients showed evidence of significant graft rejection at the time of the studies.

Hemodynamic measurements

The hemodynamic studies followed a routine endomyocardial biopsy performed through the right internal jugular vein. A flow directed thermodilution catheter was advanced into a branch of the right pulmonary artery where inflation of the balloon resulted in a satisfactory pulmonary artery wedge pressure tracing. In the supine position, the pressure transducers were positioned at the level of the mid-axillary line [16, 18]. Pressure measurements included pulmonary artery systolic, diastolic, and mean pressures, mean pulmonary capillary wedge pressure, and mean right atrial pressure. Cardiac output measurements were performed in duplicate or triplicate with a Criticon thermodilution cardiac output computer to obtain values in agreement by 10%. Both cardiac output and stroke volume were corrected for body surface area and are expressed as the indexes. Pulmonary vascular resistance was calculated with the formula: pulmonary vascular resistance (Wood units) = (mean pulmonary artery pressure − mean pulmonary capillary wedge pressure)/cardiac output. The trans-pulmonary gradient was calculated with the formula: trans-pulmonary gradient = mean pulmonary artery pressure − pulmonary capillary wedge pressure.

Data analysis

Data are expressed as mean values ± 1 standard deviation. Changes in hemodynamic parameters from 1 week to 1 year were assessed using the two-tailed paired t test. For assessment of the resting heart rate, a comparison was performed with the predicted intrinsic heart rate calculated using the formula of Jose et al. [11]: Intrinsic heart rate = 118.1 - (0.57 x donor heart age). Correlation between the predicted intrinsic heart rate and the recorded resting heart rate was assessed by linear regression analysis. Factors associated with changes in pulmonary artery pressure, pulmonary capillary wedge pressure, and pulmonary vascular resistance were also assessed by linear regression analysis. Non-parametric analysis was performed using the Chi-square test. A p value <0.05 was considered significant.

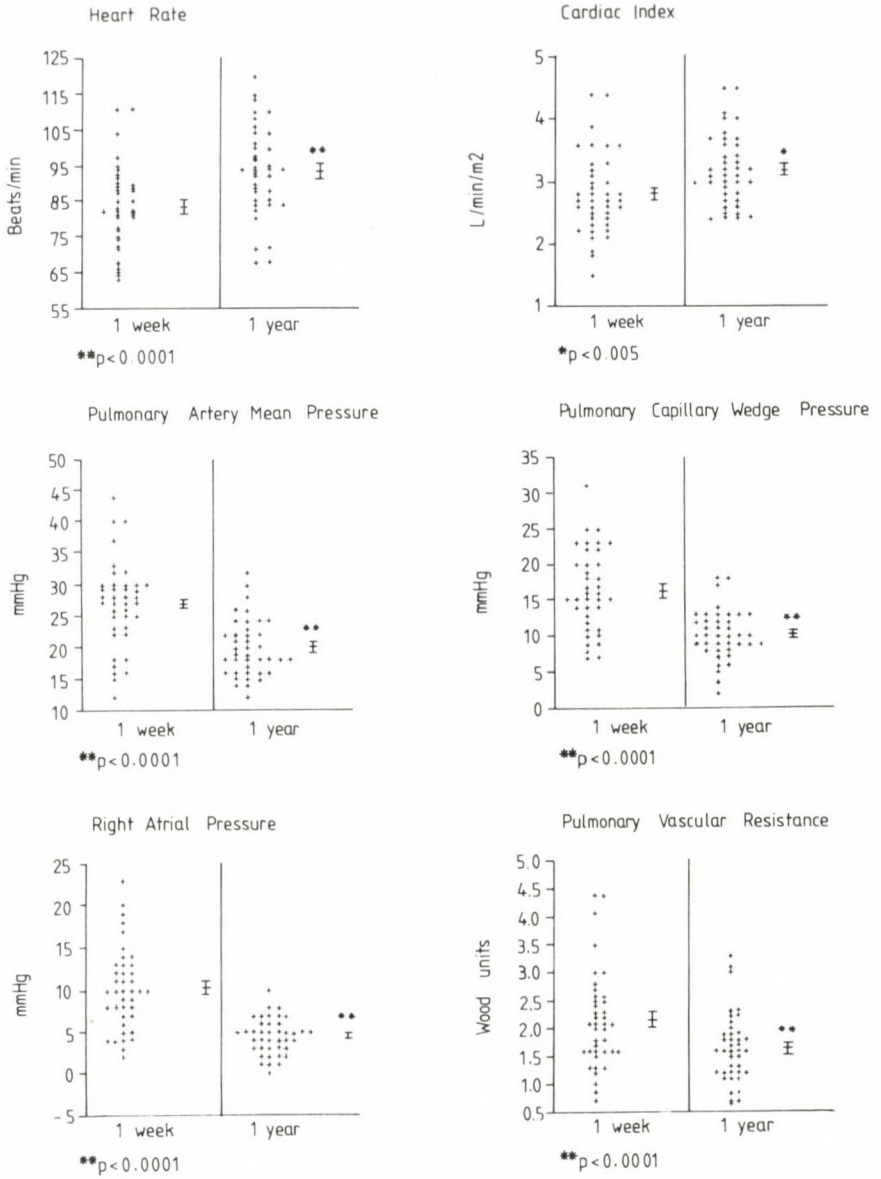

Fig. 1. Evolution of resting hemodynamics over the first year after cardiac transplantation

Results

Rest hemodynamics (Fig. 1)

The resting heart rate at 1 week was significantly lower than at 1 year and showed no correlation with the predicted intrinsic heart rate. The resting heart rate at 1 year showed only a modest correlation with the predicted intrinsic heart rate ($r = 0.37$, $p < 0.02$). Sinus node dysfunction, defined as a resting heart rate < 75% of the predicted intrinsic heart rate was present in 10 patients (25%) at 1 year and 4 patients (10%) at 1 year. The total ischemic time in subjects with sinus node dysfunction at 1 week (219±76 minutes) was not significantly different from the remainder (217±105 minutes). Similarly, the total ischemic time in those patients with sinus node dysfunction at 1 year was not different from the remainder of patients.

The stroke index at 1 week (34±7 ml/m^2) was not significantly different than that at 1 year (35±6 ml/m^2). As a result of a higher resting heart rate at 1 year therefore, cardiac index was significantly higher.

Elevated pulmonary artery systolic and diastolic pressures at 1 week decreased significantly by 1 year into the range of normal (29±7/13±3 mm Hg). A 25% reduction in pulmonary vascular resistance was observed over the first year after transplantation. Whereas at 1 week 23 patients (58%) had a resting pulmonary vascular resistance > 2 Wood units and 3 patients above 4 Wood units, by 1 year only 9 patients (23%) had a pulmonary vascular resistance > 2 Wood units and none had a pulmonary vascular resistance above 4 Wood units. By multiple regression analysis, the reduction in mean pulmonary artery pressure was significantly and independently related to both the changes in pulmonary capillary wedge pressure and pulmonary vascular resistance ($p < 0.001$). The pulmonary capillary wedge pressure and right atrial pressure both decreased significantly to a value within the normal range (10±3 mm Hg and 5±2 mm Hg, respectively) by 1 year after transplantation.

Discussion

In this study we have documented normalization of resting central hemodynamics after cardiac transplantation, including a significant decrease in ventricular filling pressures, pulmonary artery pressure, and pulmonary vascular resistance. In addition, resting heart rate increased significantly.

Heart rate

The resting heart rate of heart transplant recipients should theoretically be similar to the intrinsic heart rate (the heart rate recorded in subjects after pharmacologic autonomic blockade with atropine and propranolol) [2]. The disparity between the predicted intrinsic heart rate and actual heart rate in transplant recipients may in part be due to sinus node dysfunction. Miyamoto et al. [14] reported an 18% incidence of bradyarrhythmia (< 60 beats/min) in the first 5 days after transplantation. Bexton et al. [2] found that a significantly lower than predicted intrinsic heart rate was a reliable indicator of sinus node dysfunction in long-term survivors of cardiac transplantation, confirmed by electrophysiologic studies. The etiology of sinus node dysfunction after transplantation is complex. Pathologic studies have shown that there is a 50% chance of damage to both the donor and recipient conduction tissue at the time of surgical procurement [21]. Jacquet et al. [12] found that those patients with abnormal sinus node function had a significantly longer ischemic time. In the present study, such an association could not be confirmed. There is also some evidence that rejection episodes are associated with transient bradycardia [8]. Although early observations suggested that sinus node dysfunction soon after transplantation signaled a poor outcome [13], more recent observations indicate that sinus node dysfunction is usually a temporary problem and only a minority of patients require permanent pacemaker therapy [14]. The finding of a trend toward the predicted intrinsic heart rate by 1 year after transplantation in this study is further evidence of the reversibility of early post transplant sinus node dysfunction.

Right and left ventricular diastolic function

Prior hemodynamics studies of heart transplant recipients suggest that ventricular filling pressures begin to slowly normalize within several weeks to several months after surgery [3, 5, 6, 16, 22]. In agreement with these studies, we have found that pulmonary capillary wedge and right atrial pressures showed a significant decrease from one week to one year. However, a cautious interpretation of this "normalization" is warranted. Prior observations from this institution [15, 17] and from other centres [9, 25] have demonstrated that cardiac end-diastolic and end-systolic volumes are significantly smaller than normal controls. Thus, in the setting of lower end-diastolic volumes, even the virtually normal ventricular filling pressures imply a leftward shifted pressure/volume relationship. This view is further supported by the fact that a "restrictive" pressure wave form pattern can often be elicited by acute volume loading in these patients [10, 24]. The etiology of diastolic dysfunction of the transplanted heart is complex [18]. Among the factors implicated are the peri-

operative ischemic injury [6], the loss of the positive lusitropic effect of direct sympathetic stimulation [18], the mismatch of recipient and donor heart size [9], and episodes of rejection [19, 23]. Pericardiac constraint may also play a role in some cases [7]. None of the patients under study had histologic evidence of significant rejection in the first week after transplantation, thus the role of rejection in the restrictive hemodynamic pattern at 1 week is unlikely. The significant improvement by 1 year after transplantation emphasizes the relative importance of potentially reversible factors such as mismatch in recipient and donor size and periopertive ischemic injury.

Pulmonary artery pressure and pulmonary vascular resistance

Elevated pulmonary vascular resistance perioperatively may result in acute right ventricular overload and heart failure after transplantation and carries an increased risk of mortality [1]. A fixed elevation in pulmonary vascular resistance ≥ 6 Wood units is a contraindication to cardiac transplantation [1]. In this study, pulmonary vascular resistance fell significantly after transplantation and continued to fall to 1 year. The independent positive association between a reduction in pulmonary artery pressure and reduction in both pulmonary capillary wedge pessure and pulmonary vascular resistance indicates that pulmonary hypertension preoperatively has both active and passive components.

There is significant evolution in resting hemodynamics over the first year after transplantation. These beneficial changes help to explain the dramatic and sustained improvement in the functional capacity of these patients late after transplantation.

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IMPACT OF COMBINED HORMONAL PRETREATMENT (INSULIN+TSH) ON THE IMPRINTING OF HORMONES ADMINISTERED IN COMBINATION TO CHINESE HAMSTER OVARY CELL CULTURE

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Cultured Chinese hamster ovary (CHO) cells were treated (imprinted) with insulin and with thyrotropin (TSH) related to gonadotropins (FSH+LH). When one week later the treatment was repeated with one of the hormones, considerable differences could be observed in the binding capacity of the cells. In the hormone combination TSH was able to evoke persistent imprinting only to a markedly lesser degree than insulin, meanwhile the imprintatory effect of insulin was of greater extent even on the cell regarded to be unspecific for insulin. Hormone treatment of one hour duration – when investigated immediately after – did not extinct the binding capacity to TSH but enhanced that to insulin. With the deterioration of the conditions of culturing, the enhanced binding capacity disappeared.

Keywords: hormonal imprinting, insulin, TSH, cell culture, ovary

The first interaction of a hormone with a target cell gives rise – usually in the perinatal period – to hormonal imprinting. As an effect of the imprinting, the genetically preprogrammed receptors complete their maturation and reach the binding capacity characteristic to adulthood [3, 4, 5]. Imprinting occurs not only in whole living organisms but in unicellulars [4] and cultures of cell lines [6, 7] as well. The information of imprinting is being transferred to the descendant cells [8]. Culture of cell lines, however, represents special problem, since the cells of the culture have already experienced imprinting in the living organism. Nevertheless, even under the conditions of culturing, the interaction with the hormone gives rise to an imprinting-like phenomenon and results in enhanced binding capacity of the

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receptors [6, 7]. Or to put it in another way, both imprinting and its mechanism can be modelled in the culture of mammalian cells.

The cells (as well as their descendants) do not forget the interaction with the hormone [8]. The aim of the present experiments was to study whether the effect of another, simultaneously administered hormone can be associated to the memory imprinted into the cell. It was investigated whether the simultaneous administration of a hormone which possesses receptors on the cell and another hormone which also acts at the membrane level, but does not influence the cell directly, is able to evoke the binding characteristic to the "target hormone" even long after the imprinting. A technique of culturing was developed which enabled, on the one hand, to gain information also on the intensity of the binding and, on the other hand, to investigate how far the changes of nutritive conditions [11] can influence the already developed binding capacity.

Materials and methods

Cells of the CHO cell line were used in the experiments. The cells were maintained in Ham F12 synthetic medium supplemented with serum from colostrum-deprived calfs. The cells were pipetted into H-tubes; each tube contained 80,000 cells in 3 ml medium. The cells adhered to the coverslips placed into the tubes previously. The treatment was carried out one day later. The experimental groups were as follows:

1. Control: no treatment at all.
2. The cells were incubated with a nutrient medium containing 10^{-8}M thyrotropic hormone (TSH, Ambinon, Organon, Oss, The Netherlands) and 10^{-6}M insulin (Insulin, Semilente MC, Novo, Copenhagen, Denmark) at 37 °C for one hour. Then the nutrient medium was removed, the cells were washed in two changes of PBS and the culturing was continued for another week.
3. The treatment and rinsing were similar as described above; after one week of culture, however, the cells were assigned to two subgroups,
   a) treatment with TSH for one hour,
   b) treatment with insulin for one hour, in the concentrations described above.

In both cases the hormones were added to fresh medium. This was followed by washing in PBS anew. Some cells (slides) were fixed in 4% formol solution dissolved in PBS, whereas the cells in the other tubes were maintained further in a nutrient medium changed every 24-hour-period, and fixed 24 or 48 hours later.

Hormone binding capacity of the cells was investigated with the help of FITC (fluoresceine isothiocyanate isomer 1, BDH, Poole, England) – TSH (FITC/protein ratio, 1.39; protein concentration, 0.4 mg/l) and FITC-insulin (FITC/protein ratio, 0.42; protein concentration 0.45 mg/l). This was followed by washing in two changes of PBS and then in distilled water. The intensity of the fluorescence of the cells was measured by means of a Zeiss Fluoval cytofluorimeter. The analogous signals of the fluorimeter were converted into digital ones by a microprocessor and recorded by a Hewlett Packard 41CX microcomputer. Twenty cells were investigated in each group and the measurements were repeated three times (each column on the figures represents the average value of 60 cells). Analysis of significance was carried out by a computer using the Quatro program.

Results and discussion

The CHO (Chinese hamster ovary) cells used in the experiments possess gonadotropin (FSH+LH) receptors [11]. The related pituitary hormone, i.e. TSH [1, 2, 13, 14] overlaps the receptors [9, 11]. In our previous experiments the imprinting effect of TSH on the CHO cells was found to be more marked than that of FSH. Insulin was attempted to be used (associated) as an indifferent hormone.

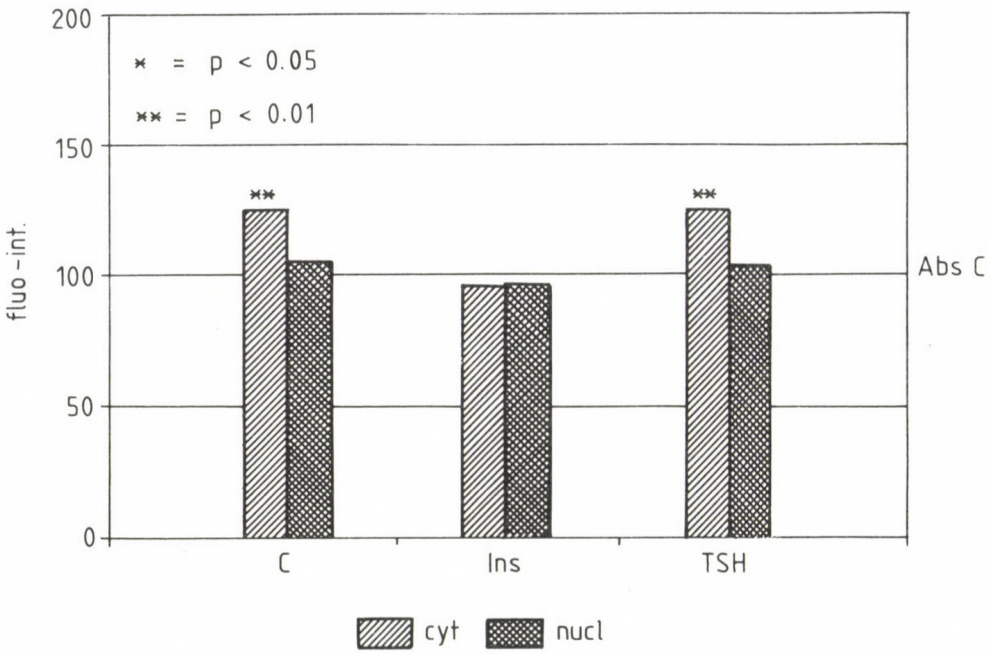

Fig. 1. Binding of FITC-TSH to untreated CHO cells and cells imprinted with insulin+TSH. Measurement immediately after insulin or TSH treatment of one hour duration (C=before the investigation untreated control imprinted with insulin+TSH)

The results of the experiments clearly indicate (Fig. 1) that one week before the investigation on hormone binding the *TSH binding* above the cytoplasm (membrane) of those cells which had been treated with both hormones was significantly higher than the binding of the control cells. Above the nucleus, however, no significant increase of hormone binding could be detected. During the 1-hour TSH treatment, the receptors of the cells should have been saturated. In contrary, the binding was exactly the same even after the 1-hour hormone treatment, which observation indicated that either the hormone concentration, or the duration of the interaction was not sufficient for the saturation of the receptors. Treatment with

insulin for one hour, however, extincted this enhancement of binding, which, otherwise, was also characteristic to the imprinted controls. We observed this phenomenon also in our previous functional investigations of *Tetrahymena*. Nevertheless, we are still unable to give explanation. The occurrence of the phenomenon of negative cooperativity can be assumed; the present experiments, however, do not provide sufficient evidence to support this assumption.

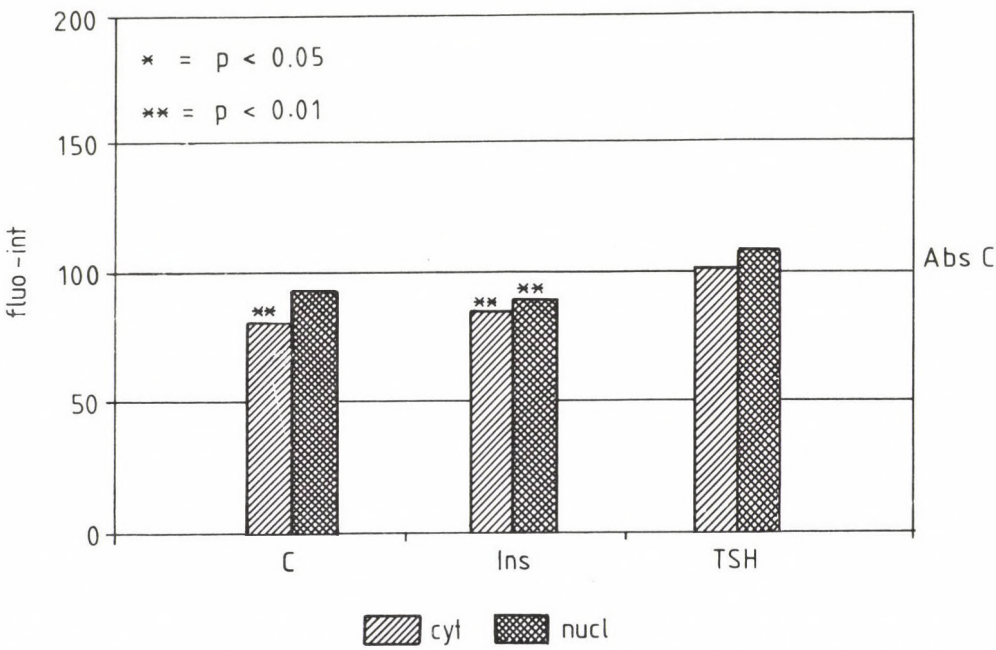

Fig. 2. Binding of FITC-TSH to untreated CHO cells and cells imprinted with insulin + TSH. Measurement 24 hours after insulin or TSH treatment of one hour duration (C = see Fig. 1)

When TSH binding was investigated 24 and 48 hours later, the enhancement of binding could not be detected (Figs 2 and 3). Since in the previous experiments [10] the effect of imprinting was persistent also under the conditions of culturing – provided that the imprinting was evoked by one hormone alone – it can be assumed with reason that when two hormones are administered simultaneously, to some extent, they may weaken the effect of one another. Thus, by the end of the experiments when the amount of nutrient available decreases (one-week-old cultures were investigated with high cell number and, thus, large amount of nutrient might be consumed during 24 hours) and the amount of degradation products increases, i.e. the general condition of the culture deteriorates, the binding capacity of the cells also gets poorer. Previous experiments [11] have provided data in this respect, too.

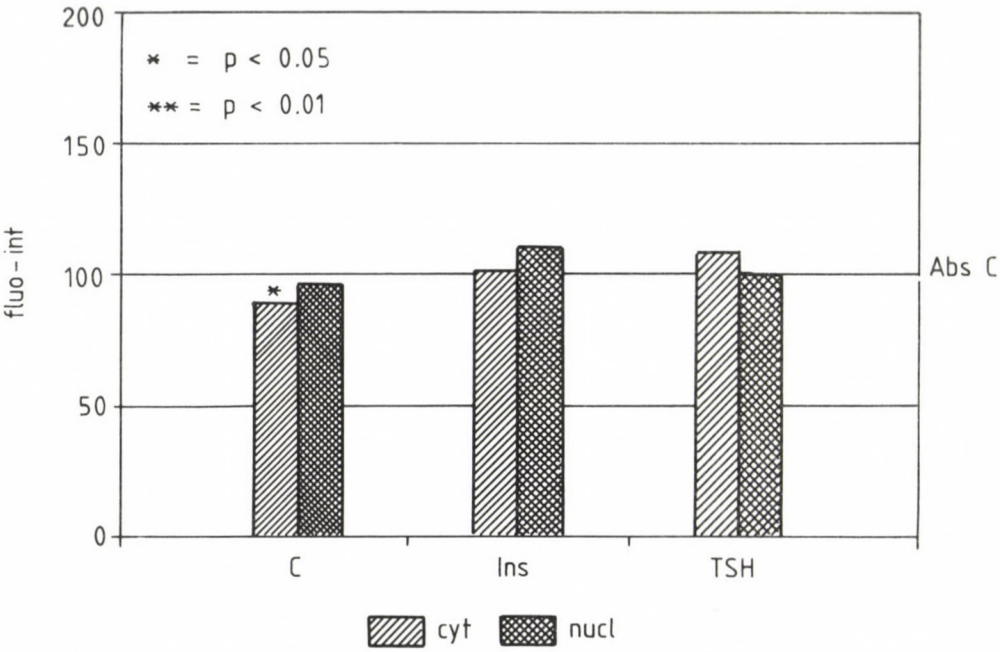

Fig. 3. Binding of FITC-TSH to untreated CHO cells and cells imprinted with insulin + TSH. Measurement 48 hours after insulin or TSH treatment of one hour duration (C = see Fig. 1)

Even when *insulin binding* of the cells was investigated, some positive effect of the double imprinting performed one week before could be detected. Insulin treatment for one hour, when administered one week after the pretreatment, led to marked increase of insulin binding (Fig. 4). This increase could be persistently detected even after 24 and 48 hours (Figs 5 and 6). Also TSH treatment of one hour duration led to some enhancement of insulin binding (Figs 4 and 5).

Numerous cells of the animal organisms posses insulin receptors which are capable of specific binding; the role of these receptors, however, remains to be elucidated [12]. The present experiments suggest that – also in the case of the ovarium-combined hormone treatment results in more explicit strengthening of insulin receptors than of the receptor sites capable to bind TSH. This observation either indicates that in the hormone combination insulin is the dominant hormone, or suggests that in the membrane of ovarian cells the insulin receptors are more dynamic than the gonadotropin receptors which are also able to bind TSH.

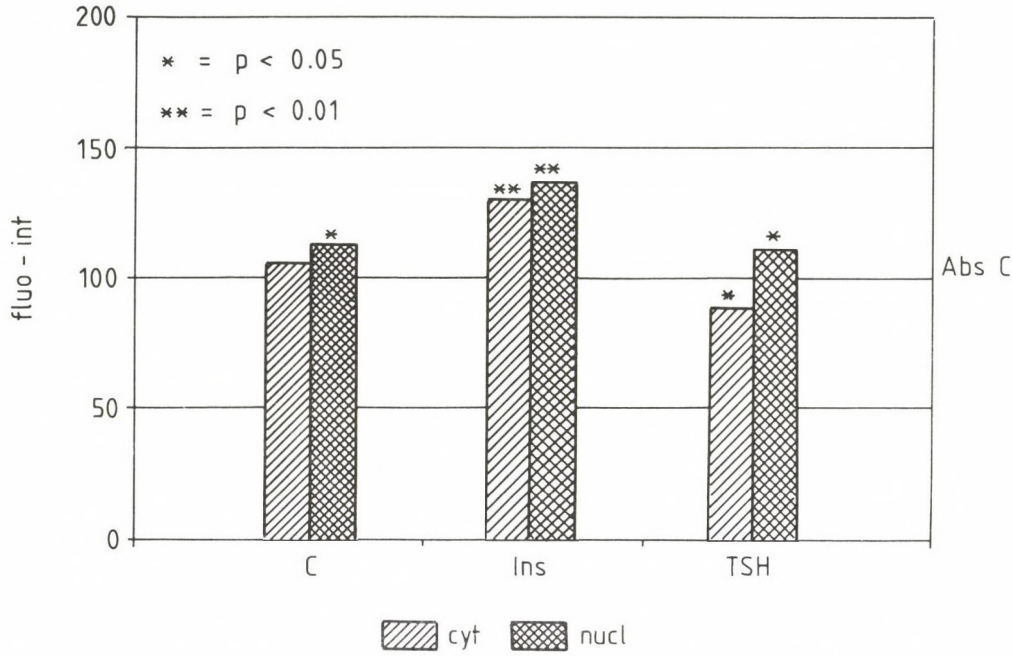

Fig. 4. Binding of FITC-insulin to untreated CHO cells and cells imprinted with insulin+TSH. Measurement immediately after insulin or TSH treatment of one hour duration (C=see Fig. 1)

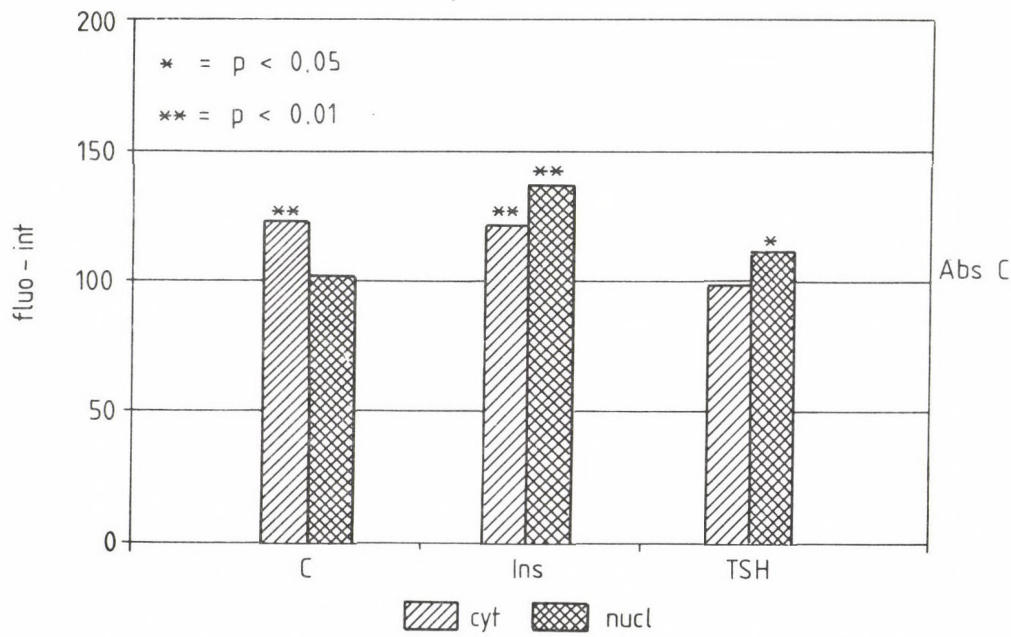

Fig. 5. Binding of FITC-insulin to untreated CHO cells and cells imprinted with insulin+TSH. Measurement 24 hours after insulin or TSH treatment of one hour duration (C=see Fig. 1)

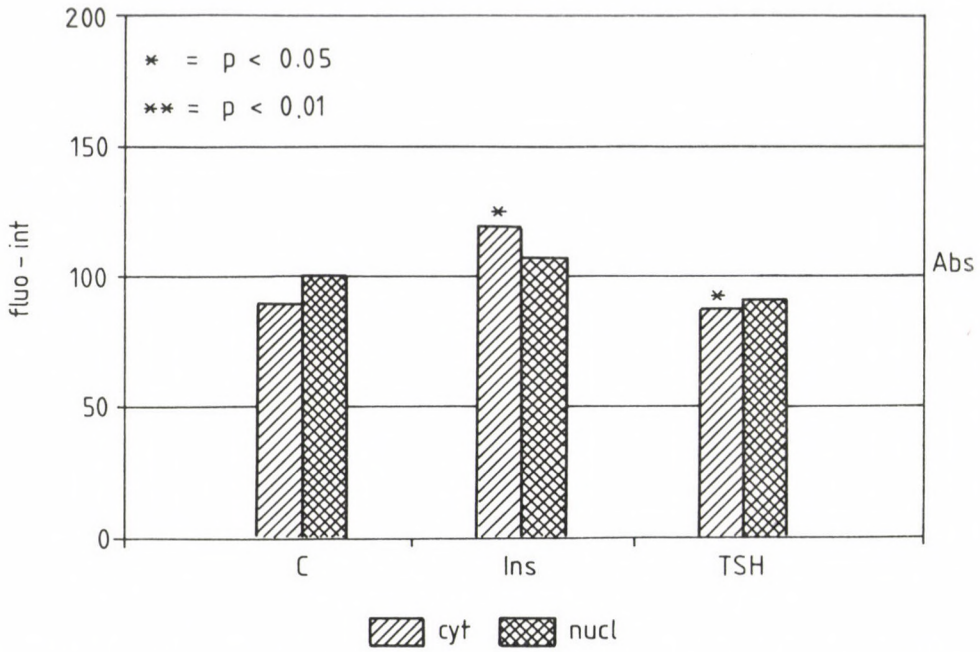

Fig. 6. Binding of FITC-insulin to untreated CHO cells and cells imprinted with insulin+TSH. Measurement 48 hours after insulin or TSH treatment of one hour duration (C=see Fig. 1)

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HISTOCHEMICAL STUDY ON ADRENAL Δ^5-3β-HYDROXYSTEROID DEHYDROGENASE ACTIVITY IN CADMIUM TREATED TOAD (*Bufo melanostictus*)

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Effect of a single subcutaneous injection of cadmium chloride at the dose of 0.5 mg/toad on adrenal Δ^5-3β-hydroxysteroid dehydrogenase (Δ^5-3β-HSD) was observed after 7 days. The activity of Δ^5-3β-HSD was measured histochemically. The experiments indicate that cadmium chloride resulted in a significant decrease in the activity of adrenal Δ^5-3β-HSD in toad during breeding season (June-July).

Keywords: cadmium, adrenal gland, Δ^5-3β-hydroxysteroid dehydrogenase

Inhibition of spermatogenesis [7, 10], testicular steroidogenesis [2, 3] and secondary sex organs or accessory sex glands [4] activities were noted earlier in cadmium treated mammalian and non-mammalian species. Studies in mammals have confirmed that both the gonad and the adrenal gland are target organs of cadmium action. Adrenal hypertrophy [8] and impairment of adrenal steroidogenesis [2] in cadmium treated rat have been reported. No study has yet been performed on non-mammalian adrenal gland after cadmium treatment. Amphibian adrenal tissue is not possible to isolate for biochemical investigation of Δ^5-3β-HSD activity, the Key enzyme for adrenal steroidogenesis [9]. So, the present study was undertaken to demonstrate histochemically the activity of adrenal Δ^5-3β-HSD in control and cadmium treated toad in breeding season.

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Materials and method

Experiments were carried out on twenty four adult male toads, *Bufo melanostictus*, weighing about 50-55 g. They were collected from their natural habitat during breeding season (June-July). They were fed with ant eggs and water ad libitum. The animals were divided into 2 groups. A single subcutaneous injection of 0.5 mg cadmium chloride, dissolved in 0.2 ml of amphibian saline was given to each animal of one group while the animals of remaining group considered as control and received vehicle only. After 7 days all the animals were sacrificed and the whole kidney with embedded adrenal tissue of each animal was taken out and processed for histochemical demonstration of Δ^5-3β-HSD activity using DHEA as substrate [1]. After 30 min incubation, sections were fixed and mounted with glycerol gelatin.

Results

Figure 1 demonstrates the location of adrenal Δ^5-3β-HSD in control toad. Cadmium injection caused a decrease in Δ^5-3β-HSD activity in the adrenal tissue compared to that seen in controls (Fig. 2).

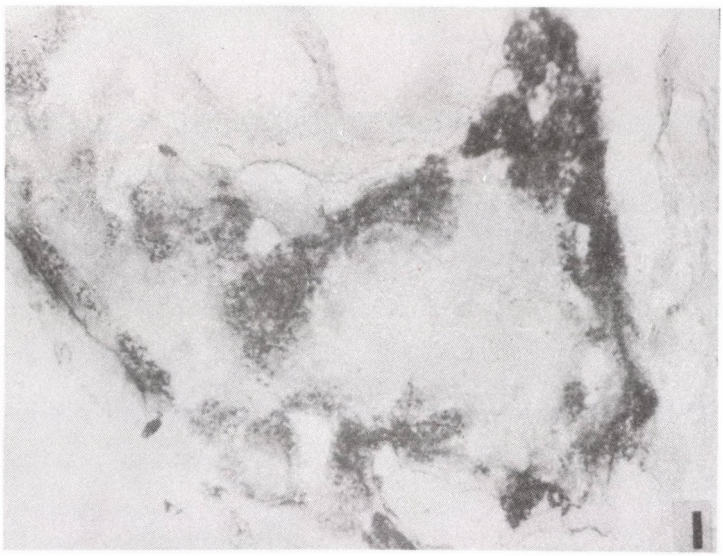

Fig. 1. Δ^5-3β-HSD activity in the adrenal gland of control toad X125

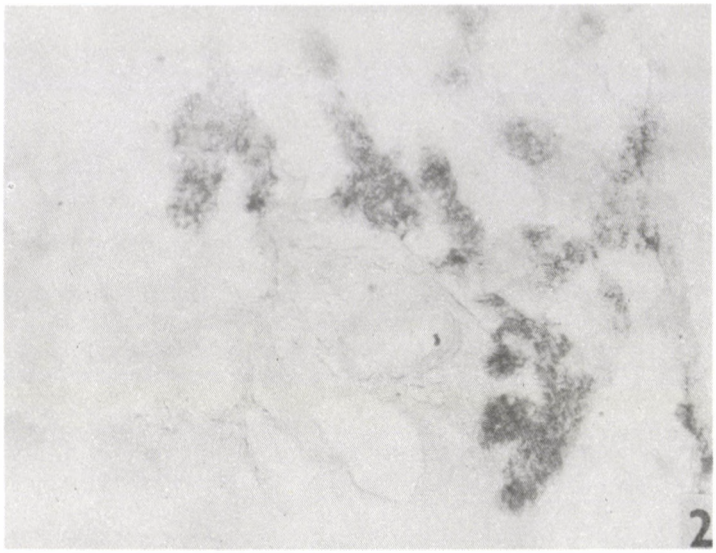

Fig. 2. Decrease in the Δ^5-3β-HSD activity in adrenal gland of cadmium treated toad X125

Discussion

In this study, impairment of adrenal steroidogenesis in toad after cadmium treatment is consistant with previous report in rat [2]. Adrenal steroidogenesis is under the control of ACTH [12]. So, it may be suggested that the observed changes in the activity of adrenal steroidogenic enzyme after cadmium treatment may be due to decreased pituitary ACTH. The cause of the inhibition of pituitary ACTH secretion in cadmium treated toad is not properly known. It has been reported that cadmium treatment in toad results in high pituitary LH [5] which may be the possible cause for low ACTH level in cadmium treatment as high rate of LH secretion is in expense of ACTH [16]. The mechanism of high LH level in cadmium treated animal is not clearly known but it may be suggested that low plasma levels of testosterone in cadmium treated animal [3] is the possible factor, that results in high level of pituitary LH by positive feedback mechanism, as observed in gonadectomy [11]. The low level of testosterone in cadmium treated animal is consistant with our observation where we have reported that cadmium treatment in toad leads to remarkable inhibition in testicular 17β-HSD activity [3], the key enzyme of androgen synthesis. So, the present findings can be explained due to low ACTH secretion from pituitary in cadmium treated toad.

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TIME COURSE OF CHANGES IN LONG-TERM POTENTIATION OF SYNAPTIC TRANSMISSION FOLLOWING SUBCORTICAL DEAFFERENTATION ON THE RAT HIPPOCAMPUS

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Brief tetanic stimulation potentiates synaptic transmission both in the CA1 and dentate area of slices cut from normal rats. This long-term potentiation (LTP) was assayed in slices made at various times from rats subjected to complete bilateral sectioning of all subcortical afferents which enter the hippocampus. Over about one week survival time, LTP is present in the CA1 region of all and also in the fascia dentata of about 50% of slices. We found no signs of LTP in the dentate area of slices cut over 8 weeks after deafferentation, while the responses were clearly potentiated in the CA1 area of the same slices. Four week was the longest period when a somewhat modified version of LTP could be produced in the subcortically deafferented dentate area. The results confirm previous reports that subcortical afferents mediate some unknown factors essential for maintenance of long-term plasticity of intrinsic synapses in the fascia dentata. This unidentified, perhaps trophic influence diminishes in about 4 weeks after severing the subcortical fibers. In contrast, maintenance of subcortical inputs are apparently not required for the LTP in the intrinsic CA1 synapses.

Keywords: slices, granule cells, CA1 pyramidal cells, plasticity, trophic influences

Electrophysiological properties of neurons can be altered by partial denervation. Various groups of afferents influence plasticity of neurons in different ways. For example partial elimination of descending control of soleus motoneurons alters their membrane properties [8], while the same properties remain unaltered after chronic section of dorsal root fibers [25]. Persistent stimulation of one kind of presynaptic input can prevent alterations induced by loss of others [8]. Another examples are Tsukahara's [40] results on the synaptic plasticity in the red nucleus.

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Apart from the long known changes involved in denervation supersensitivity, heterosynaptic influences upon synaptic transmission are also widespread. Parts of the persistent influences are mediated by cyclic AMP mechanisms and peptidergic transmission is involved in others. Well-known examples of short-term heterosynaptic changes of synaptic transmission are the presynaptic inhibition and the heterosynaptic facilitation. Most of the heterosynaptic influences can be modified by partial elimination of presynaptic fibers.

Buzsáki and Gage [6] discovered a new example of persistent modulation of synaptic transmission. They reported on the absence of long-term potentiation (LTP) in the subcortically deafferented fascia dentata. Stimulation of the angular bundle with high frequency induced the well known [4] sustained increase of the evoked population spike in the dentate area of their normal rats, but not in others which suffered removal of fibers running in the fimbria-fornix and supracallosal path. Their finding fall in line with other recent reports on the critical role of noradrenergic modulation in the long-term plasticity of the fascia dentata [5, 22, 29, 33–35].

LTP is a stable, relatively long-lasting increase in the magnitude of a post-synaptic response to a constant afferent volley following brief tetanic stimulation of the same afferents. Many papers deal with the relationship of LTP to the neuronal mechanisms underlying learning and memory [30, 38, 39]. There are also reports on blocking or eliminating the LTP [10, 21, 26, 36, 37, 41]. Buzsáki and Gage [6] found a way to alter the plastic properties of an intrinsic synapse of the hippocampal formation by eliminating an extrinsic input to the system.

We have previously asked if plastic properties of other intrinsic synapses, like the Schaffer collateral Ca1 pyramidal cell transmission can also be influenced by chronic subcortical deafferentation. Our answer for that question is no [9]. Further details and especially the timecourse of alterations of plastic properties of slices cut from partially denervated hippocampus are reported here.

Methods

The experiments were performed on Sprague-Dawley rats of 120-200 g body weight. Three to five slices were examined from each of the 18 normal and 21 operated animals. The slices were assayed 1 to 8 weeks after deafferentation.

Surgery

Under 100 mg/kg ketamine anesthesia the skull was opened and the cingulate bundle, the supracallosal stria, the corpus callosum, the dorsal fornix, the fimbria and the ventral hippocampal commissure were removed through a hole aspired near the midline from the cingulate and parietal cortex. The wound were closed and the rats were kept in individual cages providing food and water ad libitum.

Completeness of the aspirative lesion of the fimbria-fornix fibers was evident when dissecting the hippocampi to cut slices: the septal (anterior) pole of the hippocampus slipped backward since it lost its anchorage by the fornix. In addition, two further criteria were also used to verify the deafferentation of the hippocampus: one was the absence of acetylcholinesterase reaction in anatomical slices from the same animals, and the other the regular occurrence of epileptiform activity elicited by stimulation of the slices in accord with recent observations in vivo [7].

Slice techniques

Under deep ether anesthesia, the brain of the rats were quickly removed and dropped to cold oxygenated artificial cerebrospinal fluid (ACSF). The fluid contained (in mM): NaCl 124, KCl 3.5, KH_2PO_4 1.25, $NaHCO_3$ 24, $CaCl_2$ 1.8, $MgSO_4$ 1.2, D-glucose 10. Bubbling with a mixture of 95% O_2 and 5% CO_2 set the pH to 7.4. After hemisection, the hippocampus was dissected free and about 400 μm thick transverse slices were cut manually from the dorsal hippocampus. Several slices from each rats were incubated for 90 to 120 minutes in the experimental chamber at 35 °C degree and perfused with ACSF (2ml per min). We use a home-made version of the Oslo type interface chamber.

Electrophysiology

Field potentials were recorded with glass microelectrodes of 10-20 MOhms filled with 150 mM NaCl. The signals were fed into an electrometer and then to an AC amplifier (bandwidth 0.2 Hz to 10 KHz). The output of this amplifier was digitalized with a Labmaster (Tecmar) board and fed into a computer and was also monitored with a digital oscilloscope (Nicolet 310). The presynaptic fibers were stimulated monopolarly (1-10 Volt pulses of 0.1 ms duration) with a 2 MOhm stainless steel microelectrode.

Evoked potentials in the CA1 region were tested with stimulation of stratum radiatum and recording from the stratum pyramidale, and those in the fascia dentata with stimulation of the stratum moleculare and recording from the cell body layer. Position of electrodes and intensity of stimulation was adjusted to obtain at least 3 mV population spike responses to supramaximal pulses. Then we set a constant intensity of the stimulation to elicit population spikes of 40 to 60% maximal size.

Responses were initiated with single pulses delivered at a low rate (with 2 to 5 second intervals). Four evoked potentials were averaged using an IBM AT compatible computer and Aitken's [1] program. Three examples of these averaged potentials collected in a 15-min period before high frequency stimulation served as controls for both viability of the slices and stability of the responsiveness.

If the stability of responses was sufficient (i.e. amplitude fluctuation of the population spike remained within 10%), the slices were subjected to a brief high frequency stimulation with parameters we found in preliminary experiments to induce LTP regularly. Tetanic stimulation was made with 12 trains delivered in three groups within a period of 1 minute. Each train lasted for 400 ms and contained several pulses repeated with 80 to 100 Hz frequency. "Zero" time was defined at the end of the last train, when low rate single pulse stimulation was set up again. Four responses were subsequently averaged again, like during the pretetanic period, at 1, 2, 4, 6, 10, 15, 20, 25, and 30 min posttetanic time. Clear enhancements of the averaged population spikes were observed in many slices during the 10 to 30 min posttetanic period compared with the pretetanic controls. Changes in the first minutes after cessation of the train stimulation were variable and inconsistent, either depression or facilitation could occur.

We considered the enhancement of the population spike responses as representing LTP if the increase of their amplitude exceeded 20% during the 10- to 30-min posttetanic period. Over 90% of the slices from normal rats met this criterion and a total of 5 slices from lesioned rats were rejected from further analysis because potentiation remained less then 20% in the CA1 region of them. No slice was rejected because of the lack of LTP in the dentate area.

Several phenomena were considered as indicating potentiation of the population spike responses. Besides some increase in magnitude, decrease in latency, faster rate of rise of initial postsynaptic potential and shorter time to peak values for the population spike were also observed. Most of these

phenomena were either less consistent or accompanied with the size increases. In some slices with only a solitary population spike during the pretetanic control, a second population spike also developed after the high frequency stimulation. In slices from deafferented rats, with two or more population spikes before high frequency stimulation, these later components also increased substantially with tetanic stimulation. We could not set up for simultaneous monitoring of the evoked responses both in the dendritic or in the cell body layer. Although LTP is sometimes better indicated by an increase of population postsynaptic potentials recorded in the dendritic layer, separately from alterations of the evoked discharges seen in the cell body layer, we opted for monitoring the evoked discharges because we wanted to see if there was epileptiform activity in our slices.

Stability of the responsiveness was tested in keeping several slices stimulated only with low rate and averaging 4 responses at every 5 minutes during a 40-minute period. Both some slow increase or decrease of evoked responses throughout this period were noted as well as some fluctuation of the evoked responses, but these changes remained within 10-15% of the initial size.

Data processing

To compare measurements taken in different slices data were normalized as follows. First the size of the population spike was calculated in each averaged response. Conventions in measurements for that calculations are shown in Figs 1 and 6. Then the mean value from the three pretetanic measurements were determined for each slice and all the other measurements were expressed as percentage of this pretetanic mean size. Finally the per cent data representing the corresponding time periods (i.e. 5 min pretetanic time, or 30 min posttetanic time) were taken from groups of slices for calculating the mean values which were then plotted in a single graph.

Statistical analysis of the results was performed by means of Student's t tests with significance limits at $P<0.05$, or $P<0.1$. Relationship of mean values from each posttetanic time period to pretetanic means was tested in each experimental groups. In addition, selected pairs of complete time course data were compared and significance was tested between corresponding mean values from each pre- and posttetanic time class.

Results

LTP in slices taken from normal rats

A reliable way of producing LTP in the CA1 area of slices was to be found first. Neither the minimum parameters of priming high frequency stimulation which might induce LTP, nor parameters which generate the most robust changes in synaptic efficacy were looked for. A set of parameters were established in preliminary experiments not included in further analysis. Twelve trains of 400 ms duration each were delivered in groups of four in 3 second intervals. Longer resting periods were allowed after the forth train so that the whole procedure lasted for a minute. Frequency of pulses within the trains was set to near 100 Hz, but reduced to 90 or 80 Hz if the evoked responses disappeared quickly. Strong gradual depression of the evoked responses was common during train stimulations, but recovery was noted in the intertrain intervals. Some reduction of the frequency of pulses helped to avoid complete depression of postsynaptic responses. We observed in some cases, both in normal and operated rats, that the tetanic stimulation blocked all

postsynaptic responses completely for a prolonged time associated with a strong negative shift of the potential level. Therefore slices in which no recovery of evoked responses was detected in the second posttetanic minute were rejected and not counted among those where LTP failed.

Table I

Quantitative changes of evoked potential components found in slices after tetanic stimulation

	n	Mean	S.D.	Changes
From control rats				
CA1 group 1	10	4.99	2.64	+80%
CA1 group 2	12	7.02	3.15	+70%
CA1 "Best 15"	15	11.2	1.85	+90%
Fascia dentata	8	3.18	1.26	+40%
From operated rats				
– at the 2nd week				
CA1 P1	12	2.69	1.25	+70%
CA1 P2	12	1.87	1.46	+130%
CA1 P3	10	1.44	1.11	+120%
Dentate P1	6	4.74	1.59	+60%
Dentate P2	6	1.49	0.84	+340%
Dentate "No effect"	5	4.77	1.50	−10%
– at the 5th week				
CA1 P1	11	2.69	1.46	+80%
Dentate P1 All slices	9	4.55	1.79	−10%
Dentate P1 subgroup 1	3	2.43	0.32	+30%
Dentate P1 subgroup 2	6	5.61	1.17	−30%
– at the 9th week				
CA1 P1	9	2.96	0.75	+60%

n: number of slices included.
Mean: in mV, the size of pre-train values normalized within the group.
S.D.: the range of positive and negative deviations from the mean.
Change: the closest single number expression of increases over the pre-train normalized amplitudes during the 10 to 30 min period after the tetanic stimulation.
P1, P2, P3: first, second and third peak of population spikes

Since we used LTP data from normal slices as controls for the operated animals, sampling of slices from different animals could be a problem. A way to estimate errors due to sampling might be to compare subgroups of normal slices taken from different animals. Twelve slices were selected in one group from experiments made in slices from intact rats, and 10 slices into another group. These

control experiments were made on days alternating randomly with the ones when slices from operated rats were used. All slices which met our 20% potentiation criterion (see Methods) were included in these two groups. In one of them the mean amplitude of the CA1 population spike response was 4.99 mV and the mean enhancement was around 80% during the 10 to 30 min posttetanization period. See Table I for quantitative data. Then the "best" 15 slices from these two subgroups were taken and the percentage of changes was calculated again. All the three groups were plotted (Fig. 2) and significance of differences among these plots was tested. The differences were not significant even at the $P<0.1$ level. We used the "best 15" data group as controls in the subsequent comparisons.

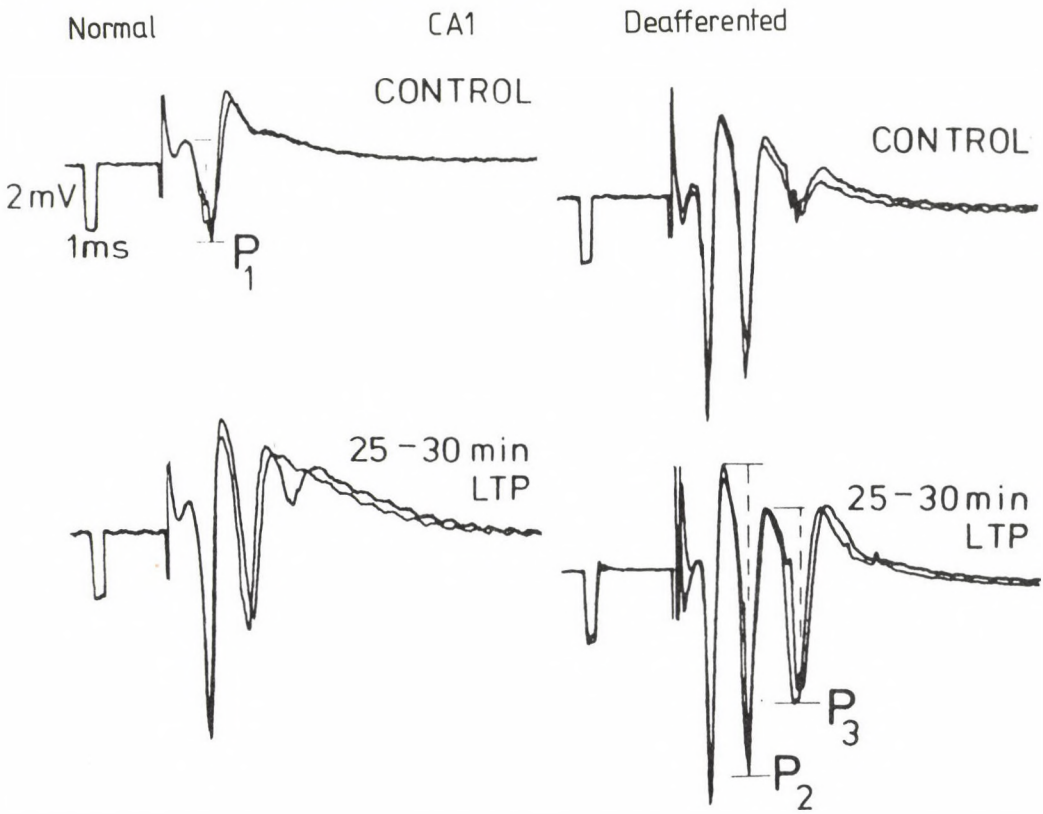

Fig. 1. Waveforms of the responses evoked in the CA1 area. On the left (normal) two superimposed responses are shown to constant, low rate stimulation of the str. radiatum at 10 and 5 min before (control, top) and at 25 and 30 min after (bottom) tetanic stimulation of the slice. This example was selected to illustrate the cases when increase of the first population spike (measured as shown) was associated with development of subsequent population spikes. On the right similar responses are shown from a slice obtained at the 9th postoperative day after subcortical deafferentation. All records indicate a 2 mV-1 ms negative calibration pulse

Our control group from the dentate area was smaller. In eight slices, the granule cell population spike response showed a potentiation of over 38%, from an initial 3.18 mV mean amplitude. We decided to use the same "12 trains in one minute" parameter in the dentate area, although preliminary experiments revealed that a somewhat different pattern of tetanic stimulation could be more effective in generating LTP in granule cells.

Subcortical deafferentation changes the behavior of the operated rats and increases the likelihood of their spontaneous epileptiform activity [7]. In accord with these findings, we observed that the postsynaptic cells in slices from lesioned rats responded with two or three subsequent population spikes, indicating high excitability, to single presynaptic pulses.

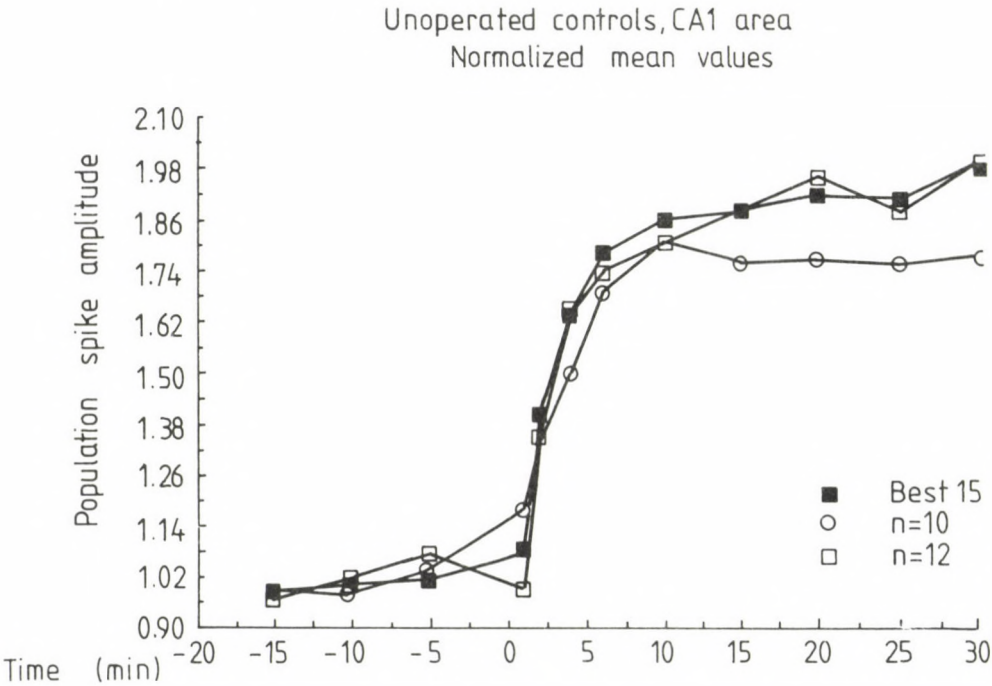

Fig. 2. Long-term potentiation of the monosynaptic activation of the CA1 pyramidal cells in slices from normal rats. The data points are obtained as follows: Averaged waveforms from each slice at each of the indicated time points were measured, and normalized according to a mean value calculated from the data collected during a period (negative time values on the graphs) before tetanic stimulation of the slices. Trains of high frequency stimulation were delivered in a 1-min long period, ending at 0 time, when data collection for LTP started. The normalized individual measurements from corresponding time points resulted in the means plotted here. Data from 22 slices from young unoperated rats are shown. Open symbols plot two subgroups, filled boxes a third made from them by selecting the 15 slices in which the responses were over 10 mV in amplitude

LTP in the second postoperative week

In 12 slices obtained from 8 rats during the period of 6 to 9 postoperative days, tetanic stimulation of the stratum radiatum produced also a large increase of the population spike in the CA1 stratum pyramidale. The mean enhancement of normalized size of the first population spike component varied between 54 and 68% during the 10 to 30 min posttetanization period (Fig. 2). Potentiated values from the 4th posttetanic minute on differed from the pretetanic means significantly at $P<0.05$ level. Differences between this set of data and that from normal ones were not significant even at the $P<0.1$ level.

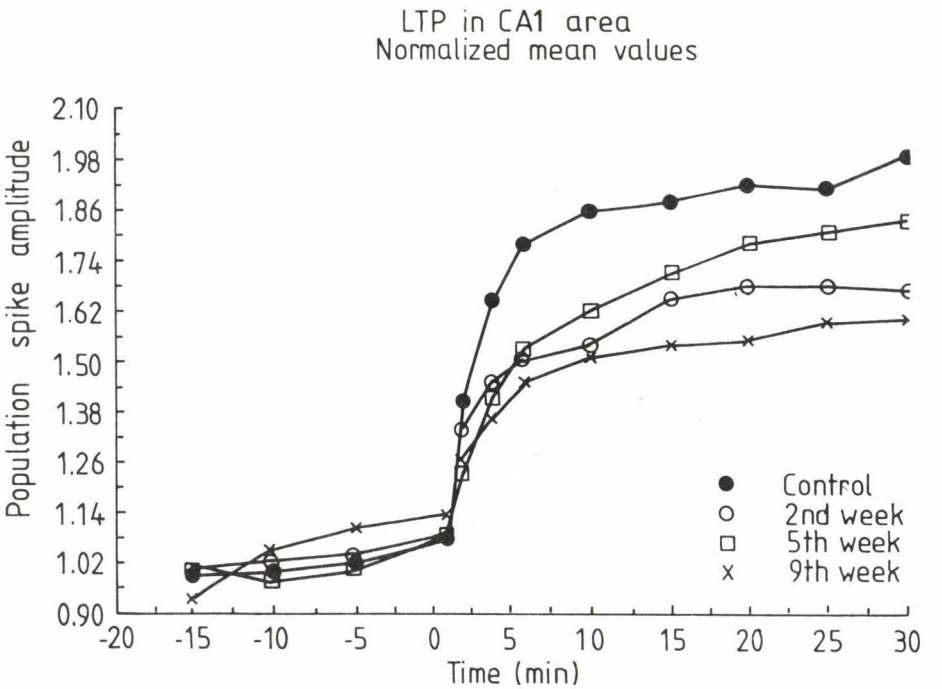

Fig. 3. Changing of LTP with time after cutting the subcortical afferent fibers. See Fig. 2 for conventions of plotting the data. Filled symbols show the best control values from unoperated rats (also shown in Fig. 2). Open symbols plot the sizes of first population spike component of the evoked response in slices made on the second, fifth and the ninth week after subcortical deafferentation of the hippocampal formation

The second and the third population spike responses increased to more than 200% of their original size following high frequency stimulation (Fig. 3). The mean amplitude of these first three population spikes was 2.69, 1.87 and 1.44 mV, respectively, during the pretetanization period. Since deviations of these set of data

from those of the first population spike were not significant ($P<0.1$), we ignored the late spikes during the further analysis.

Tetanic stimulation of the stratum moleculare in the fascia dentata of the same 12 slices also potentiated the population spike response generated by the granule cells in 6 slices. As shown in Fig. 7, the enhancement of the first population spike component over 50% (significant in the 4 to 30 posttetanic minutes at $P<0.05$) from an initial 4.74 mV was larger than the potentiation seen in control slices, but this difference was statistically not significant ($P<0.1$).

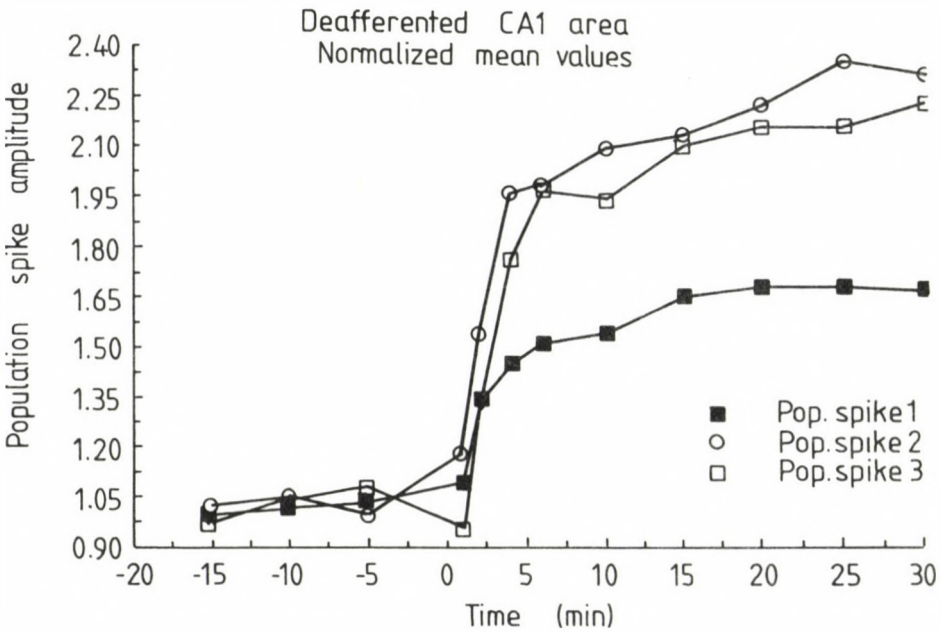

Fig. 4. Alterations of subsequent peaks of the evoked response after tetanic stimulation of the CA1 area. See Fig. 2 for conventions of plotting the data. All data from slices made during the second postoperative week. The amplitudes of the first, second and third population spikes were measured separately as shown in Fig. 1

In the remaining 5 slices of this group no potentiation was detected. The mean amplitude of the first population spike (4.77 mV), or that of the second one (3.2 mV), remained practically unaltered (Fig. 5). This set of data differed significantly ($P<0.05$) from those indicating potentiation either in the 6 slices with the same postoperative time or that in the normal rats.

LTP in the fifth postoperative week

Nine slices were studied from 7 rats in this postoperative time window. Time course of potentiation in the CA1 area at the 5th week did not significantly ($P<0.1$) deviated from that seen in the other experimental groups plotted in Fig. 2. The mean amplitude of the first population spike of pyramidal cells was 2.69 mV in this postoperative group.

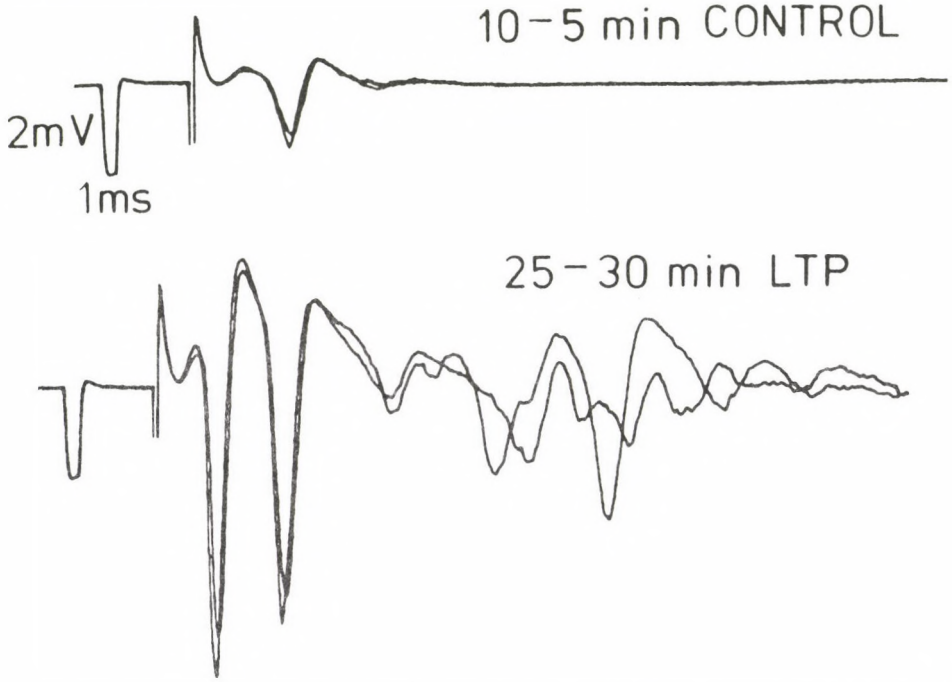

Fig. 5. Epileptiform responses observed in many slices made from subcortically deafferented hippocampal formation. In this case, the slice was made at the 5th postoperative week. The top trace shows two averaged waveforms recorded at the cell body layer of the CA1 area with low intensity stimulation of the str. radiatum, 5 and 10 minutes before tetanic stimulation. Higher intensity shocks evoked larger population spikes with 2–3 separate peaks. Bottom, two averaged responses evoked by the same weak volleys at 25 and 30 minutes after a period of tetanic stimulation. Some of the individual volleys induced prolonged after discharges following the regular 2–3 population spikes. They caused the polymorph late components of the averaged waveforms shown here

Better response (mean amplitude of first population spike 4.55 mV) was seen in the dentate area of the slices in this group. When tested for LTP, some enhancement was found in the first 10 posttetanic minutes followed by a depression not seen in other group of slices (Fig. 7). Splitting of the data into two subgroups revealed some potentiation in 3 slices, but the enhancement of amplitudes was not

significant ($P<0.05$). The more evident depression of the population spike in the other subgroup (Fig. 8) was statistically significant ($P<0.05$) when compared with the pretetanic mean amplitude.

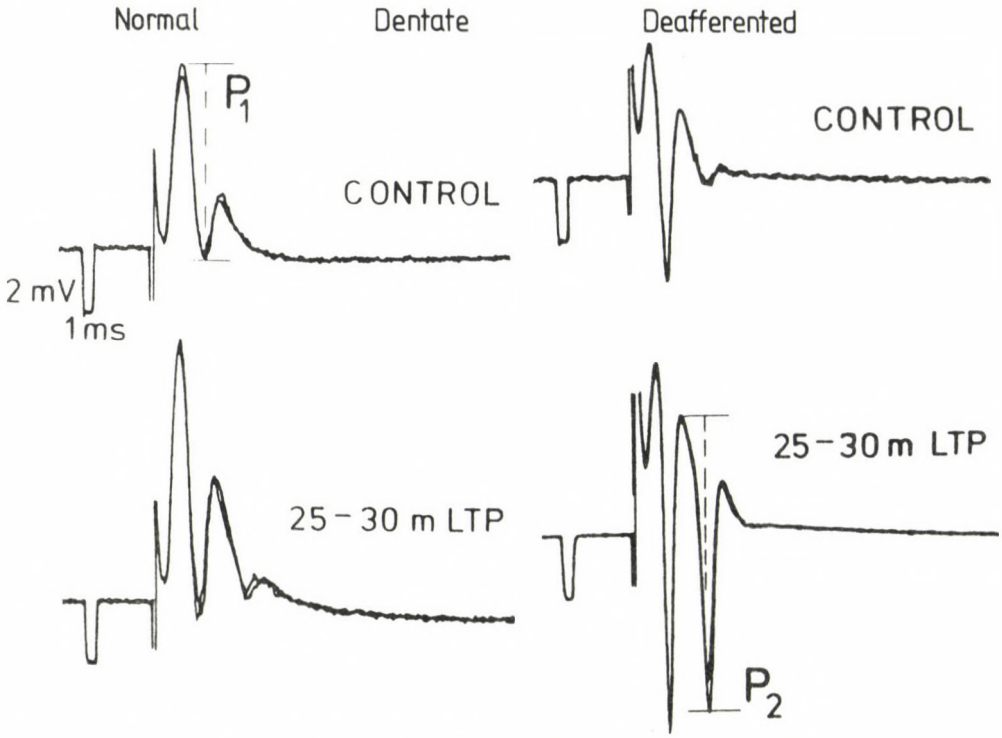

Fig. 6. Averaged waveforms from the dentate gyrus. Top left frame shows two superimposed responses to low rate single volley stimulation of the molecular layer. Below, the same stimulations induced waveforms averaged from responses taken at 25 and 30 min after tetanic stimulation of the same afferents. The two frames on the right show waveforms obtained in a similar way from a slice made 9 days after elimination of the subcortical afferents to the hippocampal formation

LTP 2 months after subcortical deafferentation

LTP was tested in 9 slices cut from 4 rats at the 9th postoperative week. The mean amplitude of the first population spike during the pretetanization period was 2.96 mV. Although the data suggested less plasticity in these slices, potentiation was statistically similar found in the others (Fig. 2) because the deviation from the time course of control LTP was not significant ($P<0.1$). This might be due to considerable fluctuation of the individual responses accompanied with a clear

tendency to epileptiform discharges (Fig. 5), as well as the worse viability of slices obtained from these relatively old rats.

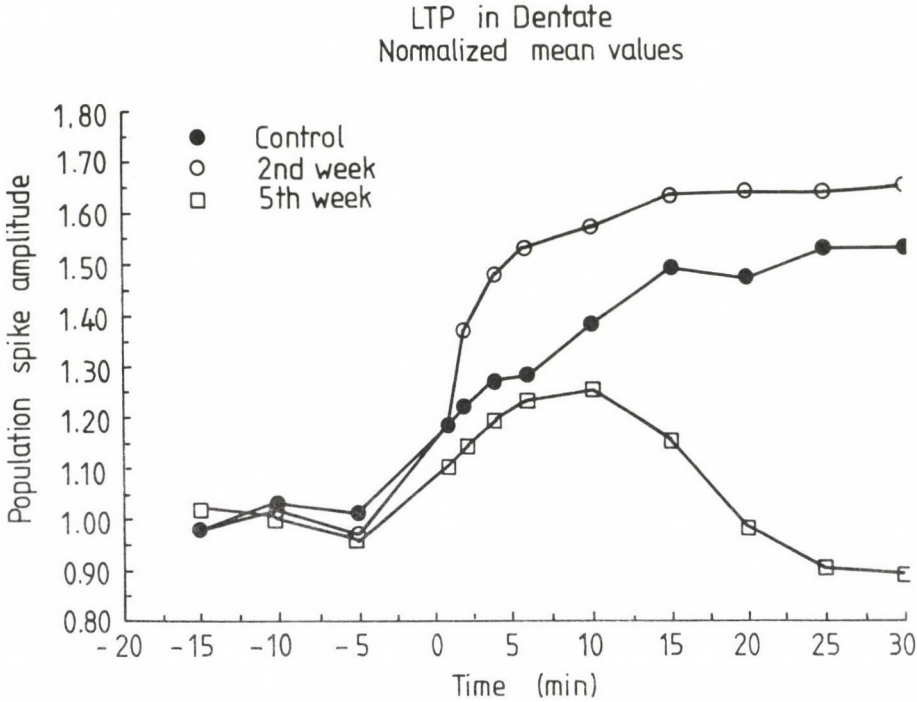

Fig. 7. Changes of postsynaptic discharges after tetanic stimulation in the fascia dentata of normal and subcortically deafferented slices. See Fig. 2 for conventions making the graph. Mean values were calculated from data on the first population spike taken as indicated in Fig. 6

Long-lasting stability of the responsiveness was followed in 3 slices in this age group without detecting significant change of the first spike of the averaged evoked potentials. Subsequent population spikes of the averaged responses changed more.

No sign of LTP was found in the fascia dentata of slices in this group. Posttetanic depression was noted in some cases which were unfortunately rejected assuming slice deterioration, before detailed analysis of the whole set of data revealed the possibility of late depression shown in Fig. 8.

Discussion

We concluded that subcortical deafferentation did not affect LTP in the CA1 area in slices where LTP in the dentate area was clearly modified or missing entirely. This is evident from the results indicating insignificant changes illustrated in Fig. 3, in

contrast with the strong modifications of LTP in the dentate area shown in Figs 7 and 8. In addition, we observed that epileptiform neuronal activity is common in slices cut from operated rats (Fig. 5). Examples of LTP failure were found in the fascia dentata as soon as about 1 week after surgery, but on the other hand some LTP appeared even 4 to 5 weeks after the operation. This may suggest that the reasons of LTP depression are complex and involve various factors in addition to simple elimination of the subcortical afferentation.

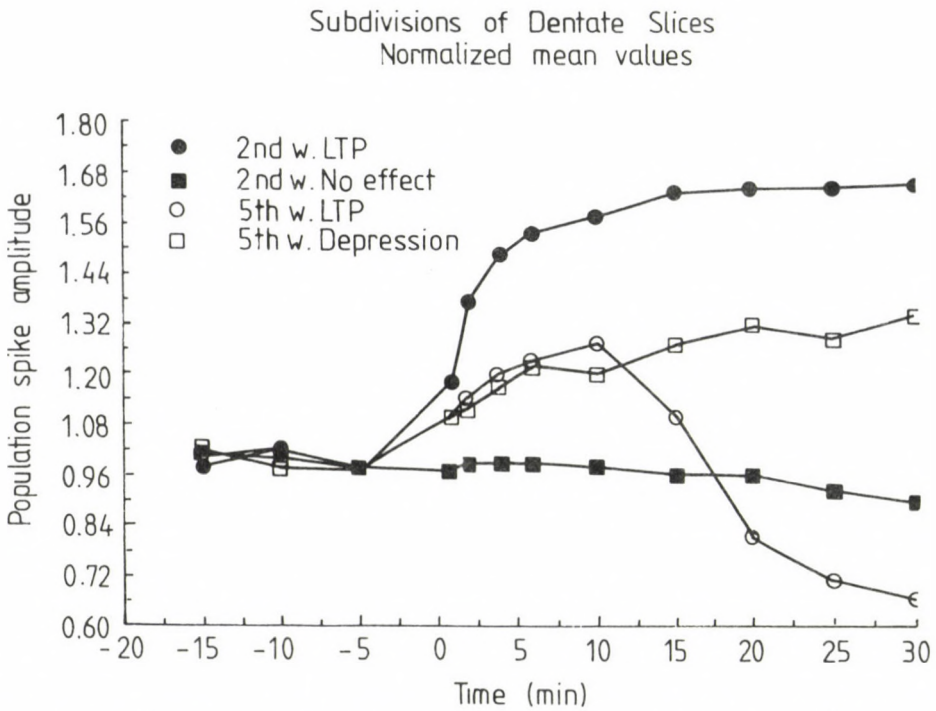

Fig. 8. Further details of effects of subcortical deafferentation upon responses evoked in the dentate area. Filled symbols show mean values calculated from the data on the first population spikes in slices made on the second postoperative week. Circles plot the data also shown in Fig. 7, squares illustrate another group of slices, in which the tetanic stimulation failed to induce potentiation. Open symbols are two subgroups separated from the data collected in the 5th postoperative week and illustrated in Fig. 7. Criterion for subdividing the data was the larger versus smaller amplitude of the population spike at 20–30 min after then before tetanic stimulation of the slices

An alternative conclusion may come from considering the statistically insignificant but visible decrease of plasticity in the CA1 area. This tendency may indicate that loosing plasticity begins much later and proceeds more slowly in the CA1 then in the dentate area. Slight tendencies toward less dynamic

electrophysiological responses may come from another complication, however. One cannot obtain slices with 2-month postoperative time from young rats, while it is our common experience that general long term viability of slices cut from older animals is less than that of those from the young ones. Our control experiments did not allow to decide that the slightly less plasticity of the CA1 synapses was an artifact, or it was due to the longer postoperative period, or to the age of rats.

Subcortical deafferentation by surgery eliminates in addition to the cholinergic, adrenergic and GABAergic pathways to the hippocampal formation [14, 15, 16, 19, 27, 31] other connections of the CA subfields as well. Buzsáki et al. [7] discussed the details of anatomical consequences of that operation and we applied the same technique. The lateral septal nucleus is a larger extra-hippocampal target for pyramidal cells, and the fimbria, fornix, ventral commissure pathway is a major route of contralateral projection of these cells [28, 32]. Sectioning of subcortical connections cuts parts of the input of CA1 pyramidal cells and the axons of all principal cells projecting through the disrupted pathways. On the other hand, axotomy is not involved in the fascia dentata because the main output of the granule cells goes to the CA3 and CA4 subfields. Therefore consequences of such kind of surgical deafferentation is expected to be more complicated in the CA1 than in the dentate area. The reason remains unclear why subcortical deafferentation affects the LTP in the CA1 area less, although the connections of this subfield is destroyed by the operation more.

Differences between mechanisms of LTP found in different synapses within the hippocampal formation (e.g. mossy fiber-CA3 connection versus Schaffer collateral and commissural fiber – CA1 connections [30]) have been recognized, including different modulations of the synaptic transfer by catecholaminerg and other pathways projecting toward the CA1 and dentate areas [11, 13, 34, 39]. The number of papers supporting the importance in LTP mechanisms of processes on the postsynaptic side of the transmission is growing continuously [2, 20, 30]. It remains to be clarified whether and how consequences of axotomy and elimination of subcortical modulation of transmission mediated by excitatory amino acids are involved in the apparent loss of LTP in the dentate but not in the CA1.

Buzsáki and Gage [6] felt reasonable to attribute the absence of LTP in the subcortically deafferented fascia dentata to their finding that the same operation induced chronic epilepsy [7]. Paroxysmal interictal discharges occurring chronically could drive the neuronal circuits on a level high enough to saturate the LTP mechanism [39], thus experimentally induced train stimulations would fail to enhance further the synaptic efficacy. We do not know if the CA1 area in our operated animals suffered also from chronic and frequent paroxysmal discharges like the subcortically deafferented fascia dentata. However, our observation (Fig. 5) suggests that the CA1 area is also more seizure prone in slices from the operated then in the

control animals. This makes unlikely an assumption that one can produce LTP in deafferented CA1 simply because these neuronal circuits are not kept in the stage of "LTP saturation".

Axotomy is well known to alter the physiological properties of the neurons [12, 24, 25], (see [3] for further references) including changes in the excitability of the dendrites which was also recognized long ago [23]. These important studies provided strong evidence for the depression of postsynaptic effects of monosynaptic excitatory inputs coming from sectioned fibers, and the complicated trophic influences involved in the consequences of fiber sections have been the subject of extensive analysis (e.g. [17, 18]). Knowledge available on the properties of the CA1 pyramidal cell dendrites and spines at present is insufficient to define the factors which may be involved in LTP modulations, axotomy and partial denervation. One may expect opposite alterations of synaptic efficacy by various factors involved in the complicated process resulting in apparent "no change" in the LTP. However, it seems quite possible that the LTP remains indeed unchanged in the CA1 area by subcortical deafferentation, which suppresses the LTP in the dentate.

Our results fall in line with the views [30] which consider the LTP as a complicated group of phenomena with several properties common in different synaptic connections but also with many aspects characteristic only of some but not other synapses. It seems likely that the rather delicate aspects of LTP, like its possible modulations addressed in our work, reveal more of the differences among neuronal systems which all share this important phenomenon.

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IMMUNOGLOBULIN-G AND CREATININE LEVELS IN RABBITS IN ALTITUDE ADAPTATION

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The changes of immunoglobulin-G and creatinine levels in mid-altitude were investigated in rabbits. The animals living at sea level were exposed to 2240 m altitude for 22 days period. When compared with sea level values; immunoglobulin-G levels were significantly low. Serum creatinine level decreased significantly in the 2nd day, then reached the sea level amount on the 12th day. On the 22nd day a significant increase was observed. It was concluded that the decrease in immunoglobulin-G values may be due to the depression of protein synthesis. The increase in plasma creatinine level would be explained by the decrease in urine.

Keywords: immunoglobulin-G, creatinine, altitude adaptation

It is well known that exposure to high altitude alters concentrations of several serum proteins as well as various electrolytes and distribution of water and salt between body compartments [3, 9, 10].

Generally the changes in the levels of some serum proteins are reported in studies on human [1, 4, 8]. However, the mechanism of these alterations have not beeen clarified yet. It has been stated by kidney function tests that altitude adaptation also effects the function of kidneys [7]. In another study the simultaneous measurement of multiple immune variables suggested that T-cell function was impaired during hypoxemia. The increased plasma immunoglobulin-G (IgG) and immunoglobulin-M (IgM) levels may have reflected in vivo polyclonal expansion due to the loss of normal T-cell regulatory influence [5].

In this study, we have concentrated on changes in serum IgG and creatinine levels at mid-altitude in an effort to compare sea-level values. In relation to this, we

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also want to discuss the alterations in serum IgG and the proteins determined in our previous study [6].

Materials and methods

Animals

Fifteen adult healthy New Zealand white rabbits weighing 1190±144.91 g (mean ± SD) and grown at Ege University Laboratory of Experimental Animals at sea level were used. They were fed by standard pellet food, normal tap water, green vegetables ad libitum. They were housed in wire-bottom cages in a room kept at 24 ± 1 °C.

Experimental procedure

Control group: Two milliliters of blood of rabbits kept at sea level were drown by cardiac puncture and transferred into clean and dry deionized tubes. The serum samples were obtained and stored at −20 °C until analyzed.

Thereafter, they were transported in approximately 10-h periods to mid-altitude and kept at Erciyes Ski House at Tekir Plateu (2240 m altitude). Animals were housed under similar conditions as it was possible. Blood samples were taken on 2nd, 5th, 9th, 12th, 15th and 22nd days during the examination period at the same time of day serum samples were prepared. In order to eliminate the travel stress the blood samples were collected on the 2nd day.

Serum IgG measurement

Serum IgG levels were determined by a modification of Sagan's method based on rivanol-precipitation [13]. For protein precipitation 1.5% Rivanol lactate per 1 ml serum was used. After gentle mixing and centrifugation the supernatant was separated. The protein concentration of the supernatant was determined by the biuret reaction [11]. After determination of the protein concentration 5 μl of supernatant were applied on Sepraphore III strips (cellulose-acetate, 2.5x7.5 cm). The separation was carried out in trisbarbital buffer, pH=8.8, ionic strength 0.1. The electrophoresis was run for 45 minutes at 2.5 (1-3) milli-ampers per strip. After that, the sepraphore strips were dried and stained with ponceaus (in 7.5% TCA). The excess stain was removed by a 5% solution of acetic acid. Two fractions obtained in these circumstances were cut out and eluted in 0.1 N NaOH solution. The absorbances of the eluates were measured at 565 nm wave length (against blank) and the percentage of the two fractions was then calculated.

The coefficient of variation was 2.75 for 20 determinations for a single sera sample. Figure 1a represents the electrophoretic pattern on cellulose-acetate strips at pH=8.8 and quick-scan pattern are shown in Fig. 1b.

Serum creatinine level measurement

Serum creatinine values were determined by Jaffé reaction [12]. This assay is based on the reaction of creatinine with an alkaline solution of sodium picrate to form a red Janovski complex.

Statistical analysis: All values are reported as means ± standard deviation (SD). Differences between paired means were determined by Student's *t* test, and two tailed, paired *t* test. Spearman rank-order correlation was used in order to discuss the results in detail in discussion.

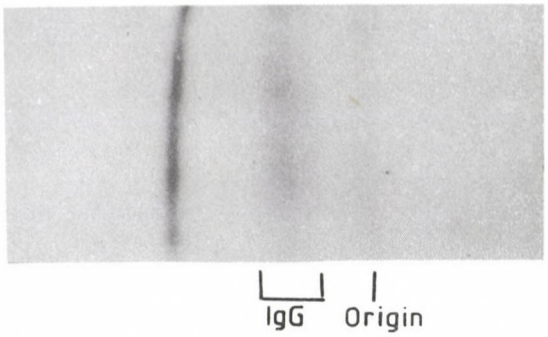

Fig. 1a. Electrophoretic pattern of sample on Sepraphore III (cellulose polyacetate) strips at pH = 8.8

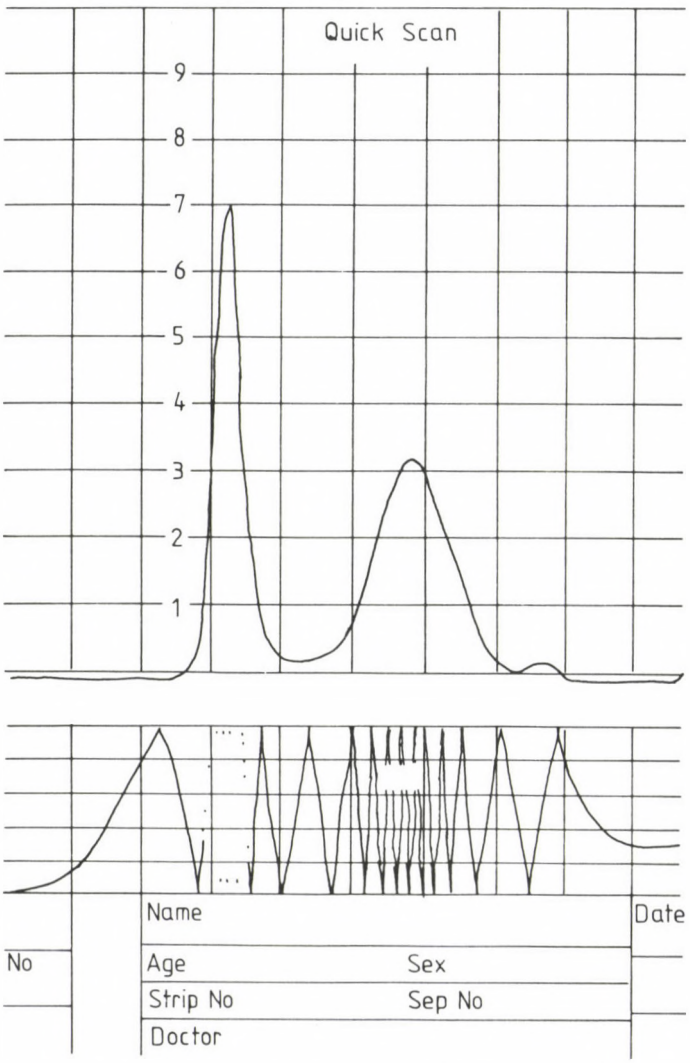

Fig. 1b. Quick-scan pattern of sample on Sepraphore III strips

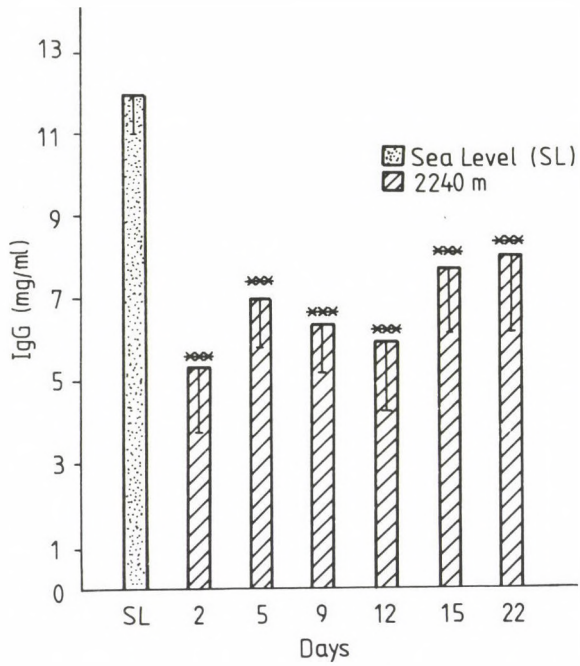

Fig. 2. IgG levels in rabbits (n = 15) at mid-altitude. Values are means ± SD, Significances: compared to sea level (SL): ***p < 0.001

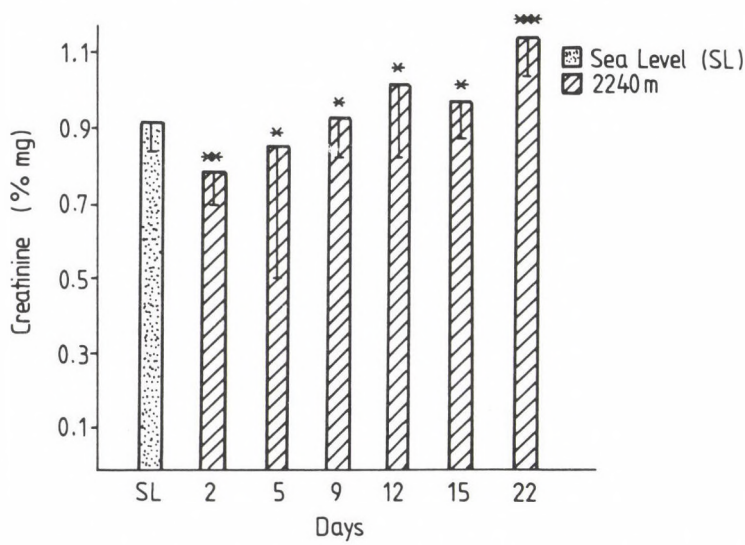

Fig. 3. Creatinine levels in rabbits (n=15) at mid-altitude. Values are means ± SD, Significances: compared to sea level (SL): *p < 0.01, **p > 0.05, ***p < 0.001

Results

The changes in IgG levels are shown in Fig. 2. A statistically significant difference was observed when the values at 2240 m altitude were compared with sea level ($p < 0.001$). There was a slight increase in IgG levels on 5th, 15th and 22nd days compared to other days.

A significant decrease was observed in serum creatinine levels well below the sea level on the second day ($p < 0.01$) (Fig. 3). This level was reached to sea level values with a slight increase for the following days (on day twelve, $p > 0.05$). A statistically significant increase was determined on day 22 ($p < 0.001$).

Discussion

Several authors have studied the changes in plasma proteins during acclimatization. Rennie and co-workers [8] measured the immunoglobulins; IgG, immunoglobulin-A (IgA) and immunoglobulin-M (IgM) plus albumin and α_2-macroglobulin, and reported that all these proteins increased significantly in concentration. On the other hand, following a decrease in IgG concentration the normal level has been reported by Bradwell [1] during acclimatization. Although Bradwell's subjects are human beings and they started to examine the biochemical changes from 1000 m altitude for 13-day period, the changes in IgG levels determined in our study are in good agreement with Bradwell's findings. In our study, the examination period is longer than Bradwell's and our starting point is sea level, so the decrease in IgG levels are more apparent than Bradwell's. It has been concluded that plasma volume changes occurred caused marked alterations in the total protein, IgG and albumin, IgG is a much larger molecule and would not be expected to parallel the movement of albumin although it would accompany water shifts to some extent [1]. Albumin is a small molecule with a molecular weight of 65,000 D. It can easily be transported in and out of vascular compartment with water. In a previous study of us, we also observed statistically significant changes in protein synthesized in liver (fibrinogen, transferrin and albumin) during acclimatization to altitude [6]. If the changes in protein levels related to our previous study are examined in association with IgG determinations in the present study; we observed that albumin, transferrin and fibrinogen changes are not dependent on IgG (Spearman rank-order correlation between mean values of albumin, $r = -0.330$, $p > 0.05$; IgG-transferrin, $r = -0.173$, $p > 0.05$; IgG-fibrinogen, $r = 0.366$, $p > 0.05$; IgG-total protein, $r = 0.123$, $p > 0.05$) (Fig. 4). Since IgG (mw = 150,000) and fibrinogen (mw = 340,000) are large molecules, their transport to the vascular

compartment is limited, even these two protein molecules follow the same alteration pattern (Fig. 4).

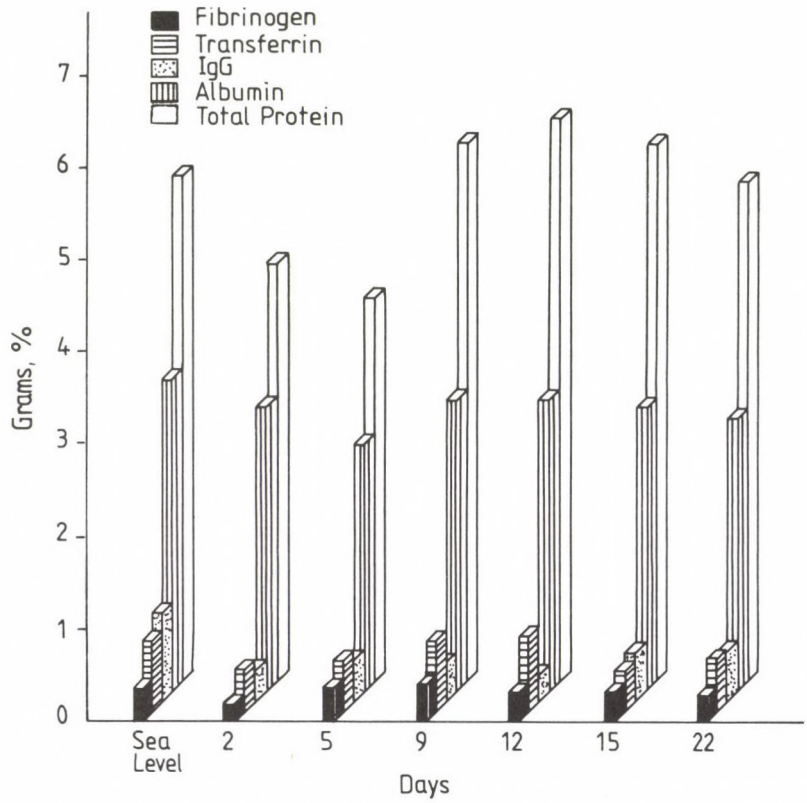

Fig. 4. Changes of fibrinogen, transferrin, IgG, albumin (Alb) and total protein during adaptation to mid-altitude. The data, except IgG belongs to a previous study [6] (Spearman rank-order correlations depending on time during adaptation between mean values of IgG-alb, $r = -0.33$, $p > 0.05$; IgG-transferrin, $r = -0.173$, $p > 0.05$; IgG-total protein $r = 0.123$, $p > 0.05$)

Our data would indicate that the decrease in IgG values may be explained by the depression of protein synthesis in liver under low oxygen tension and hypoxia may induce alterations in immune regulation [5].

It has been reported by Delamere and Jones [2] that urine output decreased during a trek to high-altitude and this phenomenon would appear to be related to a fall in glomerular filtration rate. Generally, the change in plasma creatinine level belongs to the subject's muscle mass and function. Since our animals are kept in cages with limited motion, no change in creatinine values would be expected due to muscle function. Under this condition, the increase in plasma creatinine levels on the

second day would be related to a decrease in glomerular filtration rate. Although we did not determine the urinary creatinine output, the increase in plasma creatinine level would be explained by the decrease in amount of urine. In the present study, the observed increase in plasma creatinine during adaptation to altitude could be a result of a decrease in glomerular filtration rate as concluded by Delamere and Jones [2].

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RADIOIMMUNOASSAY FOR A NEW PHENOTHIAZINE DERIVATIVE AND ITS APPLICATION*

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Fluphenazine-4-chlorophenoxy-isobutyrate ester, a new phenothiazine derivative was synthetized in the Institute for Drug Research Budapest. Radioimmunoassay was developed for the therapeutic monitoring of the drug level after intramuscular depot injection. The fluphenazine hapten was coupled to BSA by mixed-anhydride method. Antisera were produced to this conjugation in New-Zealand white rabbits and were tested for the antibody-titer. The specificity was tested by the cross-reaction with phenothiazine-analogues and other psychotropics. Strong cross-reaction was found with compounds possessing piperazine in side chain (trifluoperazine, perphenazine), but other psychotropic drugs did not react. Tritium-labelled trifluoperazine (spec. activity: 3.5 TBq/mmol) was used as a tracer in the radioimmunoassay. The detection limit was 75 pg with a CV of <5% in 50 μl plasma sample (equivalent to 1.5 ng/ml concentration) and a standard curve in the 3 ng/ml – 50 ng/ml GYKI-22441 concentration range showed a CV of <10%.

Preliminary pharmacokinetic study was performed in Beagle dogs after intramuscular depot injection with GYKI-22441 in sesame oil in a dose of 0.1 mg/kg. The GYKI-22441 concentration of the plasma samples were measured by the RIA method during a 28-day interval after the treatment and was evaluated by the MultiCalc Immunoassay Data Management program (Pharmacia).

Keywords: neuroleptic, phenothiazine analogue, radioimmunoassay, pharmacokinetics

Phenothiazine neuroleptics with a piperazino-propyl side chain are widely used in the treatment of schizophrenic disorders, and in a depot form are frequently used in the outpatient treatment.

A new phenothiazine derivative fluphenazine-4-chlorophenoxy-isobutyrate ester (GYKI-22441) was synthetized in the Institute for Drug Research and was

*Some results described in this paper were presented in the Symposium on Pharmacokinetics and Metabolism, Mátrafüred, Hungary, 18–19 October, 1991.

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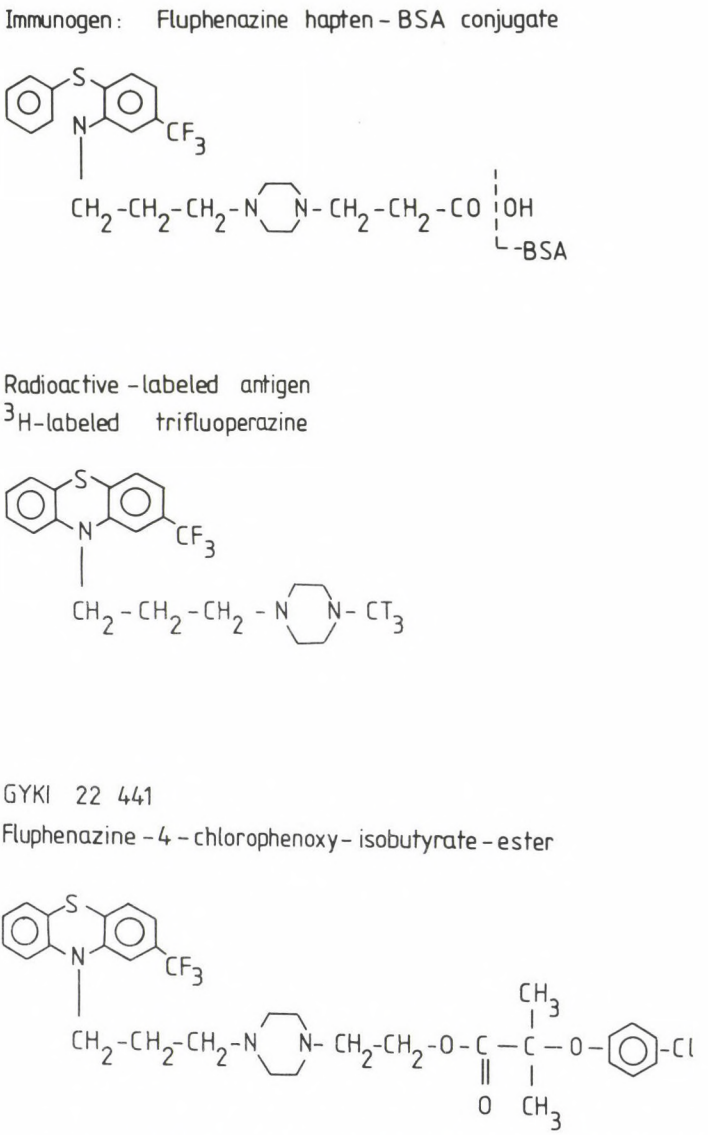

Fig. 1. Structural formulae of fluphenazine-BSA conjugate (top), H^3 trifluoperazine (middle) and GYKI-22441 (bottom)

prepared in a long acting depot injection form in sesame-oil. The preliminary pharmacological study proved the effect of the drug [8, 9] and the need of developing a sensitive and rapid method for monitoring the therapeutic level of the drug and for the determination of the optimal interval between the injections to reduce the risk.

The radioimmunoassays, described for trifluoperazine, fluphenazine [12, 13, 16] seem to be suitable for measuring the relatively low plasma level after depot injections of phenothiazine-esters.

Materials and methods

Materials

1. GYKI-22441 (Fig. 1) compound was synthetized in the Institute for Drug Research.

2. Phenothiazine analogues (trifluoperazine, perphenazine, fluphenazine) were synthetized in the Institute for Drug Research, diazepam and amitriptyline were Sigma products.

3. Radioactive trifluoperazine (Fig. 1) with a specific activity of 3.5 TBq/mmol was prepared in the Institute by methylation of N-desmethyltrifluoperazine with carrier free H3-methyl iodide [14].

Stock solutions of the phenothiazines were prepared in methanol (about 200 μg/ml) and were stored at 4 °C in dark. Dilutions were made daily for the assays.

4. Immunogen: Fluphenazine hapten was coupled to BSA (Fig. 1) by mixed anhydride method and the number of hapten per mol BSA was > 25:1 as calculated by UV spectrophotometry [5, 6, 7].

Reagents

1. Phosphate-saline buffer PBS (0.05 M pH: 7.2) containing 0.2% BSA
2. Dextran coated charcoal (2% solution in PBS)

Chemicals applied were of analytical grade.

Methods

Immunization: Antisera to hapten-BSA conjugates were raised in 10 New-Zealand white rabbits immunized with various quantity of the immunogen emulsified in 1:1 ratio with complete thereafter incomplete Freund' adjuvant. The intervals between the intradermal injections were 2 weeks and after the 3rd injection 4 or 6 weeks. The immunsera with adequate titer checked by radial immunodiffusion method were drawn and stored at −20 °C.

The specific antibody titer of the antisera were determined from the titration curves.

Specificity was tested by cross-reaction with phenothiazine analogues and other pshychotropic drugs (50% inhibition of binding at zero concentration GYKI-22441) by the criteria of Abraham [1].

Table I

Cross-reaction of phenothiazine derivates and other psychotropic drugs with antiserum F1-10

Test compound	Cross-reaction
Perphenazine	153%
Trifluoperazine	148%
Fluphenazine	162%
Amitriptyline	0 at 50 ng
Diazepam	0 at 50 ng

Treatment of dogs with GYKI-22441

Five beagle dogs were treated with GYKI-22441 depot injection in a dose of 0.1 mg/kg im. Blood samples were drawn from the brachial vein, and after centrifugation plasma samples were stored at −20 °C.

Radioimmunoassay procedure

The assay was performed in polystyrene tubes in 50 µl plasma samples (drug-free dog plasma was used for the standard curves). 350 µl PBS buffer containing the tracer (10 000–15 000 dpm/500 µl) was mixed with 100 µl antisera (in working dilution), and incubated at 4 °C overnight. Then 500 µl dextran-coated charcoal solution was added to the mixture and incubated for 10 min at 4 °C. The samples were then centrifuged at 4000 *g* for 15 min. The supernatants were decanted into 10 ml scintillation cocktail and was counted using Packard TriCarb2000CA Liquid Scintillation counter.

Results

I. Characterization of antisera

1. Antibody titer

The antisera were estimated for antibody content (titer) by the amount of bound tritiated trifluoperazine as tracer (B_0). Four antisera with an adequate (> 1:5000) titer – dilution which gave about 50% binding of tracer after the incubation at 4 °C overnight – was obtained usually after five, six injections and were tested for specificity.

2. Antibody specificity

The specificity of the antisera was detected by cross reaction with the other phenothiazine analogues and psychotropic drugs (Table I). As it can be expected we have got a strong cross-reaction (>100%) with the structurally related compounds (trifluoperazine and the fluphenazine) while there was no cross reaction with amitriptyline and diazepam.

Based on the antibody titer and cross-reaction the antiserum No. F1-10 was used for the assay (working dilution = 1:5000).

3. Standard curve

Calibration standard curves were prepared with GYKI-22441 standard solution in 300 pg–4900 pg range using 50 µl drug-free dog plasma. The assay conditions was determined at various incubation time (120 and 360 min) and temperature (37 °C and

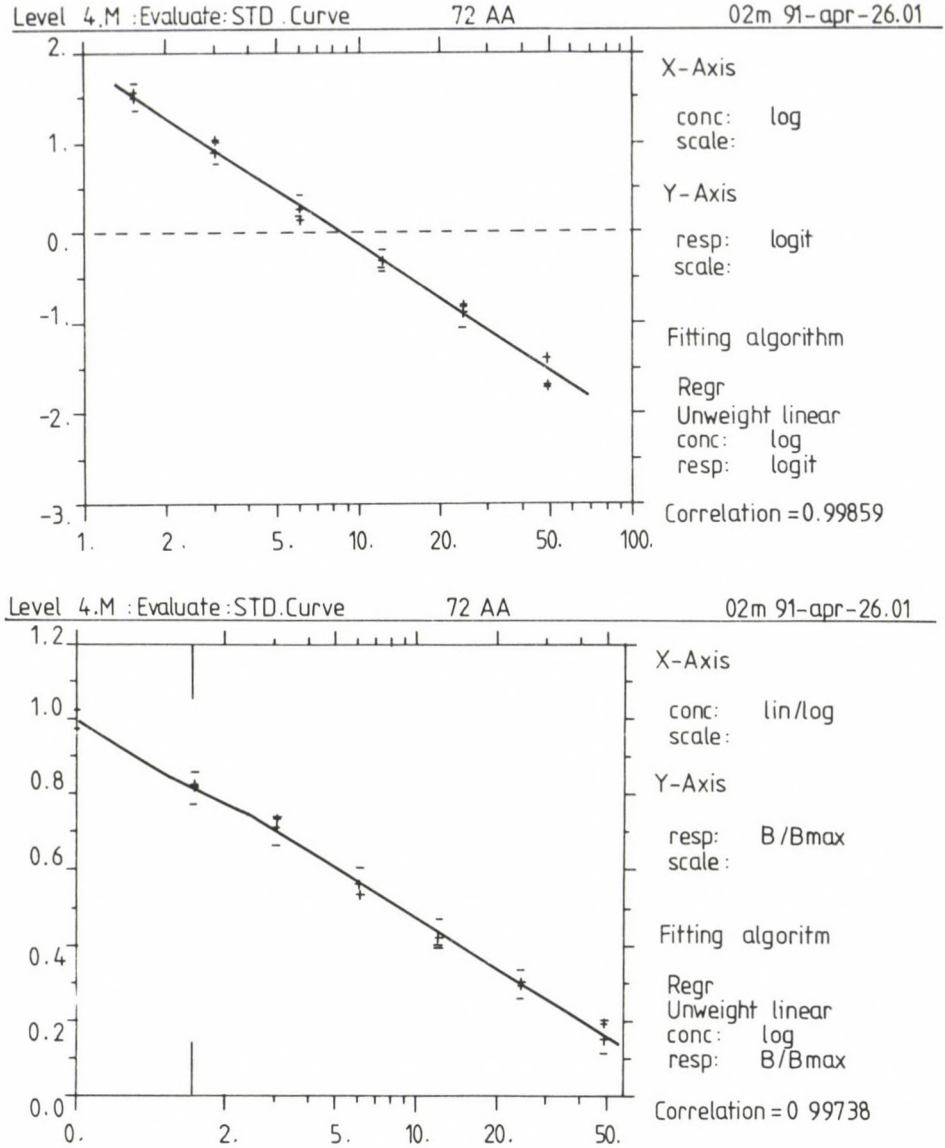

Fig. 2. Standard calibration curves for GYKI-22441 in 50 µl dog plasma logit-log (top) and B/B_0-log (bottom) equation. Each point represents the mean of 3 determinations

4 °C) and the optimal conditions were found: incubation overnight at 4 °C. Typical standard curves were constructed using the logit – log or B/B_0-log equation (Fig. 2). The least amount of GYKI-22441 which could be distinguished from the zero was 75 pg equivalent to 1.5 ng/ml using 50 μl plasma sample.

Table II

Effect of plasma
Precision and accuracy obtained by assay of known amounts of GYKI-22441 compound (range 300 pg – 4900 pg) added to 50 μl dog plasma from 3 dogs

GYKI-22441 conc. ng/ml (added)	Mesaured conc. ng/ml mean (n = 3)	S.D.	CV
3.06	3.03	±0.12	3.8
6.125	6.4	±0.69	10.8
12.25	12.0	±0.72	6.0
24.5	23.8	±1.19	4.9
49.0	49.5	±0.4	0.8

Table III

Interassay variance and accuracy. Data calculated from the standards of the assay obtained on three different days of analysis

	GYKI 22441 concentration (added) ng/ml				
	3.06	6.125	12.25	24.5	49
B/B_0 % mean n = 3	82.3	69.7	60.3	47	34.7
sd	1.5	5.5	5.5	3	4
cv	1.9	7.9	9.1	6.4	11.7

4. Precision

Known concentration of GYKI-22441 in 50 μl plasma from 3 dogs were measured to test the plasma effect. The intraassay precision and accuracy were determined (Table II) using the B/B_0 – log equation. The interassay precision was calculated of measuring the known concentration GYKI-22441 samples in 50 μl plasma on three different days (Table III).

II. Preliminary pharmacokinetic study using RIA

Five beagle dogs (weight 8–10 kg) were treated with GYKI-22441 intramuscular depot injection in sesame oil in a dose of 0.1 mg/kg.

Blood samples were taken on the 1st, 4th, 7th, 14th, 18th, 21st, 28th days after the treatment.

Plasma concentrations measured and evaluated by the standard curves and the results are presented in (Fig. 3).

After the injection on the first week we have got a slight elevation in the plasma level, thereafter a lower standard level was found which remained stable for the next three weeks.

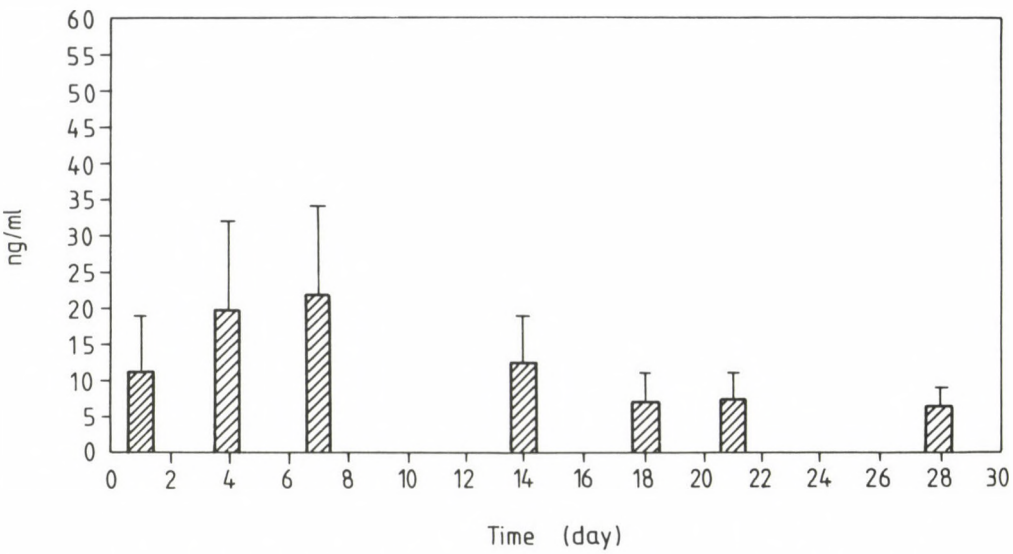

Fig. 3. Plasma GYKI-22441 mean level in five dogs receiving an im. dose of 0.1 mg/kg in a depot injection

Discussion

A specific, sensitive and suitable radioimmunoassay was developed for the pharmacokinetic and therapeutic monitoring of the new phenothiazine analogue – GYKI-22441 – synthetized in the Institute for Drug Research [10, 11]. After the treatment with GYKI-22441 depot injections the plasma samples were measured by the RIA procedure and were evaluated by the method described above. We checked the plasma effect using plasma sample from three dogs. The slope and the intercept

of the standard curves did not differ considerably. The relatively stable and standard level obtained during the 28-day investigation may be the result of the slow steady-release of the fluphenazine from the sesame-oil depot.

Observation was published that the fluphenazine-decanoate and enanthate are rapidly hydrolyzed to fluphenazine after intramuscular injection [2, 15]. The results obtained by investigation with radio-labeled compound [3, 4] showed that there was no evidence for the presence of esters in plasma, urine and faeces.

It is reasonable to suppose that in this case the main component in plasma determined by our RIA is also fluphenazine not only its 4-chlorophenoxy-isobutyrate ester.

It is also well known that the phenothiazines are extensively metabolized into numerous metabolites [2, 13, 15] including the two main (7-hydroxy and sulfoxide) which possess some pharmacological properties of the parent drug. The plasma concentration which can be determined by our assay is the immunoreactive fluphenazine level, that may include some metabolites, too.

Acknowledgements

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EFFECTS OF SYSTEMIC AND INTRACEREBRAL ADMINISTRATION OF GLUCOSE OXIDASE ON THE BLOOD SUGAR LEVEL IN RATS

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Intravenous administration of 10 to 40 U/g b.w. glucose oxidase produced hypoglycaemia in a dose-dependent manner. The enzyme-induced drop of the blood sugar level was associated with significant rise in serum potassium and the concentration of free fatty acids. Intracerebral application of glucose oxidase through chronically implanted cannula into the ventromedial, lateral hypothalamus, preoptic region and amygdaloid complex of nuclei failed to change the blood sugar level, although a moderate increase of the free fatty acids and corticosterone concentrations occurred. The local application of enzyme in the locus coeruleus region led to a significant rise of the blood sugar concentration. The observations suggest the sensitivity of brainstem catecholaminergic neuronal system to hypoglycaemia.

Keywords: glucose oxidase, blood sugar level, central regulation

Since the classical observation of the "piqure diabétique" by Claude Bernard, many investigations have supported the view that the central nervous system is implicated in the homeostatic control of peripheral carbohydrate metabolism. Evidence for the hypothalamic control of blood sugar level has emerged mainly from investigations on changes in response to chemical and electrical stimulation or lesioning of hypothalamic nuclei, and from single unit recording studies of hypothalamic neurons in response to changes in extracellular concentrations of glucose and other nutrients [9, 10, 13].

Many studies have been accumulated suggesting the essential role of noradrenergic sensitive neurons within the ventromedial and lateral hypothalamus (VMH, LH) and in the paraventricular nucleus (PVN), in the regulation of peripheral carbohydrate and lipid metabolism. Thus, local application of adrenaline

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and noradrenaline, but not dopamine, serotonine, acetylcholine or GABA facilitated the peripheral carbohydrate and lipid catabolism [3, 8, 10]. The noradrenergic neurons terminating in these hypothalamic nuclei have their origin from the cell bodies within the A1 and A2 in the brainstem [7, 9].

The present investigations provided evidence that glycoprivation in the brainstem but not in the hypothalamus results in compensatory hyperglycaemia and suggest the existence of glucose sensitive neurons in the brainstem.

Materials and methods

Male rats weighing 250-300 g were used in the study. The animals were housed in individual Plexiglas cages, at room temperature, food and water was provided *ad libitum*. The bilateral implantation of stainless steel cannulas (0.3 mm external 0.1 mm internal diameter) was performed with a stereotaxic instrument in Nembutal anaesthesia. The implantation technique and localization cannulas have been described elsewhere [5]. The experiments were performed in 3 to 5 days recovery after surgery.

Intravenous injection of glucose oxidase (EC 1.1.3.4, Aspergillus niger, SIGMA Chemicals, 250 U per mg protein) was given into the tail vein. The intracerebral administration of enzyme was performed with a Hamilton syringe, in 5 μl physiological saline. Control animals received vehicle.

Blood samples were taken from the tail cut with a heparinized capillary (100-140 μl) before and in different intervals after the administration of enzyme. The plasma glucose level was measured with GOD-POD-PAP method [13], the plasma free fatty acids with microcolorimetric technique [1], and the potassium concentration with ion-selective electrodes (Beckman ISE).

The experiments were performed in groups of 6 animals. Two way ANOVA was applied to determine the significance of differences at each sample point in comparison to the vehicle treated control values. The figures show the mean and the significance levels between the control and the enzyme treated groups.

Results

Intravenous injection of 10 to 40 U glucose oxidase per g body weight produced a dose-dependent decrease in the blood glucose concentration. High doses of enzyme led to a drop of the blood sugar concentration to undetectable levels, and the animals showed all signs of cyanosis and were lost within 30 to 40 min after the enzyme treatment. The glucose oxidase-induced hypoglycaemia was associated with a marked increase in the plasma free fatty acid and potassium concentration (Figs 1 and 2)

The intracerebral administration of glucose oxidase was performed in quantities of 5 to 10 U in 5 μl physiological saline which led to a spreading of solution at least in 1-1.5 mm area. Usually 7.5 U enzyme was used in the study, and this quantity should be effective to degrade glucose in 7.5 $\mu mol \times min^{-1}$ efficiency rate per ml extracellular fluid. The application of enzyme into the VMH,

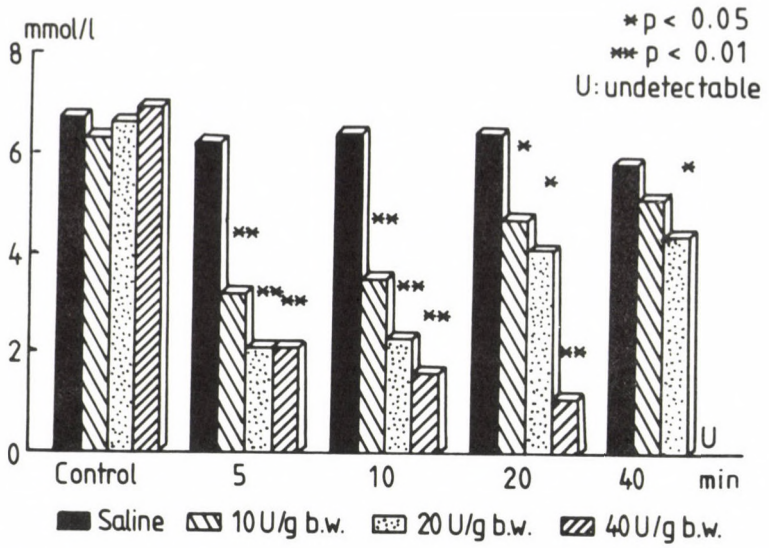

Fig. 1. Effects of intravenous injection of 10 to 40 U glucose oxidase per g body weight on the blood glucose concentration in conscious rats. Columns represent the means of groups consisting of 6 animals, and the significance of differences in comparison to the pretreated values is indicated by * above the columns. Undetectable level means less than 0.5 mmol/l glucose concentration

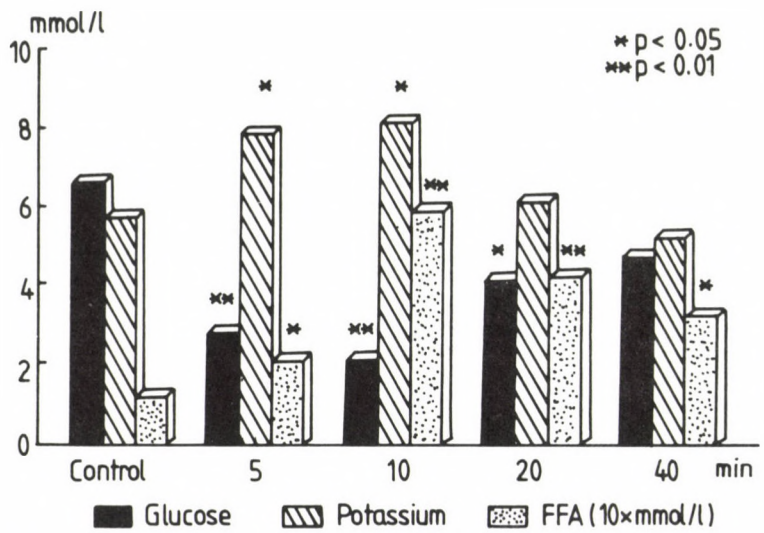

Fig. 2. Effects of intravenous injection of glucose oxidase (20 U/g b.w.) on the blood potassium and plasma FFA levels. Columns represent the means of groups consisting of 6 animals, and the significance of differences at each time interval from pretreatment values is indicated by * above the columns

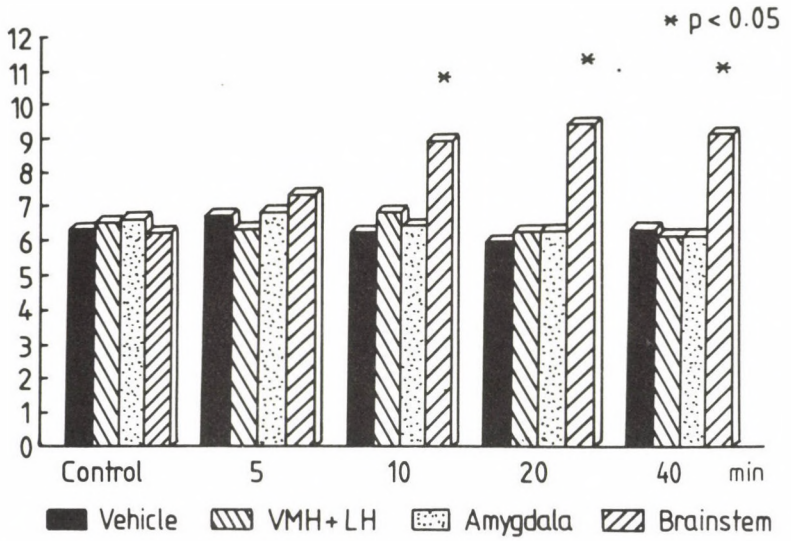

Fig. 3. Effects of glucose oxidase (7.5 U in 5 μl saline) given to the ventromedial and lateral hypothalamus (in the lack of significant difference between the two groups, the mean represents combined values), amygdaloid complex of nuclei, and into the locus coeruleus area in the brainstem. The vehicle group refers to animals with cannulas in the brainstem but they were treated with physiological saline. Statistical significance of differences in brainstem-infused rats was calculated between GOD and vehicle treated groups. In other groups, the significance of differences was calculated between pretreatment (control) and after treatment values. Significant differences are shown by * above the columns

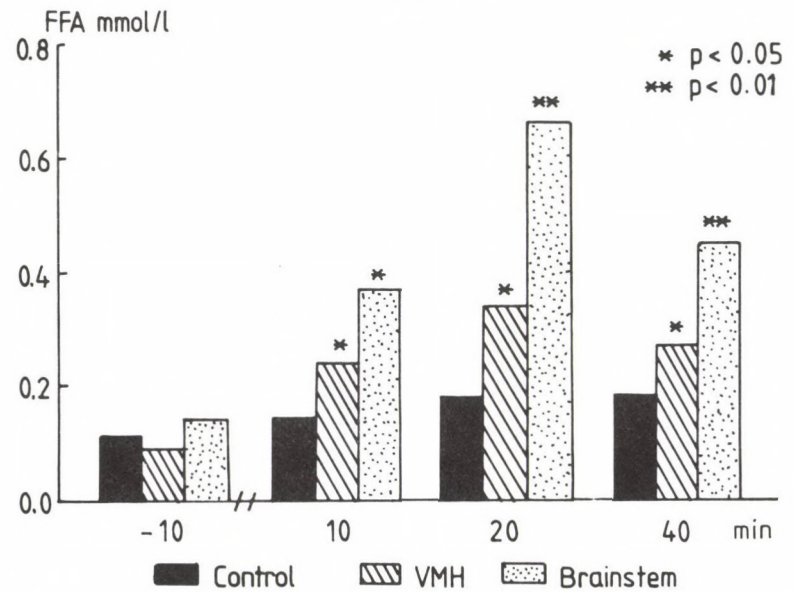

Fig. 4. Effects of glucose oxidase (7.5 U in 5 μl physiological saline) given into the VMH and the locus coeruleus area in the brainstem on the plasma FFA levels. The groups receiving vehicle alone were combined and the significance of differences was calculated in each interval following GOD administration. The significant differences are indicated by * above the columns

LH and amygdaloid complex of nuclei failed to influence the blood sugar level within 40 minutes after the injection (Fig. 3). In contrast, the local application of enzyme into the locus coeruleus area in the brainstem, led to a significant increase in blood sugar concentration. It is worth to mention that intrahypothalamic administration of glucose oxidase was followed by a characteristic restlessness of animals lasting for 15 to 20 minutes, although this gross-behavioural response was not characteristic to the vehicle-treated controls or to animals with enzyme treatment in the brainstem.

Intracerebral administration of glucose oxidase did not change the plasma potassium concentration. Intrahypothalamic administration of enzyme resulted in a moderate but significant increase in the plasma free fatty acid concentration in the absence of changes in the blood sugar level. The glucose oxidase-induced hyperglycaemia was also associated with a rise in the plasma free fatty acid concentration (Fig. 4).

Discussion

Glucose oxidase given intravenously results in a prompt development of hypoglycaemia which is associated with hyperkalemia and lipolysis. These changes appear to be opposite to those observed following insulin-induced hypoglycaemia, and they can be attributed to a metabolic hypoxic state as result of the lack of substrate important for cellular oxidative metabolic processes. Moreover, the increase of free fatty acid concentration due to hypoglycaemia may be considered as concomitant of the activation of sympathicoadrenal system [8, 13].

Local application of glucose oxidase into the glucostatic area of the hypothalamus failed to change the blood sugar level, although a moderate increase in plasma free fatty acid level suggested changes in peripheral metabolic processes. Concerning the role of hypothalamus in regulation of peripheral carbohydrate and lipid metabolism, it was found that noradrenaline sensitive neurons in the VMH play an essential role in energy expenditure-induced increase of blood sugar and free fatty acid levels [8, 11]. However, there is no consistency in alterations in the plasma catecholamine concentrations, and the blood glucose and free fatty acid levels after manipulations of hypothalamic adrenoceptors, which suggested the direct effect of the sympathetic outflow to the liver and other organs [8, 13].

The noradrenergic neuronal system seems to be involved in the transmission of signals, concerning peripheral blood glucose concentration, to the glucostatic area of the hypothalamus. The presence of glucose sensitive neurons in the lower brainstem and hindbrain has already been suggested [8, 13], and other reports questioned the significance of forebrain in glycoprivation-induced hyperglycaemia [4, 6]. The present findings with local application of glucose oxidase support the view that catecholaminergic neurons in the brainstem are sensitive to a drop of the

extracellular glucose concentration, and the compensatory hyperglycaemia and elevation of plasma free fatty acid level can be attributed to the facilitation of sympathetic outflow. These findings do not contradict to the observations that blockade of β-adrenoceptors inhibit the energy expenditure-induced increase of blood sugar and plasma free fatty acid levels [11]. The activation of neurons in the ventral noradrenergic bundle, however, seems to occur at the brainstem level in both ascending and descending directions.

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EXTENSION OF LIFESPAN IN MICE TREATED WITH DINH LANG (*POLICIAS FRUTICOSUM* L.) AND (-)DEPRENYL*

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The effect of Dinh lang root extract (DLRE) and (-)deprenyl on memory function and lifespan was examined in OFA-1 male mice. Treatments of either DLRE, (-)deprenyl or their combination were carried out 3 times a week starting at 12 months of age and following to the end of life. DLRE and (-)deprenyl significantly increased the memory function as well as surviving time of aged mice. The drug-treated animals showed a lower rate of loss of body weight than saline treated ones. It suggested that DLRE and (-)deprenyl did not prolong lifespan of mice by suppressing food intake. The combined treatment of DLRE and (-)deprenyl proved to be the most effective.

Keywords: memory function, lifespan, body weight, tumors

It has been shown in previous papers that DLRE exerts different types of Ginseng-like stimulatory activities in rodents [6, 8–10, 12, 13]. Recently, DLRE has been found to exert the effect being similar to that of small dose of (-)deprenyl [15, 16, 18].

In a new series of experiments with DLRE, it was demonstrated that the continuous administration of DLRE stimulated the sexual performance of male rats [15] and facilitated the memory function in rats and mice [16, 18].

The present paper is devoted to analyze the effect of long-term administration of DLRE and DLRE plus (-)deprenyl on the memory function and lifespan of mice.

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Akadémiai Kiadó, Budapest

Materials and methods

The dried Dinh lang rots (*Polycias fruticosum* L.) obtained from Vietnam were macerated and extracted 3 times with ethanol (50% in volume). The pooled ethanolic extracts were filtered, concentrated and dried in vacuum. 50 mg extractum siccum corresponded to 1 g of the dried roots of the plant. The preparation was dissolved in water and given subcutaneously or orally to the animals (for details, see 6 and 10).

12-month-old, male OFA-1 mice were randomly divided into 4 groups (40 mice per each). Treatment was initiated 3 weeks before starting the tests and going on 3 times a week to the end of their life. Treatments were as follows: saline, DLRE (10 mg/kg, per os), (-)deprenyl (0.25 mg/kg, s.c) and DLRE (10 mg/kg) plus (-)deprenyl (0.25 mg/kg).

One trial learning passive avoidance test [16, 19] was chosen for checking memory function of the mice. The set-up consisted of two boxes: a white box (10x10x20 cm) illuminated by a 100 W light bulb, connected with a larger dark box (23x16x20 cm) by a passage. A mouse was put into the white box. When the mouse stepped to the dark box, door was immediately closed and the unavoidable foot shock (0.2 mA, AC) was delivered through the grid. The learning test was terminated after foot shock, and the mouse was transferred into the home-cage. Retention of memory was checked 24 hours after the learning test. Retention test was conducted similarly to the learning test, except delivering the foot-shock. The "step-through" larency (the time from introducing the mouse into the white box till it stepped into the dark box) was recorded. The mouse was considered to show retention if it avoided the dark box over 60 seconds in the retention test. The retention test was terminated if the mouse stepped through the dark box or remained in the white box over 200 seconds (maximum latency).

The "step-through" latencies and number of mice showing retention were recorded once a month. Body weights were measured every two weeks. The measurements of retentions and body weights were discontinued when the number of mice in a group decreased to three. Time of the death was recorded for every animal. After death, autopsy was executed for detecting incidental tumors.

Results and discussion

Figure 1 (part 1) presents the number of mice showing retention in saline and drug treared groups. DLRE and (-)deprenyl facilitated retention. DLRE plus (-)deprenyl treatment was the most effective. At the begining of the experiment there were equal number of mice with retention in all groups. But 7 months later, only 11 saline-treated mice showed retention (loss of 50%), whereas 20-21 drug-treated mice maintained retention. 15 months later 50% of the DLRE plus (-)deprenyl treated rats showed retention whereas none of the saline-treated animals could maintain this function.

The animals in drug-treated groups showed a slower rate of loss of body weight (Fig. 1, part 2). The fact that DLRE and (-)deprenyl prevented the age-related loss of body weight in this experiment are consistent with previous work by us [14, 17, 18] in rats. The two drugs did not suppress the food intake in the aged animals.

Continuous administration of DLRE and (-)deprenyl prolonged the lifespan of mice (Fig. 2). We lost the first mouse in saline treated group during the 75th week of their life. The longest living mouse in this group lived 133 weeks, whereas, the shortest living mouse in DLRE-treated group lived 94 weeks and the longest living

one lived 171 weeks. (-)Deprenyl, too, prolonged survival time of mice. DLRE plus (-)deprenyl was the most effective. The longest living mouse in the DLRE plus (-)deprenyl treated group lived 180 weeks, i.e. twice longer than the average lifespan of the saline treated animals.

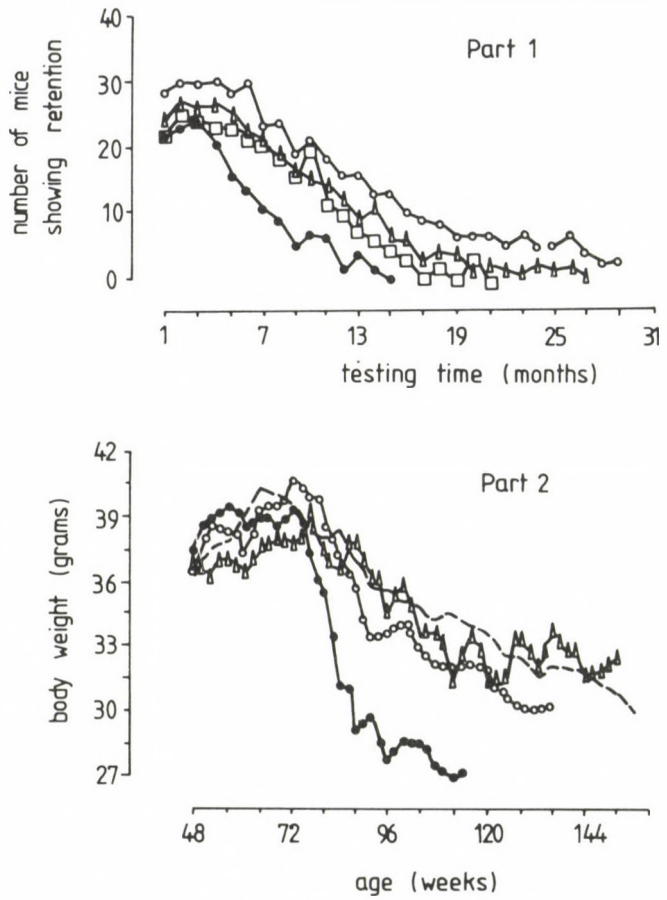

Fig. 1. Part 1: Changes in memory performance of mice treated with saline (•, n = 40), DLRE (□, 10 mg/kg, orally, n = 39), (-)deprenyl (▲, 0.25 mg/kg, s.c, n = 40 and DLRE (10 mg/kg) plus (-)deprenyl (o, 0.25 mg/kg, n = 40), 3 times a week to the end of life. Mice were tested in passive avoidance test, once a month until they died. Number of mice showing retention in every month were calculated.
Part 2: Changes in body weight of mice treated with saline or drugs. Mice were treated with saline (•), DLRE (▲), (-)deprenyl (o), DLRE plus deprenyl (- - - -). Body weight of the mice was measured in every two weeks and is presented in the Figure

Number of unclassified tumors in mice were counted via autopsy at their death. We found to our surprise that in DLRE and DLRE plus (-)deprenyl treated groups, there were much lower number of tumor-bearing mice than in the saline treated group. (-)Deprenyl was found to be less effective (Table I). The influence of DLRE on tumor development in aged mice needs further investigation.

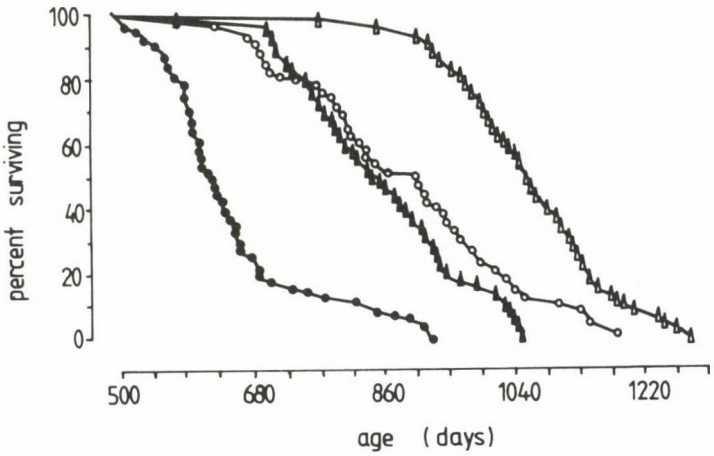

Fig. 2. Survival curves of the mice treated with saline or drugs. The mice were treated with saline (•, n = 40), DLRE (o, 10 mg/kg, orally, n = 39), (-)deprenyl (▲, 0.25 mg/kg, s.c, n = 40) an DLRE (10 mg/kg) plus (-)deprenyl (Δ, 0.25 mg/kg, n = 40), 3 times a week to the end of life. Time of death of every mouse was recorded. Each point in survival curves represents one mouse

Table I

Per cent of tumor-bearing mice in saline and drug-treated groups

Groups		Per cent of tumor-bearing mice dying out at			Total amount of tumors (in %)
		80 – 100 (weeks)	101 – 12 (weeks)	above 121 (weeks)	
Saline	(40)	5.0	17.5	15.0	37.5
DLRE	(39)	0	10.3	2.6	12.9a
DEP	(40)	0	10.0	12.5	22.5
DLRE+DEP	(40)	0	7.5	2.5	10.0a

Four groups of 12-month-old mice were treated three times a week with saline (n=40), DLRE (10 mg/kg, orally, n=39), (-)deprenyl (DEP, 0.25 mg/kg, s.c., n=40) and DLRE (10 mg/kg, orally) plus (-)deprenyl (0.25 mg/kg), respectively, to the end of life. All animals were dissected when they died to find the incidental tumor(s). Tumor frequencies were counted unclassified and compared between groups using chi-square test. a. $p<0.05$

Table II

Relation between the individual differences in memory function and in lifespan of mice treated with DLRE and DLRE Plus (-)deprenyl

Classification of memory function	Saline (40)	DLRE (39)	DEP (40)	DLRE+DEP (40)
		Average lifespan (weeks)		
Weak	84.8±1.6	102.2±1.1a	114.3±1.5b	118.3±0.9b
Good	101.4±2.1	120.9±1.8b	126.7±1.2c	141.6±3.5c
Excellent	119.1±0.9	149.4±1.6c	137.7±1.2c	157.7±2.8c
		Maximum lifespan (weeks)		
Weak	96	116	145	127
Good	107	133	131	154
Excellent	133	170	162	180

Four groups of 12-month-old mice were treated three times a week with saline (n=40), DLRE (10 mg/kg, orally, n=39), (-)deprenyl (DEP, 0.25 mg/kg, s.c., n=40) and DLRE (10 mg/kg, orally) plus (-)deprenyl (0.25 mg/kg), respectively, to the end of life. Memory function of mice was checked once a month until death. Relation of memory and lifespan are shown in the Table. a) $p<0.05$; b) $p<0.01$ and c) $p<0.001$ vs data in the saline treated group

For analyzing the correlation of memory function and lifespan in mice the animals were categorised according to their memory function as follows: Weak (no sign of retention throughout the experiments), Good (retention was detected in 1-6 tests), Excellent (retention was detected in more than 6 tests). Table II shows the effect of DLRE and (-)deprenyl treatment on the memory function and life span in aged mice. The data suggested that the good performers live longer than the weak ones in all groups. In the drug-treated animals a parallel increasing of memory function and lifespan in mice could be detected.

The results of this experiment confirm the previous finding that there was a parallel increasing of sexual performance and lifespan in the male rats with long-term treatment of DLRE and (-)deprenyl [3, 15, 18].

Thus, small-dose, long-term treatment of DLRE or (-)deprenyl exerted similar effects on restoring behavioral performance of aged rodents and prolonging their lifespan. Our results strongly contribute to Knoll's finding that (-)deprenyl slowed down the age-related decline of nigrostriatal dopaminergic machinery, the most rapidly aging system in the brain [5] and in this way a significant shift in the time of natural death can be reached [1, 4, 7].

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EFFECT OF (-)-PARA-FLUORO-DEPRENYL ON SURVIVAL AND COPULATION IN MALE RATS*

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Six-month old male rats were treated with 0.25 mg/kg, s.c., (-)p-fluoro-deprenyl (n=40) or salt solution (n=20) three times a week for 25 months. Three of the 20 saline-treated and 15 of the 40 drug-treated males survived ($p < 0.05$). Sexual activity of the survivors was tested at the end of the experiment. Three of the (-)p-fluoro-deprenyl-treated 31-month-old males proved to be sexually fully active, though, Wistar rats lose their ability to ejaculate by completing their second year of life.

Non-copulator, 13 month old male rats were treated instead of the usually used 0.25 mg/kg dose with 0.01 mg/kg, s.c., (-)deprenyl (n=9), (-)p-fluoro-deprenyl (n=9) and salt solution (n=9), three times a week, for 82 weeks and mating activity was tested weekly. The lifespan of the non-copulators was very short: 102 weeks for saline (n=9), 106 weeks for (-)deprenyl (n=8) and 104 weeks for (-)p-fluoro-deprenyl (n=7).

Survival was lightly changed by this very small dose treatment, one (-)deprenyl-treated male and two (-)p-fluoro-deprenyl-treated rats remained alive. The copulatory activity, however, was substantially improved.

Keywords: survival, sexuality, (-)para-fluoro-deprenyl; (-)deprenyl

Long-term, small dose (-)deprenyl treatment was found to enhance sexual activity and extend lifespan in male rats [2, 3]. The aim of this work was to analyse the effect of (-)p-fluoro-deprenyl (pFD) on copulatory activity and survival.

Methods

Forty Wistar male rats were treated with 0.25 mg/kg pFD s.c. three times a week (on Monday, Wednesday and Friday) and twenty males with 0.9% salt solution (1 ml/kg s.c.). The animals were six

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months old at the beginning of treatment (body weight 300-350 g). At the end of the 25th month of treatment the survivors were paired with receptive females in separated test-boxes and the male copulatory patterns (mountings, intromissions and ejaculations) were registered for 120 minutes.

Another group of males run our standard sexual activity testing procedure [1] and we selected 27 males which failed to show any copulatory patterns towards the receptive females (non-copulators), we started to treat these males at the age of 13 months (body weight: 550-600 g). Nine males were treated with 0.01 mg/kg (-)deprenyl s.c., 9 males with pFD (Chinoin, Budapest, Hungary), s.c., and 9 males with 0.9% salt solution (1 ml/kg s.c.) three times a week. The copulation of these males were checked weekly (test duration: 30 min) during 82 weeks.

Results and discussion

Table I shows that treatment increased significantly the survival rate of male Wistar rats.

Wistar rats lose their ability to ejaculate to the end of their second year of life [3]. We tested the copulatory activity of the survivors at the end of the 25th month of treatment. As expected the three survivors of the salt solution-treated group did not show any copulatory patterns. Of the 15 survivors in the pFD-treated group three males proved to be sexually fully active.

Table I

Effect of (-)para-fluoro-deprenyl (pFD) treatment (0.25 mg/kg, s.c., three times a week; (n = 40) on the survival of male rats in comparison to the salt solution treated ones (n = 20). Treatment started at the age of six months

Treatment in months	Number of survivors	
	Salt	*pFD*
0	20	40
12	15	31
25	3	15*

* $p < 0.05$; chi-square test

Table II shows the performance of these rats. The activity of Male 1 and Male 2, which displayed 6 and 4 ejaculations, respectively, may well be denoted as unusual cases of drug-induced enhancement of copulatory activity. These males were 31-month old and we never observed even in young males 6 ejaculations during one test period. Why two males out of the 15 pFD-treated survivors reacted to the treatment dramatically remains to be elucidated. The fact, however, that this is possible at all sustains good hopes for the future.

Table II

Sexual activity of three aged males treated with (-)para-fluoro-deprenyl in a dose of 0.25 mg/kg s.c. for twenty-five months

	Number of			
	mountings	intromission	ejaculations	latences in min
Male 1	32	17	6	6, 12, 20, 34, 43, 60
Male 2	9	12	4	12, 20, 28, 35
Male 3	32	15	1	44

Copulatory activity was tested at the end of treatment for 120 min.

In a series of experiments we treated a group of selected non-copulator males instead of the usually used dose of (-)deprenyl (0.25 mg/kg, s.c., three times a week) with a very low dose (0.01 mg/kg s.c., three times a week). This very low dose treatment exerted a pronounced stimulation of mating patterns in non-copulator males: ejaculations and intromissions were consistently maintained on a high level during a forty-week period. The same dose of pFD was also effective from 21-40 weeks of observation. In the saline treated group only one ejaculation occurred during the test, but mountings and intromissions were detectable (Table III).

The lifespan of these groups of males did not differ significantly. One (-)deprenyl treated male and two pFD treated males were still alive at the end of the 82 weeks of observation (Table IV).

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Table III

Total number of mountings (M), intromissions (I), and ejaculations (E) in non-copulator male rats treated with (-)deprenyl, (-)para-fluoro-deprenyl (pFD) in a dose of 0.01 mg/kg, s.c., three times a week, in comparison to salt solution treated males during an observation period of fifty weeks. Treatment started 24 hours prior to the fifth test

	M	I	E	M	I	E	M	I	E	M	I	E	M	I	E	M	I	E
Saline (n=9)	0	0	0	20	7	0	8	1	0	36	16	1	0	0	0	0	0	0
(-)deprenyl (n=9)	0	0	0	28	30	3	107	58	9	172	103	11	29	13	0	0	0	0
(-)para-fluoro-deprenyl (n=9)	0	0	0	0	0	0	42	16	3	39	14	1	2	0	0	0	0	0
Weekly mating test:	1-4			5-20			21-30			31-40			41-45			46-50		

Table IV

Lifespan of male rats in weeks treated with 0.01 mg/kg (-)deprenyl, (-)para-fluoro-deprenyl in comparison to the salt solution treated males during a 82 week observation period. Treatment started at the age of 13 months

Salt treated (n = 9)	102.00 ± 6.45 weeks
(-)deprenyl treated (n = 8)	106.50 ± 7.66 weeks
pFD treated (n = 7)	107.14 ± 6.26 weeks

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(-)DEPRENYL (SELEGILINE) IS DEVOID OF AMPHETAMINE-LIKE BEHAVIOURAL EFFECTS IN RATS*

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Central effects of high dose (-)deprenyl (50-100 mg/kg sc) was compared to that of (+)deprenyl and (±)amphetamine (AM). (+)Deprenyl and AM induced stereotyped behaviour, increased spontaneous motility in smaller and decreased it in higher doses, inhibited escape behaviour and shutlle-box avoidance. (-)Deprenyl failed to possess any of these effects, providing further evidence that AM-type metabolites have no share in the action of (-)deprenyl.

Keywords: deprenyl, amphetamine, behavioural effects

(-)Deprenyl, a close structural relative to methamphetamine developed in the 1960's [10] proved to be the first selective monoaminooxidase(MAO)-B inhibitor and is still the only one in clinical use. Whereas (+)deprenyl a strong selective inhibitor of MAO-B was found to possess amphetamine(AM)-like stimulatory properties, the levorotatory form, a stronger MAO-B inhibitor than (+)deprenyl, was almost ineffective as an AM-type stimulant [11].

Reynolds et al. [14] showed, that (-)deprenyl was metabolized to AM and methamphetamine in man and left open the possibility, that these metabolites contribute to the action of (-)deprenyl. The clinical experiences of the recent years excluded this possibility; neither AM-like unwanted effects [16], nor abuse liability [1] were observed during long-lasting (-)deprenyl treatment and the difference in the onset of the action of (-)deprenyl and AM in Alzheimer disease was also stressed [17].

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The forming of AM and methamphetamine as deprenyl metabolites was confirmed also in rats [4, 12], however, the comparison of the effects of (-)deprenyl and AM in rats following repeated administration revealed marked differences. While (-)deprenyl increased the DA content and turnover in the striatum, AM decreased it [19], the supersensitivity of DA receptors to apomorphine, which develops during AM treatment failed to develop in case of (-)deprenyl [18].

The aim of this work was to study the potential role of AM-type metabolites when applying very high doses of (-)deprenyl.

Animals and drugs

Experiments were performed on adult Wistar rats of both sexes (140-160 g), housed in groups of 5 at constant temperature (20-21 °C) under a standard 12-12 h light-dark cycle (light on at 6.00 a.m.). Water and food were available ad libitum, except for periods of tests. All the observations were made at comparable times of the day during the light period.

Drugs: (-) and (+)deprenyl HCl (Chinoin, Hungary), (±)amphetamine sulfate (AM, Chinoin). All the drugs were dissolved in 0.9% saline and administered subcutaneously. The dose of AM refers the free base.

Statistical analysis: Through the text the data are presented as mean values, with standard errors of the mean (x ± SEM). Statistical significance of difference between mean values was evaluated by two-tailed Student's *t*-test, Mann-Whitney U test and ANOVA analysis. A p value of less than 0.05 was considered to be significant.

Results

I. *Stereotyped behaviour* was assessed according to the score system of Costall et al. [2], detailed elsewhere [18]. The intensity of the stereotyped behaviour (characterized by the maximal score value) and the duration of it (characterized by the total number of the scores during the whole observation period) induced by AM and deprenyl enantiomers were compared. Following a 30 min adaptation period the animals were injected with any of these drugs and the score values were assessed every 10 min for a 60s period until the complete cessation of the stereotyped behaviour.

The intensity and the duration of the AM-induced stereotyped behaviour was increased dose-dependently. While (+)deprenyl in a high dose (50 mg/kg) induced a stereotyped behaviour corresponding to the effect of 5 mg/kg AM, the (-) form failed to produce any sign of stereotype during the 4 hour observation period even in a 100 mg/kg dose (Fig. 1).

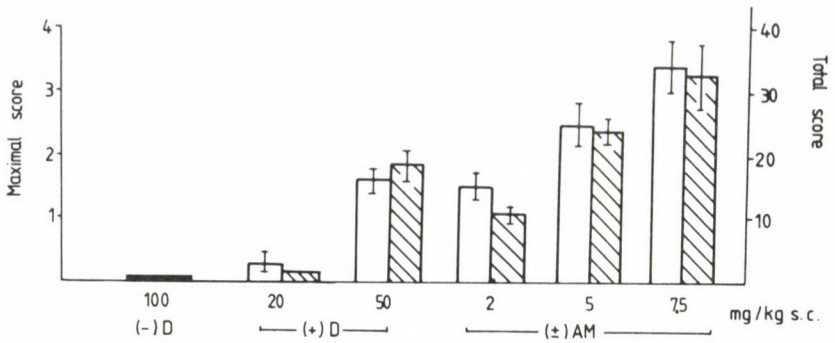

Fig. 1. Stereotyped behaviour

D - deprenyl, AM - amphetamine
Score system according to Costall et al. (3)
Observation period started immediately after s.c. drug administration, following a 30 min adaptation period
Score values were assessed every 10 min
empty bars - maximal score, hatched bars - total score
n = 6

II. *Spontaneous motility* was measured by counting the spontaneous crossings in a shuttle-box, detailed elsewhere [18]. The observation period started 30 min following the drug administration and lasted for further 30 min. AM and (+)deprenyl increased locomotion in smaller dose (0.5 and 5 mg/kg, respectively), and decreased it in higher dose (10 and 50 mg/kg). (-)Deprenyl displayed neither stimulatory nor inhibitory action (Fig. 2).

III. *Escape behaviour* was measured in the modified jumping test [9]. The animals were put under a glass cylinder on a hot plate (45 °C) 30 min after drug administration and escaped by jumping to the upper rim of it. The latency time of escape was expressed in arbitrary units. AM in a dose of 5 mg/kg and (+)deprenyl in a dose of 20 mg/kg prolonged latency time significantly, (-)deprenyl failed to inhibit escape behaviour (Fig. 3).

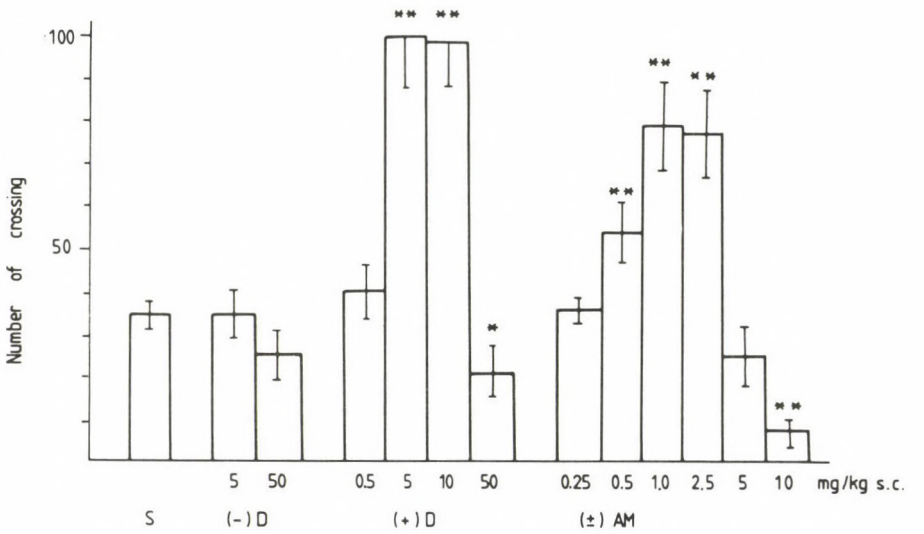

Fig. 2. Spontaneous motility
S - saline, D - deprenyl, AM - amphetamine
Observation period started 30 min after s.c. drug administration and lasted for 30 min
* $p < 0.01$, ** $p < 0.001$ compared to saline (two tailed Student's *t*-test)
n = 10

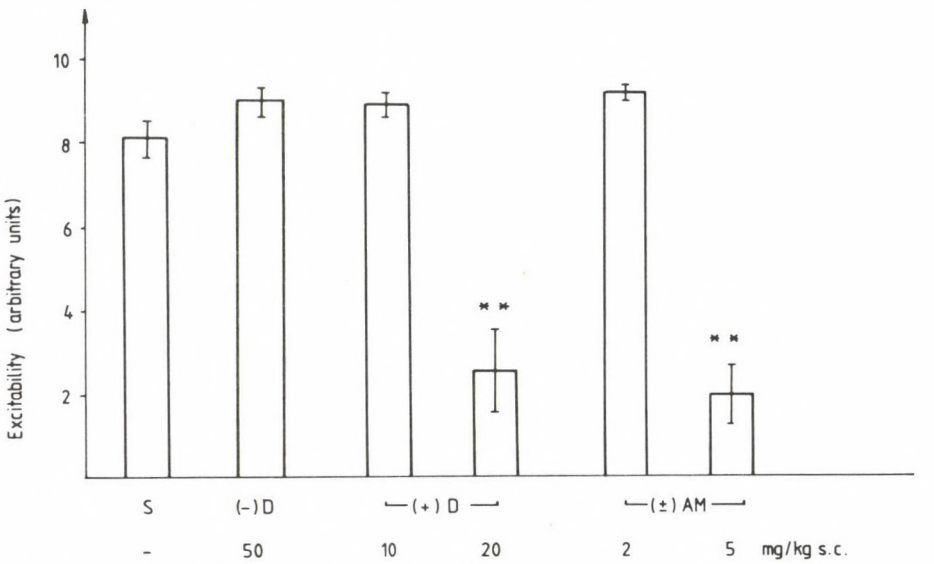

Fig. 3. Escape behaviour (Modified jumping test)
Abbreviation - see Fig. 2
Observation period started 30 min after sc drug administration; latency time is expressed in arbitrary units
* $p < 0.01$ compared to saline (Mann-Whitney U test)
n = 10

IV. *The shuttle-box avoidance* was investigated according to the method described in ref. 5. The animals were tested on five consecutive days 30 min after drug administration. AM depending on the dose increased (2 mg/kg) or decreased (5 mg/kg) the number of conditioned avoidance responses, (+)deprenyl in a dose of 20 mg/kg decreased it, whereas the same dose of (-)deprenyl was ineffective (Fig. 4).

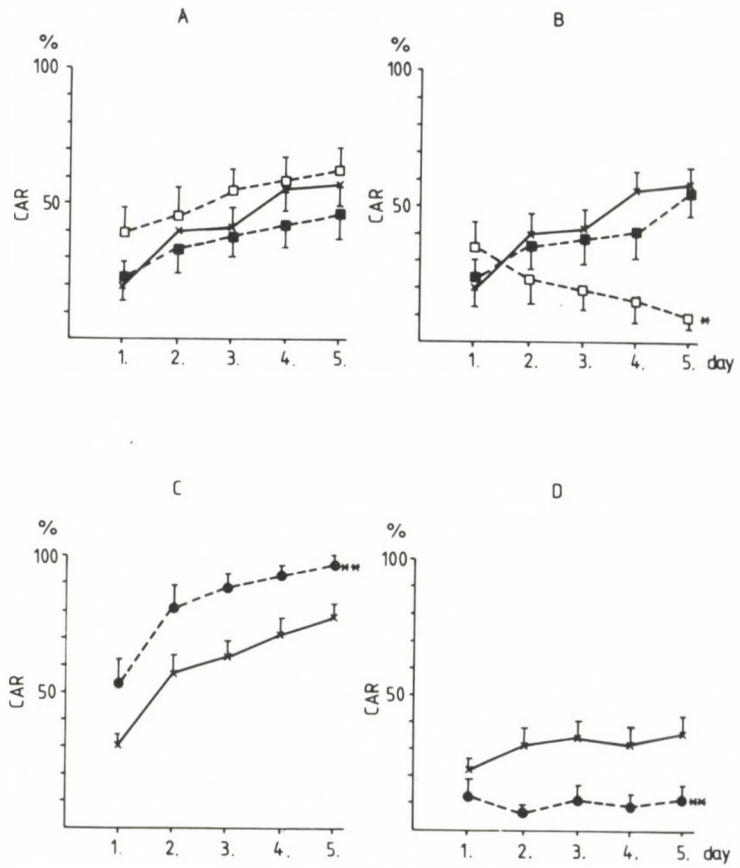

Fig. 4. Shuttle-box avoidance

CAR - conditioned avoidance response
x - - - x - saline; □ - - □ - (+)deprenyl; ■ - - ■ - (-)deprenyl; ● - - ● - amphetamine. A - 5 mg/kg s.c. daily; B - 20 mg/kg s.c. daily; C - 2 mg/kg s.c. daily; D - 5 mg/kg s.c. daily
Observation period started 30 min after s.c. drug administration
* $p < 0.05$, ** $p < 0.01$ compared to saline (ANOVA analysis)
n = 12

Discussion

The central stimulatory effects of the AM enantiomers differ from each other quantitatively, the (+) form being more potent than the (-) variant [3]. The difference between (+) and (-)deprenyl seems to be in many aspects qualitative in nature. E.g. in contrast to (-)deprenyl, (+)deprenyl, similarly to other MAO inhibitors, potentiates tyramine effect [7]. It has been published recently, that (+)deprenyl induced typical symptoms of AM-dependency, whereas physical dependency did not develop in (-)deprenyl treated rats [13]. Similar differences were now demonstrated concerning the central effects of the two enantiomers. (+)Deprenyl showed a central effect similar to AM, (-)deprenyl was devoid of it.

The metabolites of (-)deprenyl are the (-) variants. In patients treated with 5 mg (-)deprenyl twice daily, less than 6.5% of the AM and methamphetamine metabolites proved to be (+)isomers [15].

In our rat experiments we did not observe AM-like central stimulatory or inhibitory effects by administering (-)deprenyl up to 100 mg/kg, i.e. about a 400 times higher than the usually used dose, and which can be expected to form high quantity of metabolites.

^{14}C-Deprenyl was published to show a very rapid penetration into the mouse brain after iv administration, followed immediately by a sudden decrease in the radioactivity of the brain. This very rapid crossing of blood-brain barrier can be explained by the lower polarity of deprenyl compared for example to AM [12]. Our results are in good agreement with these data showing that even applying extremely high doses of (-)deprenyl the AM-type metabolites do not accumulate in the brain in a quantity high enough to induce central effects.

It was demonstrated recently, that the multiple dose effects of (-)deprenyl on the nigrostriatal dopaminergic system is not due to its MAO-B inhibitory and uptake inhibitory effects [6, 8]. The data presented in this paper exclude the possibility that the metabolites of (-)deprenyl play any role in the pharmacological effects of this drug.

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REGULATION OF PROTEIN KINASE C AFTER STIMULATION OF α_1-ADRENOCEPTORS IN RAT HIPPOCAMPUS*

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Endogenous inhibitor of protein kinases (type II inhibitor, GABA-modulin) blocks the phosphorylation catalyzed by cAMP-dependent protein kinase (PKA) and protein kinase C (PKC) as a competitive inhibitor of substrate proteins when histone is used as a substrate. Moreover, type II inhibitor blocks the phosphorylation of endogenous membrane proteins by PKC. Stimulation of α_1-adrenoceptors induced rapid redistribution of PKC from cytosol to membrane fraction which lasted at least 3 h, accompanied by rapid and short-lasting translocation of type II inhibitor from membrane to cytosol fraction. The cytosol content of type II inhibitor reached maximal level 10 and 20 min and became normal again 40 min after i.p. administration of methoxamine. The above actions of methoxamine were completely blocked by pretreatment with prazosine.

It seems that short-lasting redistribution of type II inhibitor from membrane to cytosol fraction allows the effective phosphorylation of membrane proteins by PKC after stimulation of α_1-adrenoceptors.

Keywords: protein kinase C, alpha-1-adrenoreceptor, hippocampus GABA-modulin, cAMP-dependent protein kinase, histone

Phosphorylation catalyzed by protein kinases is regulated by several endogenous protein inhibitors. Type I inhibitor (Walsh inhibitor) is specific for cAMP dependent protein kinase (PKA) and acts competitively with respect to catalytic subunits of the enzyme [2]. Protein kinase C (PKC) is regulated by PKC-inhibitor. Both PKC- and PKC-inhibitor are zinc-binding proteins and it seems that they may be co-regulated by an intracellular zinc ion flux [12]. Type II inhibitor (GABA-modulin) is able to block phosphorylation mediated by PKA, cGMP-

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dependent and cyclic nucleotide-independent protein kinases and acts competitively in respect to substrate-phosphate acceptor proteins [7, 14]. It is known that stimulation of benzodiazepine receptors induces translocation of type II inhibitor from membrane to cytosol fraction [6, 7].

Since type II inhibitor is located in membrane fraction and is a competitive inhibitor of substrate-phosphate acceptor proteins, we have studied the role of this inhibitor in regulating the activity of PKC after stimulation of α_1-adrenoceptors.

Methods

Male Wistar rats (180-220 g) were killed by decapitation. Hippocampi were quickly removed and homogenized in 20 mM Tris-HCl, pH 7.4, containing 2 mM EDTA, 0.5 mM EGTA, 2 mM PMSF, 50 mM dimercaptoethanol (buffer A) and 0.33 M sucrose. The mixture was centrifuged at 100 000 x g for 60 min at 0-4 °C. The resulting supernatant was used as a cytosolic fraction and the pellet was resuspended in buffer A containing 0.15% Triton X-100 and centrifuged again. The resulted supernatant was used as a membrane fraction. Protein determinations were according to Lowry et al. [11]. PKC was partially purified by DE-52 chromatography by to Ginsberg et al. [5].

PKC was assayed by measuring the incorporation of 32-P from [γ-32P] ATP into histone according to Guy et al. [8] with minor modifications. The standard incubation mixture (120 μl) contained 20 mM HEPES buffer, pH 7.4, 10 mM Mg^{+2}, 20 μg histone, 30 μl sample, 10 μM [γ-32P] ATP (specific activity 3.7 MBq/μmol), 5 mM Ca^{+2}, 12.5 μg phosphatidylserine and 0.5 μg 1,2/diolein.

The mixtures were incubated for 10 min at 30 °C.

The incubation was terminated by pipetting a 25 μl aliquot of the incubation mixture onto phosphocellulose paper (Whatman P-81) (2.5 x 2.5 cm). The amount of 32-P incorporated into histone was measured according to Witt and Roskoski [16].

One unit of the activity of PKC was defined as the amount of the enzyme that in 1 min at 30°C catalyzed the transfer of 1 pmol of phosphate from [γ-32P] ATP into histone.

Type II inhibitor was partially purified as previously described [15] by steps of heat treatment at 90-95 °C for 10 min and Sephadex G-50 chromatography.

The assay of type II inhibitor was based on its ability to inhibit the phosphorylation catalyzed by protein kinases [13]. The standard assay system (100 μl) contained 150 mM sodium acetate buffer, pH 6.0, mM Mg^{+2}, 10 μl of histone, 30 μl of the Sephadex G-50 eluate, 0.1 μM [γ-32P] ATP (specific activity 3.7 MBq/μmol), 0.2 μM cAMP and 20 units of partially purified cAMP-dependent protein kinase from beef heart prepared according to Kuo an Greengard [10]. The reaction was carried out at 30 °C for 10 min and was terminated by pipetting a 25 μl aliquot of the incubation mixture onto phosphocellulose paper and the amount of 32-P-histone was measured as described above. One unit of type II inhibitor activity was defined as the amount of the inhibitor which blocked the phosphorylation of histone by 20% under conditions described above. The results were expressed as units of PKC or type II inhibitor per mg of cytosol or membrane proteins.

Results

Type II inhibitor equally blocked the phosphorylation of histone catalyzed by protein kinases A and C (Fig. 1A) and decreased in a concentration-dependent manner the phosphorylation of endogenous membrane-bound proteins catalyzed by protein kinase C (Fig. 1B). Kinetic analyses have shown that different concentrations

of type II inhibitor change K_m value for histone without changing V_{max} value for the enzyme in the presence of both protein kinase A and protein kinase C (Fig. 1C and 1D). Methoxamine induced a dose-dependent decrease in the activity of cytosol protein kinase C in rat hippocampus followed by an increase in the enzyme activity in membrane fraction. Statistically significant changes were observed when

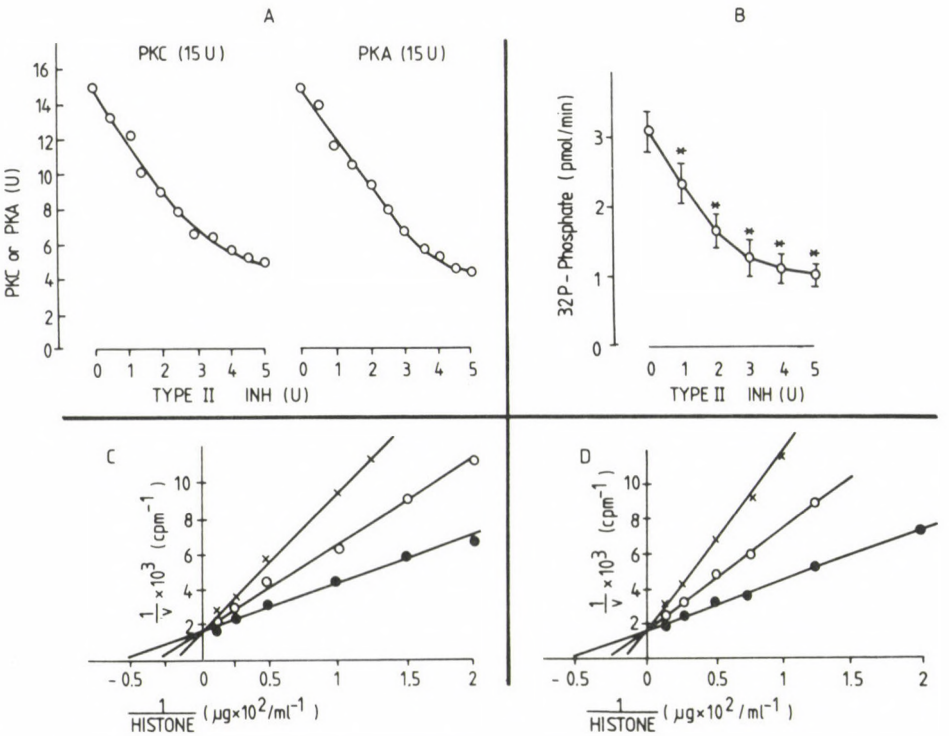

Fig. 1. The effect of partially purified type II inhibitor on the phosphorylation of histone in the presence of partially purified PKA and PKC (A) and on the phosphorylation of endogenous membrane proteins by PKC (B). Double reciprocal plots for initial velocity of phosphorylation of histone by PKA (C) and PKC (D) versus concentrations of histone at constant levels of partially purified type II inhibitor (● ----- ● 0, o-----o 1 U/ml, x-----x 2 U/ml). B - The membrane fraction was incubated with partially purified PKC, Ca^{+2}, phosphatidylserine, diolein and [γ-32P] ATP (see Methods). 32-P phosphoproteins were precipitated with 5% TCA

methoxamine was used in doses 5-50 mg/kg (Fig. 2A). The translocation of protein kinase C from cytosol to membrane fraction begins already 10 min after i.p. injection of methoxamine and lasts for at least 3 h (Fig. 2B). In contrast, methoxamine induced a dose dependent translocation of type II inhibitor from membrane to cytosol

fraction (Fig. 2C). The maximal increase in the activity of cytosol type II inhibitor was seen 10 and 20 min after injection of methoxamine. Type II inhibitor activity in cytosol fraction came back to normal value 40 min after administration of methoxamine. The methoxamine induced translocations of protein kinase C and type II inhibitor were complety blocked by pretreatment with prazosine (Tables I and II).

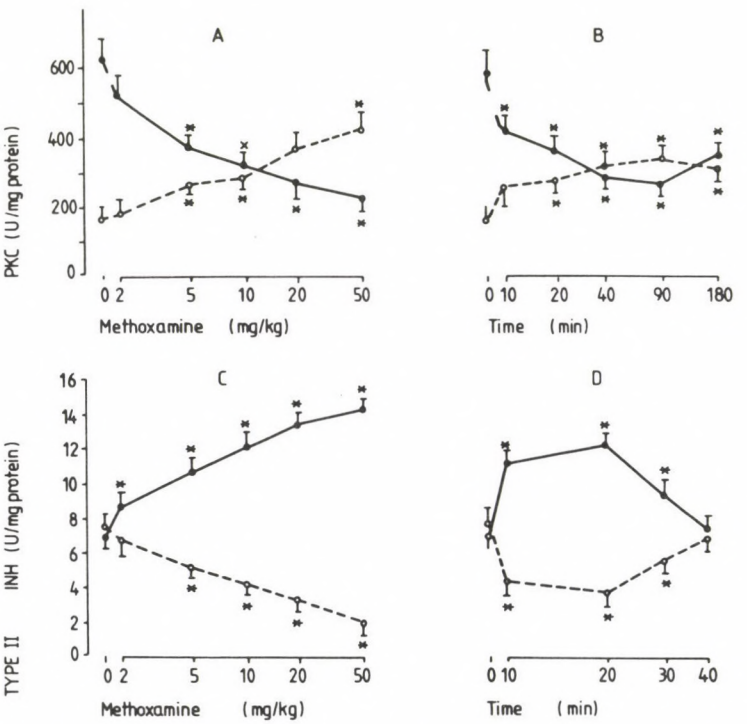

Fig. 2. The effect of various doses of methoxamine on the cytosol (● ----- ●) and membrane (o- - - -o) PKC (A) and type II inhibitor (C) activities and the effect of methoxamine (10 mg/kg i.p.) on the cytosol (● ----- ●) and membrane (o- - - -o) PKC (B) and type II inhibitor (D) activities in rat hippocampus at various time after i.p. injection of the drug. Methoxamine was injected i.p. 20 min (C) and 40 min (A) before killing the rats

Each point represents mean +SEM of 6-7 experiments

* $p < 0.05$ in comparison to the control group

Table I

The effect of prazosine on the methoxamine induced changes in PKC activity in cytosol and membrane fraction of rat hippocampus

Treatment	Dose (mg/kg)	PKC activity (U/mg protein)	
		Cytosol	Membrane
Saline		634 ± 30	161 ± 9
Prazosine	1.0	579 ± 32	155 ± 12
Methoxamine	5.0	376 ± 24*	242 ± 10*
Prazosine	1.0		
+		597 ± 43	158 ± 13
Methoxamine	5.0		

The rats were killed by decapitation 60 min after i.p. injection of prazosine and 40 min after i.p. administration of methoxamine. Each value represents mean ±SEM from 6–7 experiments
* $p < 0.05$ in comparison to the saline-treated group

Table II

The effect of prazosine on the methoxamine induced changes in type II activity in cytosol and membrane fraction in hippocampus of rats

Treatment	Dose (mg/kg)	Type II inhibitor (U/mg protein)	
		Cytosol	Membrane
Saline		7.3 ± 0.3	7.8 ± 0.5
Prazosine	1.0	6.8 ± 0.2	7.6 ± 0.4
Methoxamine	5.0	10.8 ± 0.4*	5.3 ± 0.2*
Prazosine	1.0		
+		7.6 ± 0.4	8.0 ± 0.6
Methoxamine	5.0		

The rats were killed by decapitation 60 min after i.p. injection of prazosine and 20 min after i.p. administration of methoxamine. Each value represents mean ±SEM from 6–7 experiments
* $p < 0.05$ in comparison to the saline-treated group

Discussion

Type II inhibitor blocks the activities of PKA and PKC and is competitive in respect to substrate-phosphate acceptor proteins. Kinetic analysis has shown that in the presence of PKA and PKC type II inhibitor changes K_m for histone without affecting V_{max} for the enzymes. Moreover, type II inhibitor blocks phosphorylation of endogenous membrane proteins catalysed by PKC. Thus it seems that type II inhibitor might be involved in regulating the activity of PKC. This concept is supported by the observation that stimulation of α_1-adrenoceptors induced a translocation of type II inhibitor from membrane to cytosol fraction.

Stimulation of α_1-adrenoceptors by methoxamine resulted in a translocation of PKC from cytosol to membrane fraction in rat hippocampus, which was completely blocked by pretreatment with prazosine, a specific antagonist, of those receptors. The above results are in accordance with findings that stimulation of α_1-adrenoceptors induces the activation of phospoholipase C and hydrolysis of inositol containing phospholipids [1, 4, 9]. At least two second messengers are formed, inositol triphosphate which modulates the intracellular concentration of calcium and diacylglycerol which modulates the activity of PKC [3]. Redistribution of PKC from cytosol to membrane fraction is the major mechanism of activation of the enzyme [8]. Methoxamine induced a rapid and long-lasting redistribution of PKC, associated with rapid but short-lasting translocation of type II inhibitor from membrane to cytosol fraction. An increase in PKC activity in membrane fraction was seen 20 min after methoxamine administration and lasted for at least 3 h. In contrast, the lowest activity of type II inhibitor was observed 10 and 20 min after i.p. injection of methoxamine. Forty min after treatment with the drug the activity of type II inhibitor in cytosol as well as in membrane fraction returned to control values. It is known that diacylglycerol, a physiological stimulant of PKC is rapidly metabolised [3, 4]. Thus it seems that short-lasting translocation of type II inhibitor from membrane to cytosol fraction allows the effective phosphorylation of membrane proteins catalyzed by PKC after stimulation of α_1-adrenoceptors.

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CROSS REACTIVITY AMONG THE LIGANDS OF THE 7 HELIX FAMILY*

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"Specific" drugs for receptors of the 7 helix family inhibited in high doses other receptors of the same family, as well. Atropine inhibited [3-H]-clonidine binding, beta-adrenergic drugs: propranolol, betoxalol, ICI 118.551 inhibited labelled QNB binding to all the 3 main subtypes of muscarinic receptors.

Authors propose the existence of common features in the ligand binding sites of the receptors of 7 helix family.

Keywords: muscarinic receptor, alpha adrenergic receptor, beta adrenergic receptor, 7 helix receptor family, membrane receptor evolution

The aspects for the classification of membrane receptors has changed recently. Instead of functional groups as hormone-receptors, neurotransmitter receptors, etc. a classification according to the structure of receptors, or according to the receptor-evolution is employed [5, 11].

"Seven-helix receptor family" is one of these structurally-related receptor groups. A hydrophobic, long amino acid sequence is the common feature of the members of this group. This part of the receptor protein chain is located mostly within the membrane, producing there 7 membrane spanning region (7 helix) [3]. The regions of the receptor protein within the membrane are connected to each other forming a continuous protein chain. These connecting parts, the N terminal part - outside the membrane on the intercellular surface- and the COOH terminal part in the cytoplasmic surface have hydrophylic character. The members of this family share certain amino acid sequences in the hydrophobic regions, where 20-40

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percent of the amino acids are identical, but within some of the helices this analogy is even higher [5, 9].

Three functions of the membrane receptors can be distinguished. First: the anchoring function stabilizing the molecule within the plasma membrane (a function of the hydrophobic regions of the seven helix). Second: the signal transducing function is provided by different G proteins in the 7 helix family [5] and third: the ligand binding function. Since a considerable analogy exist among the members of this receptor family in the large helical anchor, and also, only a few classes of G proteins are producing the signal transfer – the individual features of various receptors must be provided by the ligand binding site.

Considering the analogies between the receptors of this family, Venter raised a hypothesis about the evolution of receptors [11]: a common ancestor of the family was supposed, an ancient receptor. This ancient receptor following many mutations, selections and perhaps gene fusions, did develop to the existing members of this family.

More than 20 members of the 7 helix family is recognised, as yet. The muscarinic cholinergic (mAChR), the alpha-1, alpha-2, beta-1, beta-2 adrenergic, H-1, H-2 histaminergic receptors, the 5-OH tryptamine, the dopamine receptor, the tachykinin receptor (substance K receptor), yeast mating factor receptor, rhodopsins, opsins are members of this family [5, 8].

The steps of evolution of the ligand binding site is unknown. We are describing a hypothesis, that some part of the ancient receptor ligand binding site still exists in the binding locus of the modern receptors and additional points and/or conformational changes are responsible for the specific, individual binding properties.

The competitive inhibiting effect of alpha-1, alpha-2 adrenergic drugs on the labelled ligand binding of the 3 main subtypes of muscarinic cholinergic receptor (mAChR) is known [10]. The aim of present study is to prove, that "specific ligands" for one member of 7 helix family binds (in high concentration) to other receptor(s) of this family, as well.

Since in the previous studies of the authors a competitive type of inhibition was demonstrated by alpha adrenergic drugs on the binding of cholinergic ligand [3-H]-QNB to muscarinic receptors: the effect of the typical cholinergic drug atropine on the binding of [3-H]-clonidine to alpha receptors, as well, as the effect of various beta adrenergic drugs on labelled cholinergic ligand binding is examined.

Materials and methods

Materials: The following substances were purchased: [3-H]-quinuclidinyl benzoate (QNB; Amersham), specific activity 1.65 TBq/mmol, purity: 99%; [3-H]-clonidine (Amersham, specific activity

858 GBq/mmol, purity: 97%) atropine sulfate (Serva), clonidine (Sigma), ICI 118,551 (ICI), betoxalol (ICI), propranolol. Preparation of crude membrane fractions and binding studies for [3-H]-QNB was described earlier [10]. Binding studies for [3-H]-clonidine [2] or [3-H]-rauwolscine: following preincubation (5 min, 25°C) with atropine, membrane suspensions were incubated at 25°C for 90 min with the labelled ligand in the presence or absence of atropine. For the determination of non-specific binding clonidine in 600 nM concentration were used. Following incubation, bound and free ligands were separated by filtration on Whatman GF/B disk filters. The filters were washed twice with 4.0 ml cold buffer, dried, put into 20 ml vials and overlaid with 10 ml of toluene-Triton X-100 scintillation cocktail. All assays were performed in triplicates. Radioactivity was measured in a Beckman LS 9000 counter. Radioligand binding parameters were calculated by the method of Mc Pherson [7]. Protein concentration was determined by the Lowry method [6].

Results

Different drugs, as glutamic acid, dopamine, GABA, isoproterenol – tested up to 0.2–1.0 mM – did not affect [3-H]-QNB binding either to brain membrane, or to heart membrane (Table I). Atropine inhibited both labelled rauwolscine and labelled

Table I

Effect of neurotransmitters on [3-H] QNB binding, in vitro

	Tissue	Highest concentration tested (mmol/l)	% inhibition of [3-H] QNB binding
Dopamine	brain cortex	0.5	<10
	heart	0.5	<10
GABA	brain cortex	1	<5
	heart	1	<5
Glutaminacid	brain cortex	0.2	<5
	heart	0.2	<5
Histamine	brain cortex	0.2	<5
	heart	0.2	<5
Isoproterenol	brain cortex	0.5	<10
	heart	0.5	<10
PAF	brain cortex	0.2	<5
	heart	0.2	<5
Serotonin	brain cortex	0.5	<5
	heart	0.5	<5

clonidine binding to rat brain membrane preparate. Bmax values did not differ significantly in the presence or absence of atropine, Hill coefficients were between 0.90–0.99. Inhibition constant of atropine for [3-H]-clonidine binding was 16 micromole, for [3-H]-rauwolscine binding was 60 micromole (Table II).

Table II

Effect of atropine on the 3-H labelled ligand binding to rats' brain cortex membrane (X ± S.D.)

Ligand		[3-H] clonidine	[3-H] rauwolscine
No inhibitor	Kd (pM)	854 ± 219	1.35
	Bmax (pM)	70 ± 25	0.12
	nHill	0.97 ± 0.07	0.99
Atropine	Ki (μM)	16 ± 7	60
	Bmax (pM)	85 ± 25	0.11
	nHill	0.90 ± 0.06	0.96

Beta adrenergic drugs inhibited only in very high doses [3-H]-QNB binding to cholinergic receptor containing tissue homogenates. Ki values were between 53–557 micromole. Bmax values slightly differed from each other, the type of inhibition is uncertain. Hill coefficients did not differ significantly from 1.0 (Table II).

Table III

The impact of beta adrenergic drugs on [3-H]-QNB binding to crude membrane preparates of rat organs

		Kd(nM)	Bmax(nM)	nHill	IC50(μM)	Ki(μM)
Brain	control	0.868	1.711	0.88	–	–
(M-1)	propranolol	1.764	1.332	0.99	1113	557
	betoxalol	15.200	1.688	0.94	289	194
	control	0.369	0.400	0.83	–	–
	ICI1 18551	2.000	0.090	0.95	408	88
Heart	control	0.410	0.344	0.95	–	–
(M-2)	propranolol	4.139	0.441	0.99	417	99
	betoxalol	4.060	0.582	0.99	226	65
	ICI1 18551	2.004	0.447	0.92	529	146
Parotid	control	0.838	0.123	0.98	–	–
(M-3)	propanolol	2.417	0.120	0.96	543	149
	betoxalol	44.530	0.822	0.97	317	341
	ICI1 18551	6.933	0.243	0.97	246	53

Discussion

Ligand binding of muscarinic cholinergic receptors was not inhibited by high doses of various drugs, giving evidence that not every neurotransmitter inhibits this receptor in the test system employed (Table I).

The number of analogue amino acids in the membrane spanning region of the 7 helix is high in alpha adrenergic versus muscarinic cholinergic receptors [5]. Alpha-1 and alpha-2 adrenergic drugs inhibited all of the 3 main subtypes of muscarinic cholinergic receptors, however, the effective doses were high [10]. Since the type of these inhibitions were competitive, similarities were supposed between the ligand binding sites of alpha adrenergic and muscarinic cholinergic receptors. A further evidence for this similarity is the inhibition of alpha-2 adrenergic receptor (assayed by labelled clonidine) by the cholinergic drug atropine. The inhibitory effect of atropine could be demonstrated only in low ligand- and high inhibitor concentrations (Table II). Beta-1 "specific", beta-2 "specific", inhibitors, as well as propranolol in high doses inhibited all the subtypes of muscarinic receptors (Table III). Ki values were lower for ICI 118.551 with the M-1 and the M-3 receptor subtype and for betoxalol with M-2 subtype.

The exact structure of the active sites of the 7 helix receptors is unknown. The peptid chain of the receptor molecules has an alpha helical organisation, with a single turn for every 3.6 residues, so, the neighbours of a certain amino acid will be residues 3 or 4 places above or below of it [1]. Some amino acids of different membrane spanning regions of the 7 helix are also in the proximity of each other. So, the amino acid residues of the binding site could be in distinct places of the peptide chain. All types of adrenergic receptors and muscarinic cholinergic receptors has an aspartic acid and a serine residue in their ligand binding site. Other residues and the spherical organisation of the molecule are responsible for the different properties of these receptors. The cross-reaction observed (even in high concentrations) provides further evidence of the common origin of the binding sites of the 7 helix receptors.

The very high doses employed limit the practical importance of this inhibition.

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ANXIOLYTIC PROFILE OF GIRISOPAM AND GYKI 52 322 (EGIS 6775). COMPARISON WITH CHLORDIAZEPOXIDE AND BUSPIRONE*

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The anxiolytic action of two 2,3-benzodiazepines: girisopam: GYKI 51 189 (EGIS 5810): (1-(3-chlorophenyl)-4-methyl-7,8-dimethoxy-5H-2,3-benzodiazepine), and GYKI 52 322 (EGIS 6775): (1-(4-aminophenyl)-4-methyl-7,8-dimethoxy-5H-2,3-benzodiazepine) was investigated in comparison to chlordiazepoxide and buspirone using three different animal models of anxiety: the lick conflict, the elevated plus maze and the open field methods in rats. Both 2,3-benzoidazepines exerted anxiolytic effect in all three tests used, however their pharmacological profile differs considerably from that of either chlordiazepoxide or buspirone.

Using the animal models mentioned above the order of potency was GYKI 52 322 (EGIS 6775) > chlordiazepoxide > girisopam > buspirone.

Keywords: 2,3-benzodiazepines, girisopam, chlordiazepoxide, buspirone, anxiolytic effect

The 2,3-benzodiazepines (2,3-BZs): girisopam (GYKI 51 189 – EGIS 5810) and GYKI 52 322 (EGIS 6775) synthetized by Láng & Kőrösi and their co-workers in the Institute for Drug Research and sponsored by EGIS Pharmaceutical Works, Budapest; at the first superficial approach seem to be structurally related to the classical benzodiazepines, but they represent quite different and unrelated chemical entities.

We have already reported earlier, that the general pharmacological actions of these drugs – and also the most extensively investigated member of this structure-family, tofisopam – differ essentially from those of the classical 1-4 benzodiazepines [1, 7, 8].

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In the present experiments in order to characterize the activity of girisopam and GYKI 52 322 (EGIS 6775) in some further animal models detecting anxiolytic activity, we investigated their effect both in punished and nonpunished procedures, in comparison to chlordiazepoxide and buspirone.

Materials and methods

The reference substance chlordiazepoxide (Hoffman-La Roche) and buspirone (Bristol Myers) were dissolved in distilled water, while the 2,3-BZs were suspended in phys.saline containing 2% (v/v) of Tween 80.

Male SPRD rats purchased from LATI (Gödöllő, Hungary) weighing about 200 g were used for the experiments. Animals were treated in a volume of 0.25 ml/kg body weight intraperitoneally.

Vogel's lick conflict test

The experiments were performed as described earlier [19]. Rats were deprived of water for 48 h as well as of food for 24 h before the measurements. Drugs or vehicle were administered 30 min before testing. Upon placement into the test box (20x20x15 cm) each animal was allowed to drink water and every 20th licks were punished by electric current (2mA, 0.3 ms) delivered between the drinking tube and the metal grid floor during 3-min testing period. The number of shocks tolerated was registered in drug and vehicle-treated rats.

Elevated plus maze test

Experiments were carried out as described previously [12]. The maze consisted of two open arms (50x10 cm) painted white and two closed arms (50x10x40 cm) painted black. The maze was elevated to a height of 60 cm. After 20-min pretreatment the naive animals were put individually into the center of the maze facing a closed arm. The following measures were taken during a 5-min testing period: number of total and open arm entries, and the time spent in open arms. Rats were tested via TV circuit in a sound-attenuated room, between 12.00 and 16.00 p.m. The behaviour of drug treated animals was compared to that of vehicle-treated controls.

Small open field test

The measurements were performed as described earlier with slight modifications [18].

The test area was a 40x30x30 cm transparent box having a floor divided into 6 equal parts. The animals were put on the field after 30-min i.p. pretreatment and their behaviour was observed for 10 min. The number of crossings and rearings was registered. Changes were compared to controls and expressed in percent, too.

Statistics

In all the three experiments statistical significance between vehicle and drug treated groups was calculated by ANOVA analysis followed by Duncan's test.

Results

In the lick conflict test in rats chlordiazepoxide and both 2,3-BZs showed bell-shaped dose response curves concerning the number of shocks tolerated, GYKI 52 322 (EGIS 6775) displayed the most pronounced activity. Buspirone was found to be active only at one dose level, albeit as low as 0.04 mg/kg i.p. Based on the corresponding data of $5HT_{1A}$ partial agonists in the literature [6, 11], this effect was probably due to the influence of buspirone on praesynaptic $5HT_{1A}$ receptors (Fig. 1).

Increase in open arm entries and the enhancement of the time spent in open arms indicate anxiolytic activity in the elevated plus maze paradigm, while total arm entries reflect the overall influence on motor activity. Neither chlordiazepoxide, nor the 2,3-BZs induced significant decrease of total arm entries at the doses applied.

Chlordiazepoxide could not be tested at higher dose than 5 mg/kg i.p., since the animals dropped off the open arms due to motor disturbances. Buspirone caused a significant decrease in total arm entries in doses higher than 1.25 mg/kg i.p. (data not shown).

The open arm entries were found to be enhanced significantly by chlordiazepoxide and GYKI 52 322 (EGIS 6775) in doses of 0.16-0.31 mg/kg i.p. The increases observed by the other two compounds were not significant (Fig. 2).

Chlordiazepoxide and the two 2,3-BZs increased the number of crossings and rearings compared to controls in the small open field method, the dose-response curves being bell-shaped in this case, too. These increases may reflect the decreased fear of animals in the new environment. Buspirone exerted an enhancing effect only at the dose of 0.6 mg/kg i.p., at higher dose levels it diminished both the number of crossings and rearings (Fig. 3).

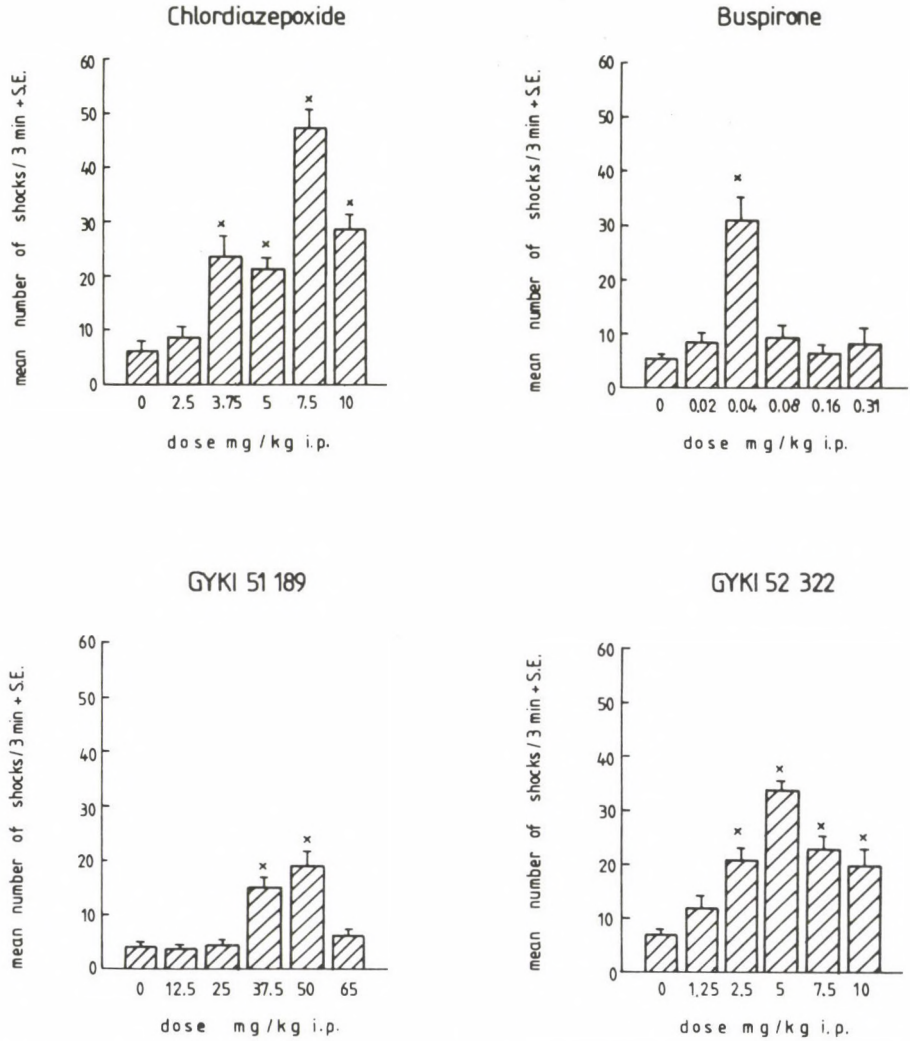

Fig. 1. Effect of chlordiazepoxide, buspirone, GYKI 51 189 and GYKI 52 322 on the number of shocks tolerated in the lick conflict test after 30 min intraperitoneal treatment in rats during 3 min testing. Minimal number of animals/group = 10. Data represent the means ± S.E.

* $p < 0.01$ by Duncan's test after ANOVA upon comparing the groups treated with test compounds and with vehicle

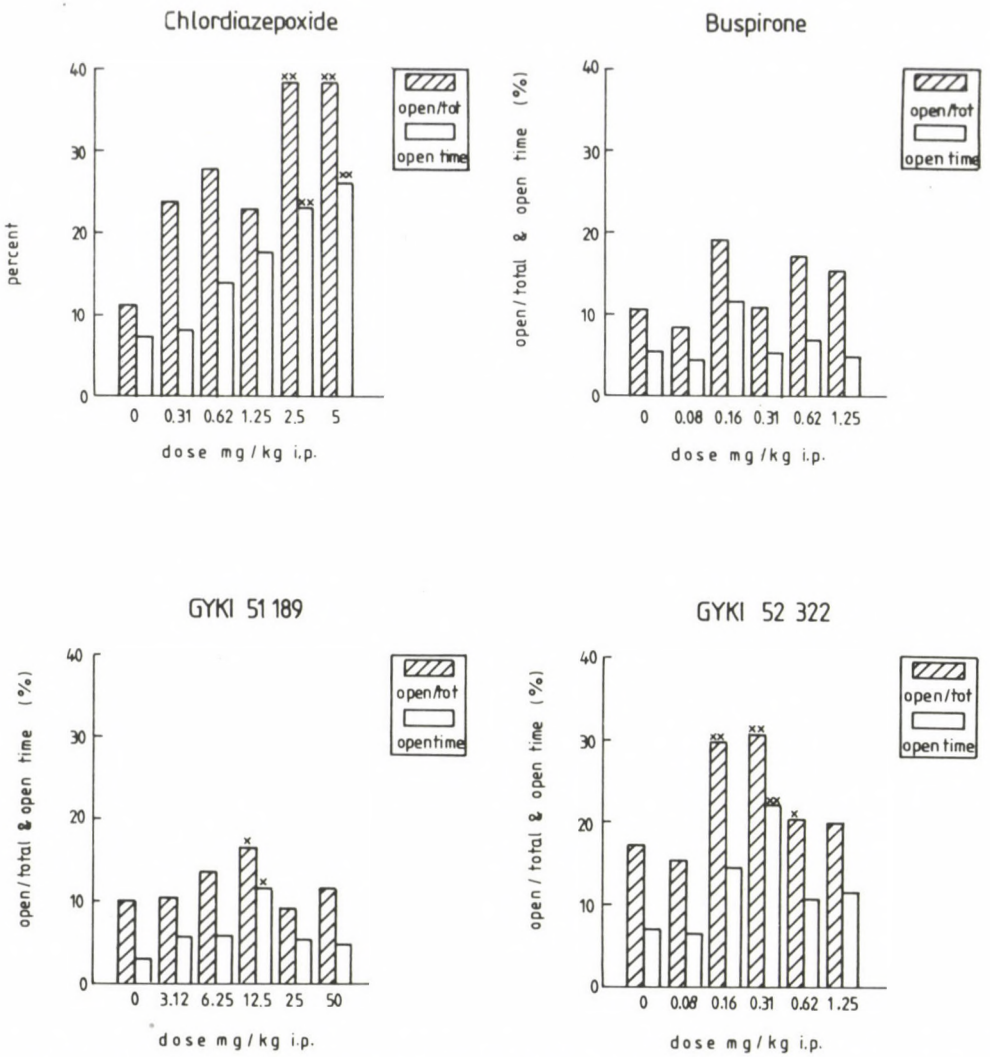

Fig. 2. Effect of chlordiazepoxide, buspirone, GYKI 51 189 and GYKI 52 322 in the elevated plus maze test in rats after 20 min i.p. treatment during 5 min testing period. Open columns show the effect of the compounds on open/total arm entries, while cross-hatched columns represent the time spent in open arms compared to control group in percent. Values were calculated from the number of open and total arm entries and the time spent in open arms. Minimal number of animals/group = 10

* $p < 0.05$, ** $p < 0.01$ by Duncan's test after ANOVA upon comparing the group treated with test compound and with vehicle

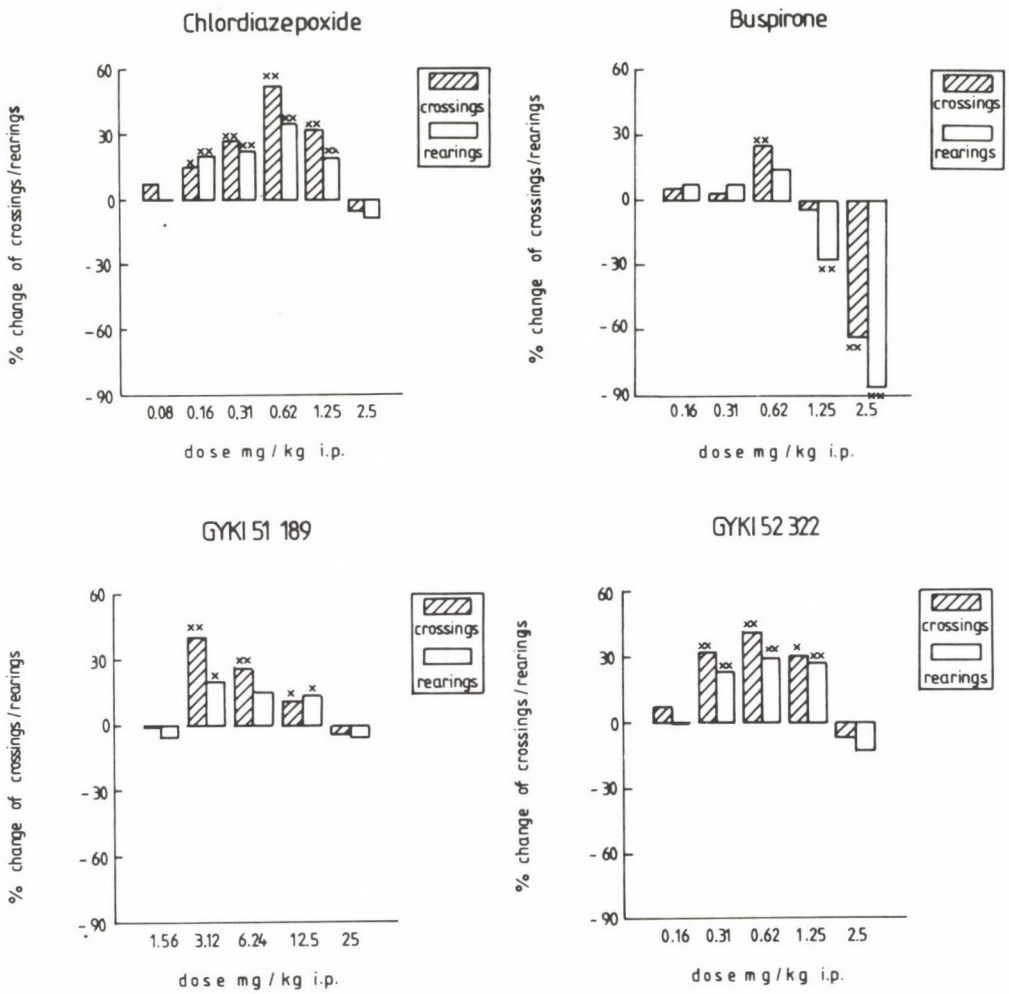

Fig. 3. Effect of chlordiazepoxide, buspirone, GYKI 51 189 and GYKI 52 322 on vertical (crossings, cross-hatched columns) and horizontal (rearings, open columns) locomotor activity in the small open field test in rats after 30 min i.p. treatment during 10 min testing. Changes compared to vehicle-treated control group are expressed in percent. Minimal number of animals/group = 10

* $p < 0.05$, ** = < 0.01 calculated from the number of crossings and rearings by Duncan's test after ANOVA upon comparing the groups treated with test compounds and with vehicle

Discussion

The classical benzodiazepines, like chlordiazepoxide or diazepam and all other structure-related compounds show sedative and muscle relaxant properties beside their anxiolytic activity, and unfortunately they have dependence capacity, too [4, 9, 14, 17, 20].

That is the reason for the growing need to find anxioselective, nonsedative anxiolytics without the hazard of drug abuse. This general need draw the attention to buspirone, a compound, which seemed to the active in humans but quite interestingly only in a few animal models considered to have predictive value for anxiolysis in man. Besides its activity was detected inconsequently and only by several authors in animals [2, 3, 5, 10–13, 15, 16].

In order to demonstrate the anxiolytic profile of girisopam and GYKI 52 322 (EGIS 6775), we investigated their effects in three different animal models of anxiety using chlordiazepoxide and buspirone as reference compounds.

In the lick conflict test GYKI 52 322 (EGIS 6775) seemed to be the most active compound among the tested four drugs. Similarly to chlordiazepoxide, this effect could be observed in different doses. Girisopam exerted a rather similar effect, but at much higher dose levels. On the other hand, buspirone showed only an occasional activity.

In the elevated plus maze test the effect of buspirone was not statistically significant, neither concerning the open arm entries, nor the time spent in the open arms compared to controls. At the same time, the other three drugs tested – the 2,3-BZ derivatives and the reference chlordiazepoxide – showed statistically significant anxiolytic action.

The same tendency could be observed in the third method applied. Using the methods described above, the anxiolytic action of buspirone in rodents is weak and only occasional.

In the contrary, chlordiazepoxide and the GYKI compounds exerted well-demonstrable effects in both punished and unpunished conflict situations in rats. In all cases the dose response curves were bell-shaped. By chlordiazepoxide, the descending part of the dose-response curves corresponds to doses causing impairment of motor performance and decreasing the muscle tone.

In spite of the finding that 2,3-BZs did not cause these unwanted side effects [1, 7], the pharmacological picture was similar.

The classical 1,4-BZ-s and 2,3-BZs are different concerning both their biochemical and pharmacological properties. For example, the latter compounds are inactive in the Geller-Seifter conflict procedure, do not bind to the central benzodiazepine receptors [1, 7].

At the same time the pharmacological activity of 2,3-BZ-s differs profoundly from buspirone, too: their anxiolytic effect is more pronounced in rodents and they do not induce anxiogenesis even at higher dose levels.

According to these data girisopam and GYKI 52 322 (EGIS 6775) may represent a special subclass of the anxiolytics. Moreover these compounds lack any appreciable effect either on the central benzodiazepine or on serotonin receptors.

Further pharmacological and biochemical experiments are needed to clarify how these compounds induce anxiolysis in rodents.

The clinical trials, which are under way with girisopam and GYKI 52 322 (EGIS 6775), may provide further evidence concerning their anxiolytic activity.

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INHIBITION OF HIPPOCAMPAL FIELD POTENTIALS BY GYKI 52466 *IN VITRO* AND *IN VIVO*[1]

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GYKI 52466 is a specific antagonist of the neuronal excitation mediated by the non-NMDA type excitatory amino acid receptors, at several sites in the central nervous system. The experiments presented here show that the drug has a dose-dependent, slowly developing, long-lasting and reversible inhibitory action on the field potentials recorded from the CA1 region of the rat hippocampus, *in vitro*. Its action is similar to that of the well-known non-NMDA receptor blocker, CNQX. When the stimulus intensity-dependence of the population spikes was investigated, both drugs shifted the input-output curves in a parallel manner, while the maximum responses were only slightly depressed at the doses applied. With i.v. application, GYKI 52466 also inhibited the hippocampal field potentials recorded from the CA1 region of anesthetized rats dose-dependently. The inhibition was relatively weak compared to the effect found in earlier studies in the spinal cord, by the same doses. Four mg/kg i.v., a doses which is able to block spinal reflexes completely, caused an only about 20% depression of the recorded responses in the hippocampal CA1 area.

Keywords: GYKI 52466, excitatory amino acids, non-NMDA receptors, hippocampus, field potentials

GYKI 52466, a 2,3-benzodiazepine compound synthesized at the Institute for Drug Research, Budapest, is a potent centrally acting muscle relaxant. It strongly inhibits both the monosynaptic patellar, and the polysynaptic flexor reflexes in cats [1]. GYKI 52466 is also a broad spectrum anticonvulsant in a variety of animal models. It protects the animals against seizures induced by electroshock, or by various chemical convulsants [3]. Smith, Dürmüller and Meldrum reported recently that GYKI 52466 is effective in two reflex epilepsy models: genetically epilepsy-prone animals (sound-sensitive rats and photosensitive baboons) were protected against seizure attacks [4]. The drug was antiepileptic also in DBA/2 mice [5]. According to

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several recent congress presentations, GYKI 52466 is neuroprotective in different situations. It decreases the number of damaged cells in the hippocampus following a local injection of the neurotoxin kainate [6], and it protects also against the neurotoxicity evoked by both focal and global cerebral ischaemia in rats [7–10].

Our earlier studies on the cat spinal cord [2] revealed that GYKI 52466 does not act like 1,4-benzodiazepines: i.e. through a potentiation of the inhibitory action of GABA [10], but it seemed to inhibit excitation directly. Thus we have investigated its effect on the excitatory processes, and in 1989, we reported that GYKI 52466 inhibited neuronal excitation evoked by iontophoretic application of glutamate, quisqualate and kainate, but not of NMDA, *in vitro*, in rat neocortical slices [11]. At the same concentration range (i.e. in 10-50 μM) it also inhibited stimulation evoked excitatory postsynaptic potentials in the same preparation. Similar antagonism of the glutamate – but not NMDA – responses and EPSPs by GYKI 52466 was reported by Ouardouz and Durand recently, *in vivo*, in rat abducens motoneurons [12].

The aim of the present study was to obtain quantitative data about the depressant action of GYKI 52466 in the rat hippocampus *in vitro* and *in vivo*.

Methods

Brain slice studies

Transverse slices (450 μm thick) were cut from the hippocampus of albino rats weighing 160±20 g, using a McIlwain tissue chopper. Slices were then transferred into an interface-type recording chamber. The Schaffer collateral-commissural pathway was stimulated continuously at 0.1 Hz with a stimulus strength (typically 4.5 V, 0.2 ms) that gave a near-maximal response. When input-output curves were obtained (just before and at the end of the drug application), the stimulus intensity was gradually elevated from the threshold up to an intensity which gave a maximal (plateau) response (Fig. 2). Field potentials were recorded from the pyramidal layer of the CA1 area. Drugs (GYKI 52466.HCl, Institute for Drug Research, and CNQX, Tocris) were dissolved in the perfusing medium and perfused for 30 min in concentrations of 10, 20, 40 and 80 μM and 0.5, 1, 2 and 4 μM, respectively. Drug application started only when the response had stabilized. The amplitude of the population spikes was evaluated using a Gould digital oscilloscope.

In vivo experiments

Male Sprague-Dawley rats (body weight: 280-320 g) were anesthetized with urethane (1.3 g/kg, i.p.) and placed in a David Kopf stereotaxic apparatus. The body temperature was maintained at 37 °C by using a heating pad. A bipolar stimulating electrode (diameter 0.1 mm, tip separation 0.3 mm, insulated except for the tip) was inserted into the CA3 field of the hippocampus contralateral to the site of the recording, and monophasic square wave pulses (0.1 ms, 0.1 Hz) were delivered continuously during the experiment. A recording glass microelectrode (3-6 MOhm) filled with 2M NaCl, saturated with Fast Green, was then positioned above the hippocampal CA1 area and lowered until it reached the stratum pyramidale, i.e. until the stimulation evoked a characteristic field potential consisting of a positive going wave (the population EPSP) followed by a narrow, negative going component (the population spike) of a reasonable amplitude. The depth of the stimulating electrode was then adjusted to maximize the

amplitude of the population spike. The stimulus intensity was adjusted to evoke a population spike with an amplitude of about 90 percent of the maximal response. The amplitudes of the population spikes were recorded on-line, using a modified version of a computerized measurement system [13]. GYKI 52466 was administered in cumulative doses (1 mg/kg, 1 mg/kg, 2 mg/kg and 4 mg/kg, i.v.) in ten minutes intervals. At the end of each experiment the position of the electrodes was verified histologically.

Results

Brain slice studies

Both GYKI 52466 and CNQX exerted a doses-dependent inhibition of the population spikes. The effect developed very slowly, even the 30 min application time did not seem enough for the drugs to reach their maximum effect, especially in the case of GYKI 52466. The action was reversible, but the recovery took a long time (sometimes several hours with the highest concentrations). IC_{50} values calculated from the maximum inhibition after 30 min application of GYKI 52466 and CNQX were 24.1 and 1.38 μM, respectively (Fig. 1).

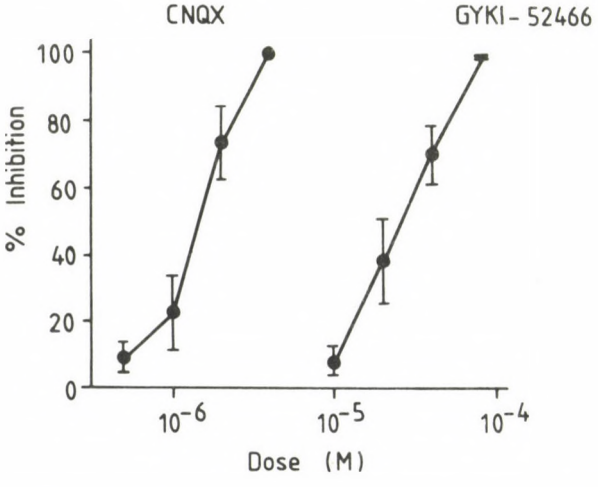

Fig. 1. Dose-response curves for inhibition by CNQX and GYKI 52466 of the population spikes in the hippocampal CA1 area, *in vitro*. Each point represents the mean ± S.E.M. of 4-10 experiments

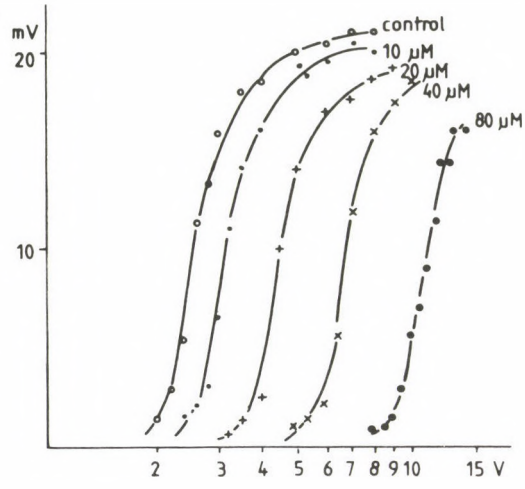

Fig. 2. Dose dependent shifts of the control input-output curve by GYKI 52466, in the hippocampal CA1 area, in an *in vitro* experiment

Figure 2 illustrates the stimulus intensity dependence of the response and the effect of GYKI 52466. At low intensities no population spikes, only EPSPs were evoked. When the stimulus strength was elevated the amplitude of the population spike increased gradually until reaching a plateau. For IC_{50} determination, a fixed stimulus intensity was used, which gave a near-maximal response. It was obvious, however, that the percentage inhibition which could be achieved by a given drug concentration was dependent on the stimulus strength used. Thus, the effects of the two drugs were also investigated at varying stimulus intensities. On Fig. 2, the so-called input-output curves (the population spike amplitude plotted against the stimulus intensity) were taken in the absence of GYKI 52466 and in the presence of different concentrations of the drug. GYKI 52466 applied at different concentrations, caused nearly parallel shifts of the control input-output curve. CNQX caused very similar changes in the input-output curves (not illustrated). Plateau responses were also depressed by both drugs, but only slightly at the concentration ranges we used. The first parts of the shifted curves are running quite parallel to the control curve. This made possible to calculate intensity-ratio (IR) values. IR values were defined on the analogy of the DR values in the Schild analysis (the ratio of the stimulus intensities which were necessary to evoke a half-maximum response in the presence, and in the absence of the drug, respectively) and were calculated at each concentration. These values offered a more reliable comparison of the effects of the two drugs. The efficacy ratio, based on DR comparisons, between CNQX and GYKI 52466 was 15.5.

In vivo experiments

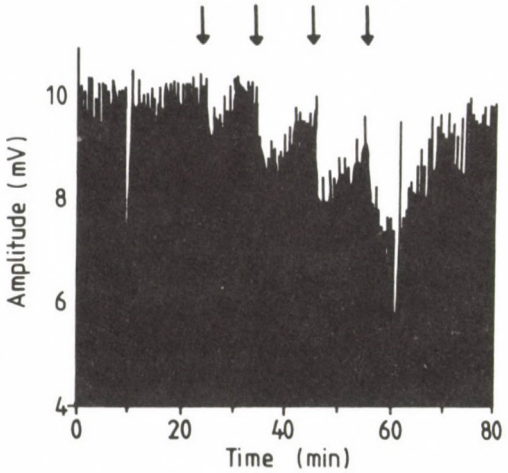

Fig. 3. Inhibition of the population spike amplitude recorded from the CA1 area of the hippocampus by increasing doses of GYKI 52466, in an *in vivo* experiment. Arrows indicate i.v. injections of 1, 1, 2 and 4 mg/kg GYKI 52466

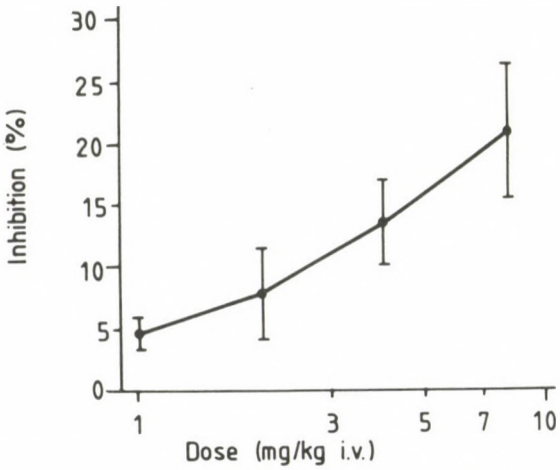

Fig. 4. Cumulative dose-response curve of GYKI 52466 in the hippocampal CA1 area, *in vivo*. Each point represents the mean ± S.E.M. of 5-8 experiments

Figure 3 illustrates the effect of GYKI 52466 on the evoked population activity in the hippocampal CA1 area, *in vivo*. The effect of GYKI 52466 was dose-dependent. The effect developed very quickly, and it had a rather short duration:

more than half of the effect disappeared as soon as ten minutes after the application. It was not possible to elevate the dose further up, as the highest dose was limited by the solubility of the drug and by the interaction with the anesthetics, causing a depression of the respiratory function (Fig. 4).

Discussion

We have found that the non-NMDA antagonist GYKI 52466 decreased the amplitude of the population spikes in the hippocampus dose-dependently, both *in vitro* and *in vivo*. Its *in vitro* action was qualitatively similar, but about 15 times weaker than that of the competitive non-NMDA antagonist CNQX. Both substances shifted input-output curves (response amplitude vs stimulus intensity) in a nearly parallel manner. The effective concentration of CNQX was at the low μM range and this is in accordance with the results of other laboratories [15, 16]. Although CNQX is much more effective than GYKI 52466 *in vitro*, it is not able to cross blood brain barrier, so it is difficult to use it *in vivo*, in contrast to GYKI 52466.

In anesthetized rats, the inhibitory action of GYKI 52466 in the hippocampus was weaker than that was found in the earlier spinal reflex studies [1, 2]. Four mg/kg GYKI 52466 is enough to inhibit spinal reflexes by 100%, but caused only about 20% decrease in the hippocampal evoked potentials. In both cases, the time-course of action was similar: the effect developed very quickly following the i.v. injections of the drug and lasted only for a short time. In a former study [1] the brain concentration of GYKI 52466 was investigated. It was found, that the assessed concentration following an i.v. injection of 4 mg/kg ^{14}C-labelled GYKI 52466, reached near 30 μM rapidly, and after this it decreased very quickly. Thus, the time course of action which was seen in the *in vivo* studies can be explained by the changes in the available concentration of the drug in the brain. In the present *in vitro* studies we found that 25-30 μM GYKI 52466 (i.e. the concentration which can be detected in the brain after 4 mg/kg, i.v.), is able to cause less than 50% inhibition of the responses, and this roughly corresponds to the effect measured in the hippocampus *in vivo*. At this dose GYKI 52466 exerts a strong muscle relaxant effect, suggesting some preference of the drug towards the spinal cord.

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CONVENTIONAL AND PROSPECTIVE MOLECULAR TARGETS IN ANTITUMOUR DRUG DESIGN.* CONCEPTS IN ANTITUMOUR RESEARCH

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To introduce a rationale in a drug development program the molecular base of the pathological lesion must be carefully considered both for selecting test compounds and to apply the most appropriate assay systems. From the beginning of antitumour drug research the principal aim has always been to select chemical compounds which could selectively inhibit tumour growth. This strategy was in full harmony with the concept that tumours are build up by fast proliferating cells. Research based on this concept has resulted in the development of more than 40 cytostatic agents, which are rather diverse in their chemical properties, but all act on one of the molecular mechanisms participating in cell proliferation. However the unsatisfactory therapeutic responses which could be obtained by the cytostatic agents focused the attention on those molecular events in the tumour cells which may be more closely related to the progression of the malignant disease.

Keywords: cytostatic drugs, mode of action, tumour-biochemistry

As pharmacological agents act at the molecular level to achieve favourably therapeutic response in a given disease the knowledge on the biochemistry of the pathological lesion has paramount importance in the drug development program. It is desirable to have information on the qualitative or quantitative metabolic differences between the pathological lesion and the normal tissues [4, 7, 9]. Some of these differences are more significant than others in the development and progression of the disease. Consequently only those changes which are specific to a pathological entity should be regarded as molecular target for therapy. The updated

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information on the biological and biochemical nature of a pathological lesion provide guidelines for the rationale outlined in any drug developmental program. The pharmacological assays for the measurement of the efficacy of the test compounds must include at least some of the characteristic features of a clinical situation; from disease related symptoms to the underlying molecular pathological alterations. Although elimination of the sometimes intolerable symptoms (pain, fever, etc.) should not be neglected, complete cure can be expected only if the molecular basis of the disease is eradicated.

From the beginning of the cancer chemotherapeutic studies the prevailing concept was that cure in malignant disease could be achieved by the inhibition of tumour growth. It has been assumed that different cell proliferation mechanism may be implicated in tumour and in normal tissues which would make possible to develop drugs selectively acting on tumour growth. The strategy followed in cancer chemotherapy was in full harmony with the pathomorphological definition of tumours, which holds that unrestricted growth is the most important property of the tumour cells. It is, however, a strong belief that the time has come when the progression in our knowledge on tumour biochemistry represents a great challenge to address the question whether our concept in antitumour drug research is still updated. In this review the site of action of the cytostatic agents will be confronted with our present knowledge on the biochemistry of the tumours to decide whether the currently available drugs act on the most appropriate molecular targets. In addition some new prospective targets for antitumour drug research will be recommended. The cell proliferation oriented antitumour drug research has provided more than 40 clinically useful drugs with remarkable activity on certain malignant diseases; such as the lymphomas [8]. However the very poor responses of the most frequent tumours of epithelial origin justifies the need to apply novel concept and methods in cancer chemotherapy.

Conventional targets of antitumour drugs

Cytostatic agents represent those family of drugs which show variable selective cell-proliferation inhibitory action against tumours compared to normal tissues. They exert their action directly on the tumour through damaging the structure and the function of a cell constituent closely associated to cell proliferation [8]. On the ground of their mode of action they can be classified as follows: 1. DNA damaging agents which includes the alkylating agents and other compounds mainly of natural origin which bind noncovalently to DNA (antibiotics). 2. Compounds reducing the nucleotide biosynthetic capacity of the cells (antimetabolites). 3. Mitotic spindle poisons (vinca-alkaloids). 4. Hormones (i.e. glucocorticoids) which inhibit cell

proliferation presumably through the modification of a receptor mediated gene regulatory process.

In the last decade a great deal of promise has been assigned to the biological response modifiers which act mainly on the antitumour defense mechanism of the organism but direct antitumour action must be also taken into account. This latter mode of action of the biological response modifiers most probably includes the interference with the defects in the cell regulatory mechanism. Until now the biological response modifiers showed no real advantage to the cytostatic agents concerning both antitumour and untowards effects. Nevertheless it is expected that the better understanding of their mode of action will make it possible to apply them more effectively. In addition, since most of the biological response modifiers are normal regulatory substances of the mammalian cells, we hope that from studies on their mode of action in tumours valuable knowledge will be obtained about the defects of regulatory process in the tumours. The rather detailed investigations on the mode of action of the cytostatic agents do not indicate any tumour specific molecular alterations [2]. In fact the cellular site of actions of the cytostatic agents showed only quantitative differences between the tumour and the normal cells [8]. More interestingly the variable effects of a cytostatic drug on a series of tumour cell lines is determined by the same mechanism as the differential sensitivity of the normal and tumour cells. These and other related evidence clearly indicates that the currently available cytostatic drug do not have tumour specific target molecules i.e. the site of their action is not qualitatively different between normal and tumour cells. This also implies that no diverse mechanisms are operating for the antitumour and for the toxic side effects. The available data indicate that the marginal selective antitumour actions of the cytostatic agents are due to differences in the metabolic conversion and also in the repair mechanism between normal and tumour cells. Nevertheless it can not be excluded that studies in the near future could provide evidence for a tumour specific site of action by applying more advanced technique. Certainly the detection of binding sites to biopolymers (i.e. DNA, mitotic spindle) at the submolecular level may point to some tumour specific action. This notion has received some support from those studies which raised the possibility that antitumour alkylating agents preferentially attack the GC rich promoter region of the oncogen in DNA which are undermethylated in tumour cells [6]. The fact is, however, that the cytostatic agents in general appear to have rather antiproliferative potency in general and they exert only slight preference to tumour cells compared to normal ones.

The biological and molecular aspects of malignant diseases

Although it has been very well known for more than hundred years that tumours could be classified according to their histogenesis, heterogeneity as a

tumour related property has not long ago received attention [4]. This means variability within tumours of similar classification and it also includes the clonal evolution of the cell populations. In addition the solid tumours in the frame of their three dimensional structure seem to outgrow their vascular supply, consequently tumour cells are located in various distances from the blood vessels. Substantial proportion of the tumour cell population is not sufficiently supplied by nutrients and oxygen [5]. The tumour cells could survive in hypoxic environment which favours the development of resistance against various harmful attacks including drug treatment. The microenvironment within solid tumours may be hypertonic as a result of the build up of catabolites due to the poor vascular supply of the tumour. An elevated interstitial pressure within the center of the tumour has been also reported, therefore the bulk flow of the interstitial fluid tend to carry the macromolecules into the normal tissues surrounding the tumours. Solid tumours contain area of low pH arising primarily from the ability of tumour cells to produce lactic acid even in the presence of oxygen. The extracellular matrix of the tumours has a very important function in the malignant process, therefore it deserves much more attention as before in the judgment of both disease prognosis and drug access to the tumour cells. Local matrix chemistry affects tumour growth by mediating growth factor presentation to the cell surface and it also contribute to the invasive character of the tumour.

In spite of the traditional view the growth rate in the tumours is not higher than in the rapidly dividing normal cell population. It is noteworthy, however, that the intermitotic time shows a very wild scatter and the % of cells in DNA synthetic (S) phase is rather variable [8]. Although the biochemistry of tumour cells has been a topic of great interest in oncology in the last 60 years, most of the observations proved to be valid for the experimental model systems applied and until the present time no general conclusion could be drawn. Noteworthy is the concept introduced by Weber that the imbalance between the anabolic and the catabolic metabolic pathway confers selective advantage to cancer cells [17]. The pattern of the metabolic changes indicated the ordered reprogramming of the gene-expression during malignant transformation. The last 10 year period has wittnessed a tremendous progress in the understanding of the various modality of genetic alterations in tumour cells. These results have been excellently reviewed by two prominent scientists working in this research field P.C. Nowell [11] and R.A. Weinberg [18]. It has been well documented that both tumour formation and progression are considered to be the consequence of multiple sequential alterations in growth regulatory genes. The numerous proteins coded by the regulatory genes participate at all levels in the cell regulatory pathways; growth factors, growth receptors, second messengers that transduce the signal through the cytoplasm and also nuclear responder molecules including transcriptional factors. The regulatory genes are either involved in stimulation

(protooncogenes) or in down regulation (suppressor gene) of cell growth and differentiation. As a result of changes in the structure of the genome the growth promoting genes are hyperactive whereas the growth constraining genes are inactive in tumour cells. Consequently the tumours are always ready to divide, beside the cells respond to the signals received from their environment in a radically different way as the normal cells. Depending on the type of the affected genes – i.e. the cellular function of the gene products – regulatory processes could be deficient at any sites. In this respect there is no uniformity among the tumours, more interestingly the growth regulatory changes are subjects of remarkable variation during tumour progression. Vogelstein's group reported the multiple sequential somatic gene alterations during tumour progression [16]. It appears that oncogene activation precedes inactivation of suppressor genes, which is then followed by amplification and instability of the genome. The complexity in the molecular-biological bases of tumour pathogenesis is in good agreement with the diverse pathobiological features of the malignant disease. In malignant tumours deregulated growth is not only accompanied by invasion into the surrounding tissue and metastasis into various organs, but biologically active substances are overproduced exerting toxic action on normal cells [19].

Prospective molecular targets

It is felt that the above briefly summarized information on tumour biology and biochemistry has substantial value to those working in antitumour drug design. Perhaps the most important for the pharmacologist is to understand that there are multiple critical targets in the tumour and they are not stabile during the whole course of the malignant disease.

Surveying the available data on the biochemical aspects of malignant diseases those molecules which show defects in one of the following cellular events may be regarded as prospective targets for antitumour drugs:

1. Metabolic imbalance [17]
2. Signal transduction (endocrine, paracrin, autocrin) [13]
3. Regulation in gene expression [18]
4. Regulation of mitotic cycle [1]
5. Genome stability [11, 16]
6. Production of biologically active substances [19]
7. Intercellular contacts [13]

To achieve this highly ambitious goal appropriate assays should be introduced to select compounds acting on one of these pathobiological processes. Obviously the wide scale of the pathobiological events associated to the maintenance of the

malignant disease offer only a menu for a research team to chose the most promising targets on antitumour drug design. Some examples for the possible pharmacological utilization of these pathobiological events are given below.

Metabolic imbalance are investigated by comparing the activities of the key enzymes in the opposing metabolic pathways (i.e. thymidine kinase and thymidine phosphorylase). The recognition of tiazofurin as an inhibitor of IMP dehydrogenase which is a key enzyme in purin metabolism proves the value of the suggestion that metabolic imbalance could be also a drug target [17]. Recently in many studies [10, 12, 13] signal transduction has been considered as an important topic in tumour biochemistry and it has been suggested that drugs are required to inhibit phospholipase and protein kinase C (Fig. 1). Figure 2 shows that genome and the surrounding protein designated as chromatin are the site of action of various antitumour drugs [5]. Applying more sophisticated technique it is expected that gene locus more closely related to the malignant alterations could be affected in the near future. The present trend in this area involves the elimination of the oncogene expression by antisense deoxy-oligonucleotides or the neutralization of the oncogene products with monoclonal antibody [14].

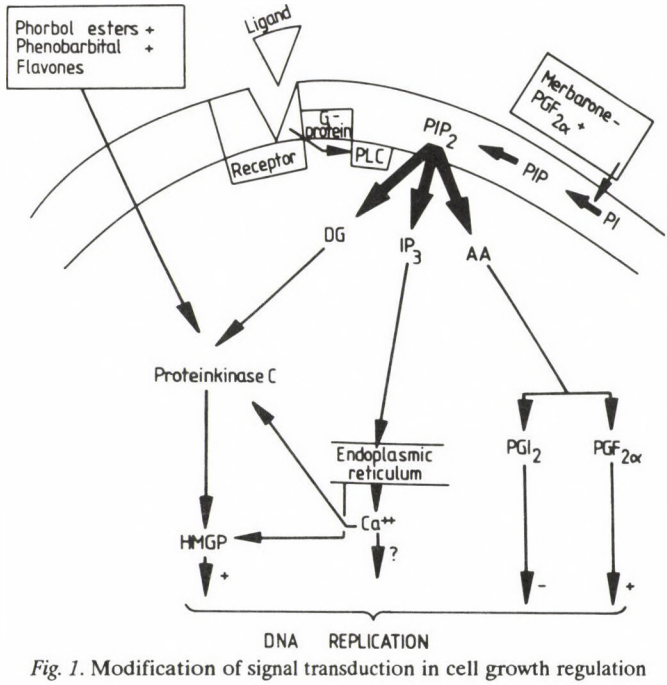

Fig. 1. Modification of signal transduction in cell growth regulation

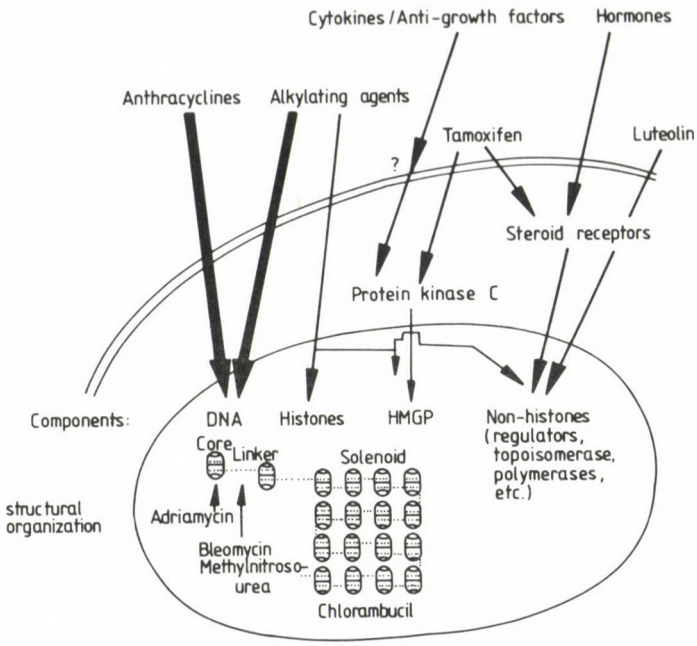

Fig. 2. Chromatin targeted antitumour drug action

To reduce the metastatic capacity of the tumour cells the modification of tumour cell surface appears to be a promising approach. Our own studies showed that 5-hexyl-2'-deoxyuridine could be a prototype of a new antimetastatic agent. This new pyrimidine derivative has no inhibition on nucleotide biosynthesis and does not incorporate into nucleic acid, but is able to reduce glycosylation of proteins and to inhibit proteoglycan biosynthesis [3].

Conclusions

The necessity to apply new model systems to identify compounds which may have more selective antitumour activity is justified by the recent data indicating that abnormal regulation is more characteristic for the malignant cells than the high rate in the proliferation activity. The great obstacle in antitumour design is the multiplicity and the variability of the critical molecular lesion. This implies that a battery of agents acting on various pathobiological events are required in the armamentary of

cancer chemotherapy. Furthermore the pathobiological events of each tumour to be treated must be determined to decide the nature of the molecular target for drug therapy. These complex studies would allow to apply the most effective antitumour agent in each clinical situation.

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EFFECT OF CYCLOSPORIN-A TREATMENT ON MICROSOMAL ENZYME ACTIVITIES IN RAT LIVER[1]

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The effect of orally administered fixed dose cyclosporin-A (CsA) on rat liver monooxygenase activities was studied. Group I was treated for 3, group II for 7 and group III for 17 consecutive days.

A time dependence in the degree of inhibition and number of microsomal enzyme activities inhibited was observed.

Keywords: cyclosporin-A, microsomal enzyme inhibition, rat liver

The clinical use of cyclosporin-A (CsA) contributed to the success in organ transplantation. A decline in the hepatic drug metabolizing capacity during CsA treatment was previously observed [3, 7]. CsA gives a type I binding spectrum with cytochrome P-450 and is metabolized by the cytochrome P-450 dependent hepatic microsomal monooxygenase system. The isozyme responsible for CsA metabolism is identical with nifedipine oxidase.

Several clinically important drug interactions are known and proved [15]. These interactions may be directly life threatening in the case of drugs with narrow therapeutic ratio [6].

There are contradictions in animal data obtained [2, 9]. The aim of the present study was to investigate the effect of a fixed CsA dose regimen of different duration on rat liver monooxygenase activities.

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[1]Some results described in this paper were presented in the Symposium on Pharmacokinetics and Metabolism, Mátrafüred, Hungary, 18–19 October, 1991.

Akadémiai Kiadó, Budapest

Animals and methods

Male albino Wistar rats weighing 150-250 g were used. The rats were kept in plastic cages and fed with LATI food pellets. The room temperature was constant and there was a fixed rhythm light regulation, too. The animals were kept fasting for 16 hours before decapitation.

The rats were divided into three groups. A 60 mg/kg bw. CsA dose was administered orally via gastric tube daily for 3 (Group I), 7 (Group II) and 17 (Group III) days. Controls received physiological saline (0.1 ml/100 g bw.) via gastric tube. The animals were treated between 8 and 9 hours a.m. every day. Decapitation was performed 24 h after the last treatment.

The liver was removed, washed in ice cold isolating solution, weighed, homogenized and ultracentrifuged in order to prepare the microsomal fractions according to Omura and Sato [4, 12].

The cytochrome P-450 and b_5 contents were determined according to Rutten [14]. NADPH cytochrome c reductase [5], aniline hydroxylase [1], aminopyrine N-demethylase [10] and p-nitroanizole 0-demethylase [11] activities were also measured.

The cytochrome P-448 activity was measured by the determination of 7-ethoxyresorufin O-deethylase activity [13].

The microsomal protein content was measured according to Lowry et al. [8].

The measurements were performed on SPECORD-M-40 spectrophotometer and HITACHI-650-10S spectrofluorimeter.

The Student's *t*-test for two means was used for statistical analysis.

Results

The results are summarized in Table I.

Only the p-nitroanizole O-demethylase activity decreased significantly compared to controls in group I (3-day treatment). There were significant decreases in cytochrome P-450 contents, aminopyrine N-demethylase and p-nitroanizole O-demethylase activities in group II (7-day treatment). The decrease in cytochrome b_5 content, p-nitroanizole O-demethylase and 7-ethoxyresorufin O-deethylase activities was significant in group III (17-day treatment).

Discussion

There are conflicting results regarding the effect of CsA on hepatic microsomal monooxygenase activities. Machková et al. [9] demonstrated an increase in cytochrome P-450 content and aniline hydroxylase activity after a three-day administration of CsA in 50 mg/kg bw./day dose. The same dose administered for 9 days resulted in a significant decrease of cytochrome P-450 content, NADPH cytochrome c reductase and other activities in an other study [2], and our data obtained from group II are in good correlation with them.

Table I

Effect of treatment with Cyclosporin-A on rat liver microsomal activity

Parameter $\bar{x} \pm SE$ n =	I. 3-day treatment Control NaCl 0.9% 0.1 ml/100g 7	 treated CsA 60 mg/kg daily 8	II. 7-day treatment Control NaCl 0.9% 0.1 ml/100g 6	 treated CsA 60 mg/kg daily 6	III. 17-day treatment Control NaCl 0.9% 0.1 ml/100g 6	 treated CsA 60 mg/kg daily 6
Cytochrome p-450 (nmol/ mg prot.)	0.70 ± 0.05	0.61 ± 0.03	0.83 ± 0.04	0.57 ± 0.05^{x} -32%	0.63 ± 0.03	0.56 ± 0.01
Cytochrome b5 (nmol/mg prot.)	0.69 ± 0.02	0.70 ± 0.03	0.67 ± 0.06	0.62 ± 0.05	0.59 ± 0.02	0.49 ± 0.01^{xx} -17%
NADPH cytochrome c reductase (nmol red. cyt. c/min/mg prot.)	58.4 ± 4.0	64.6 ± 4.3	100.6 ± 6.5	95.9 ± 21.1	56.5 ± 4.8	47.2 ± 5.0
NADPH aminopyrine N demethylase (nmol HCHO/min/mg prot.)	3.39 ± 0.4	3.30 ± 0.3	9.76 ± 0.4	6.62 ± 1.2 -32%	6.54 ± 0.6	5.46 ± 0.5
Aniline hydroxylase (nmol p-aminophenol/min/mg prot.)	0.69 ± 0.07	0.58 ± 0.06	0.78 ± 0.02	0.73 ± 0.01	0.60 ± 0.05	0.51 ± 0.09
P-nitroanisole O-demethylase (nmol p-nitrophenol/min/mg prot.)	1.37 ± 0.11	0.71 ± 0.08^{xxxx} -48%	1.42 ± 0.04	0.77 ± 0.09^{xxx} -46%	1.31 ± 0.09	1.00 ± 0.06^{x} -24%
7-ethoxyresorufin O-deethylase (pmol/min/mg prot.)	24.4 ± 6.0	10.7 ± 3.0	28.0 ± 2.9	27.6 ± 11.1	36.8 ± 3.9	20.5 ± 3.8^{X} -44%

Changes relative to control: ^{x} = $p<0.05$, ^{xx} = $p<0.02$, ^{xxx} = $p<0.01$, ^{xxxx} = $p<0.001$

There was a decrease in 7-ethoxyresorufin O-deethylase activity reflecting cytochrome P-448 (cytochrome P-450IA) activity after the 17-day treatment with CsA. Cytochrome P-448 activity is closely linked with the production of carcinogenic intermediates.

According to our data presented in this paper not only the dose but also the duration of CsA administration have an impact on hepatic microsomal enzyme activities in rats.

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PHARMACOKINETICS OF RADIOPHARMACEUTICALS*

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The pharmacokinetics of various radiopharmaceuticals following i.v. administration in mice and rats has been studied and compared. Before injection the radiochemical purity (RP) of the compounds were determined by HPLC and PAGE. In all cases RP-s were higher than 90%. The biodistribution of 99mTc labelled anti CEA IgG was studied in mice bearing human colon carcinoma xenografts. Animals with different tumour weights showed different blood kinetic and tumor uptake.

The pilot clinical study of the 99mTc labelled anti melanoma Fab and 111-In-DTPA labelled anti melanoma $F(ab')_2$ showed differences in the pharmacokinetic parameters. (99mTc labelled: comp.A. 84.6%; $T_{1/2}$:0.6 h, 111-In-labelled: comp.A.: 46%, $T_{1/2}$ 1.5 h.)

The various isonitril derivatives synthetized in our laboratory were labelled with 99mTc and the biodistribution were tested in rats.

The kinetic study showed that all the three molecules have different half lifes in the heart and liver ($T_{1/2}$ for heart ranged 5.1–18.6 h, for liver $T_{1/2}$:1.2-2.5 h). Reversed phase HPLC study of the collected bile showed the 15-100% of injected compounds are metabolized during hepatic excretion.

Literature date and our recent observations confirm that the knowledge of pharmacokinetics of radiolabelled compounds both in research, development and clinical practice is of basic importance.

Keywords: radiopharmaceuticals, pharmacokinetics, radiolabelled monoclonal antibodies, 99mTc labelled isonitril derivatives, 99mTc-anti CEA monoclonal antibody

Both in diagnostics and therapy it is essential to know the biodistribution and elimination patterns of drugs labelled with different isotopes.

The clinical use of a radiopharmaceuticals for the determination of organ localization and function seems to be subject only to one condition, namely that the uptake of the target organ and the accumulation and elimination kinetics of the

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radioactive molecule in this organ be different from those of the neighbouring organs and tissues.

The varying sensitivity of scintillation cameras used for the external detection of the biodistribution of radioactivity within the body stresses the importance of the precise understanding of pharmacokinetics. In examinations with planar cameras the target: background ratio should fall between 2-5, while in Single Photon Emission Computerized Tomography (SPECT) examinations even a 1.5 fold ratio is sufficient to obtain an adequate scintigraphic imaging. Monoclonal antibodies and their $F(ab')_2$ and Fab fragments raised against various tumour associated antigens and labelled by radioactive isotopes well demonstrate the diagnostic value of the pharmacokinetic parameters [3]. The precise knowledge of pharmacokinetics is likewise indispensable in the testing and clinical trial of new isonitrile derivatives suitable for myocardial perfusion.

In this study we examined 99mTc-anti-carcinoembryonic antigen (CEA) (BW 431/26) for biodistribution and tumour localization in a CEA positive human colon carcinoma transplanted to immunosuppressed CBA mice. The radiochemical purity of the molecule and the stability of radioactivity were determined by High Performance Liquid Chromatography (HPLC) and Polyacrylamide Gel Electrophoresis (PAGE) examinations. Studies with antimelanoma diethylenetriaminepentaacetic acid-$F(ab')_2$[(DTPA-$F(ab')_2$] fragment of labelled with 111-In and clinical testing of 99mTc-labelled antimelanoma Fab fragment (National Institute of Oncology, Licence No. of National Institute of Pharmacy (OGYI) 5368/77/86 and 2431/77/88), respectively, clearly demonstrate the differences in the pharmacokinetic parameters of the individual fragments of the antibody molecule [4].

Jones et al. [7] were among the firsts who suggested that the different isonitrile derivatives would probably be the most potent radioactive agents for myocardial scintigraphy. We have synthetized three isonitrile derivatives, BE 75, BE 77 and BE 84 [1]. The pharmacokinetics of isonitrile complexes labelled with 99mTc was determined in rats. The measure of possible metabolization during biliary excretion was also examined.

Material and methods

Determination of the radiochemical purity and stability of monoclonal antibodies

Analytical studies were carried out with a BIO-RAD HPLC 800 system using Bio-Sil TSK 250 column. Samples of 20 µl volume were eluted in 0.02 M NaH_2PO_4 buffer solution (pH 6.8) at 1 ml/min flow rate. Protein absorption was measured at 280 nm. Activity values of 0.5 ml samples obtained with an ISCO 568 type fraction collector served for the determination of radioactivity. The same samples were also examined by polyacrylamide gel electrophoresis. The non-reducing PAGE method with 7.5% gel was

applied. Samples of 40 μl volume were run at 4 mA/gel current intensity over 24 hours in 0.2 M phosphate buffer solution.

Organ distribution of 99mTc anti-CEA IgG

Experiments were conducted with CBA mice rendered immunosuppressed by thymectomy, at the age of 5 weeks. Thymectomy was followed in week 6 by whole body irradiation with gamma rays in a dose of 8.5 Gy and bone marrow reconstruction. The human colon carcinoma xenografts were implanted after the 7th week of life. Animals showing satisfactory tumour grow 2, 3 and 6 weeks after transplantation were divided into 2 groups according to tumour weight. Group A contained animals with 0.3-0.5 g tumour and Group B those with 2.0-3 g tumour. Each group comprised 25 animals. They were injected via the tial vein with 100 μl of labelled antibody (22 MBq, 0.04 mg) using a Hamilton micro-syringe. The animals were killed under ether anaesthesia 1, 6, 12 and 48 hours after injection. The following organs were weighed and examined for radioactivity: blood, tumour, thyroid, heart, lung, liver, spleen, kidney, stomach, small intestine, colon, bone, muscle, urinary bladder and brain.

Blood clearance of 99mTc anti melanoma Fab fragment and 111-In-DTPA anti melanoma $F(ab')_2$ fragment

Clinical trial of monoclonal antibody fragments of identical clone but labelled with different radioactive isotopes was performed at the Department of Nuclear Medicine, National Institute of Oncology, Budapest. The study population included 44 patients. Thirty-three of them were given technetium and 111-In labelled antibody. Highly different organ distribution patterns were obtained for the two molecules. Blood clearance parameters were determined in blood samples taken at different points of time after the intraveneous injection of the labelled substance. In case of technetium sampling was made with 6 patients and in that of indium with 5 patients.

Determination of the radiochemical purity of 99mTc-labelled isonitrile derivatives

Thin layer chromatography was made with 2.5 x 20 cm MERCK DC alufolien aluminium 60 plates. Samples were mounted in 2.5 to 5.0 μl volume, their activity ranged from 0.37 to 4.8 MBq. The chromatographic plates were placed into running tanks with ethanol saturated air space. After 30 min development the plates were dried and cut into 1 cm stripes. Radioactivity was measured in a GAMMA NK 350 type spectrometer coupled with an automatic well counter. The Rf values were as follows: for technetium labelled isonitrile complexes Rf = 0.3–0.5; for unbound pertechnetate Rf = 0.7–0.8 and for reduced hydrolized technetium Rf = 0.0–0.15.

HPLC studies

In HPLC analysis a BIO-RAD 800 equipment with RP 318 type reverse phase column was used. The chromatograph was coupled with a flow-through radioactivity detector. After injection of the sample (20 μl), a linear solvent gradient program [0.05 M $(NH_4)_2SO_4$ + MeOH] was used at a flow rate of 1.0 ml/min.

Organ distribution patterns of 99mTc labelled isonitrile derivatives

DCFY male rats of 110-130 g body weight were used. Animals were fasting for 24 hours prior to examination. All the three labelled molecules were injected in 0.2 ml dose (3.7 MBq activity, 1 μg isonitrile) via the tail vein. Rats were killed under ether anaesthesia 5, 30 min and 1, 3 and 5 hours, respectively, after the injection of the labelled compounds. Radioactivity was expressed in per cent of the injected dose or in the ratio of injected dose to g organ weight, respectively. Organs weighed and

examined for radioactivity were: heart, blood, liver, lung and kidney. Possibly arising metabolites due to the basicly biliary excretion of the molecules were examined in bile samples collected after the cannulation of the bile duct in 3 animals per test molecule. Conditions of HPLC study were the same as specified above. The radiochromatograms of the various bile samples were compared to the radiochromatograms and retention times of the injected molecules. Metabolization was expressed in the ratio of components with different retention times.

Mathematical, statistical analysis

Time-series of activity and activity concentrations in different organs and tissues (blood, liver, lung, etc.) were investigated by non-linear fitting techniques [10]. Restricted by the number of data points in some cases a single compartment model was used. Otherwise the retention curve was described by two-exponential function. To obtain the best fit weight factors of $1/A^2$, where A is the activity or activity concentration were applied in some cases [11].

Results

Radio-analytical studies performed before injection revealed high labelling efficiency for 99mTc anti-CEA IgG. The HPLC (Fig. 1) and polyacrylamide gel electrophoresis (Fig. 2) studies indicated that the molecule stored at + 4 °C showed 99.5% labelling efficiency 10 min after labelling, 96.6% 4 hours later and 96.1% after 24 hours. Organ distribution values obtained after labelling and analysis of animals with different tumour weights are represented in Tables I and II. Out of the organs examined only the data of blood, tumour, liver and kidney are demonstrated in these tables. The blood kinetic and intratumoural accumulation values of animals with different tumour weight are given in parts *a* and *b* of Fig. 3. Human blood clearance values are given in Fig. 4. It makes clear that the first fast component of 99mTc Fab fragment, presenting 85% proportion, eliminates with a very short, 0.62 h half life from the blood, while the first fast component of 111-In Fab fragment, representing a 46% proportion eliminates only with 1.5 h half life. The proportion and half life values of the slower components also show significant differences. In the case of 99mTc labelled antibody the proportion of the slower component is 15% and half life is 13.4 hours. In case of 111-In labelled derivative the same values are 54% and 28.2 hours.

The radiochemical purity data of the three isonitrile derivatives of our laboratory determined by thin layer chromatography are demonstrated in Table III. The radiochemical studies also controlled by reverse phase HPLC (Table IV) show good correlation with the results of TLC studies. The biodistribution of isonitrile derivatives was examined in relation to activity changes in the target and neighbouring organs in time and their kinetics (Fig. 5). The kinetic figures show that all the three molecules have different half lifes in the heart and liver. This is of primary importance if the actual imaging with these substances is considered. Differences in half lifes in the lung are not so expressed. HPLC data of collected bile (Fig. 6) compared to the corresponding retention times have indicated metabolization to range from 15% to 100%.

Table I

*Biodistribution of 99mTc-labelled anti CEA IgG in mice bearing human colon carcinoma.**
Size of tumor: 0.3 – 0.5 g

Organs	Time post injection (hour) 1		6		12		24		48	
	$\bar{x}$	±SD	$\bar{x}$	±SD	$\bar{x}$	±SD	$\bar{x}$	±SD	$\bar{x}$	±SD
blood	34.01	4.23	16.57	6.88	16.37	1.74	15.79	2.31	9.01	1.32
liver	10.65	1.01	7.01	3.37	4.38	0.55	4.96	0.42	2.99	0.56
kidneys	11.46	1.03	10.63	4.02	8.14	1.42	9.74	1.69	6.07	1.57
tumor	4.54	1.49	3.07	0.59	13.71	2.17	11.27	3.79	9.45	1.27

* % injected dose/g tissue (±SD) n=5/mice/point of injected

Table II

*Biodistribution of 99mTc-labelled anti CEA IgG in mice bearing human colon carcinoma.**
Size of tumor: 2 – 3 g

Organs	Time post injection (hour) 1		6		12		24		48	
	$\bar{x}$	±SD	$\bar{x}$	±SD	$\bar{x}$	±SD	$\bar{x}$	±SD	$\bar{x}$	±SD
blood	27.81	5.82	18.09	5.22	7.77	0.78	6.86	2.99	4.95	1.04
liver	8.38	3.03	7.09	1.51	4.15	0.61	3.84	0.87	3.15	0.31
kidneys	10.06	0.74	11.54	3.10	11.69	3.51	6.84	1.77	4.72	0.93
tumor	2.26	0.60	3.23	1.35	3.69	1.59	8.53	0.98	6.57	0.87

* % injected dose/g tissue (±SD) n=5/mice/point of injected

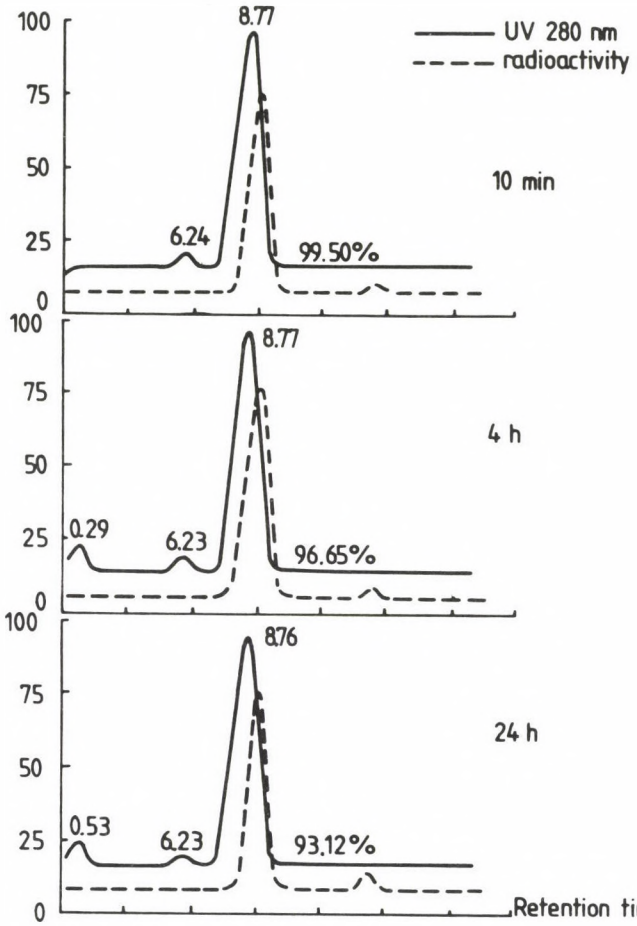


Fig. 1. HPLC Chromatographic profiles of 99mTc anti CEA IgG, 10 min, 4 and 24 h after labelling (storage at 4 °C)

Table III

*Radiochemical purity and stability of various 99mTc-labelled isonitrile derivatives determined by TLC**

Compounds (%)	BE-75		BE-77		BE-84	
	25 min	8 h	25 min	8 h	25 min	8 h
Free $^{99m}TcO_4$	0.2±0.07	0.2±0.06	0.3±0.08	0.4±0.07	0.2±0.06	0.3±0.08
Hydrolized ^{99m}Tc	1.3±0.12	3.1±0.28	0.6±0.02	1.4±0.03	0.4±0.10	1.3±0.12
^{99m}Tc-isonitrile complex	98.5±2.17	96.7±2.04	99.1±1.80	98.2±2.10	99.4±1.93	98.4±1.72

*X±SD n=5

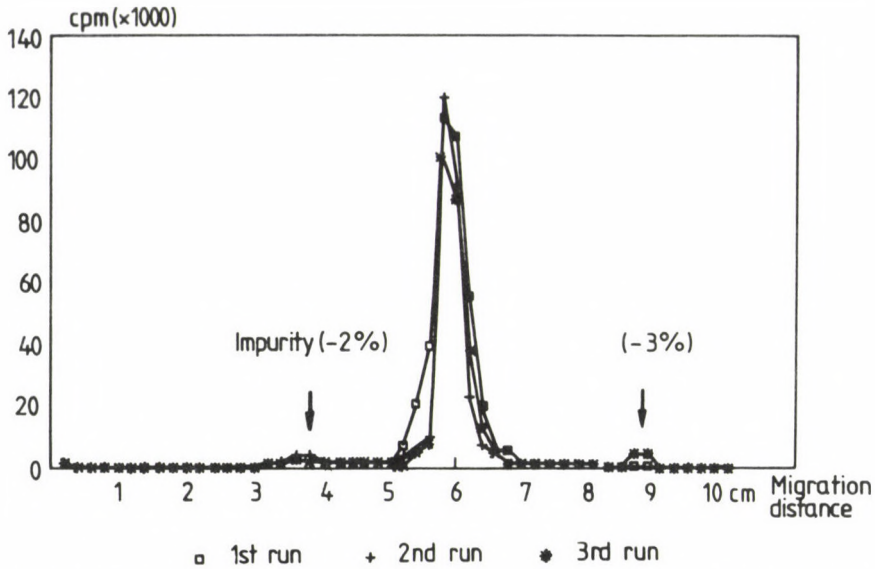


Fig. 2. Polyacrylamide gel electrophoresis of 99mTc-anti CEA IgG (gel conc.: 7.5% non-reduced 0.2 phosphate buffer, pH = 6, time: 24 hours, current: 4 mA/gel)

Table IV

*Radiochemical purity and stability of various 99mTc-labelled isonitrile derivatives determined by HPLC**

Compounds	Retention time (min)	Time of examination 15 min (% + SD)	6 h (% + SD)
BE-75	11.4	96.8 ± 1.3	94.9 ± 1.2
BE-77	13.4	97.4 ± 1.2	95.8 ± 1.4
BE-84	15.5	97.7 ± 1.2	96.2 ± 1.3

*X ± SD n = 3

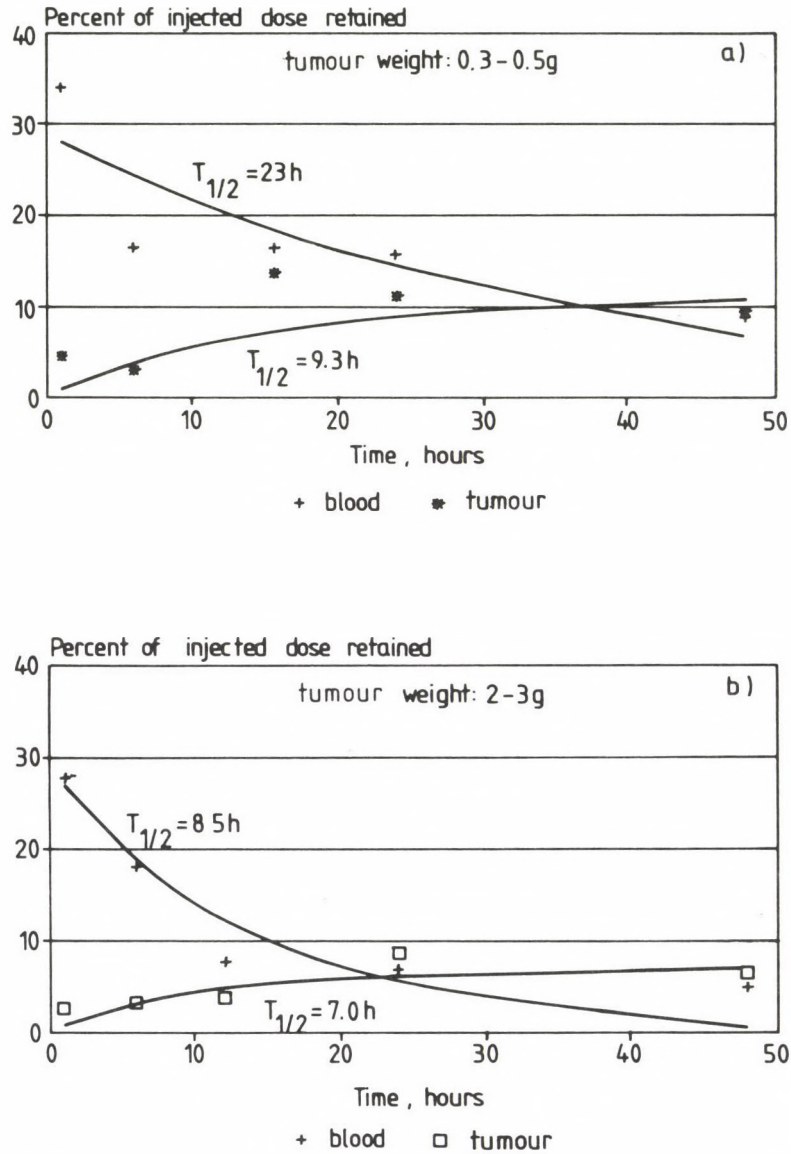
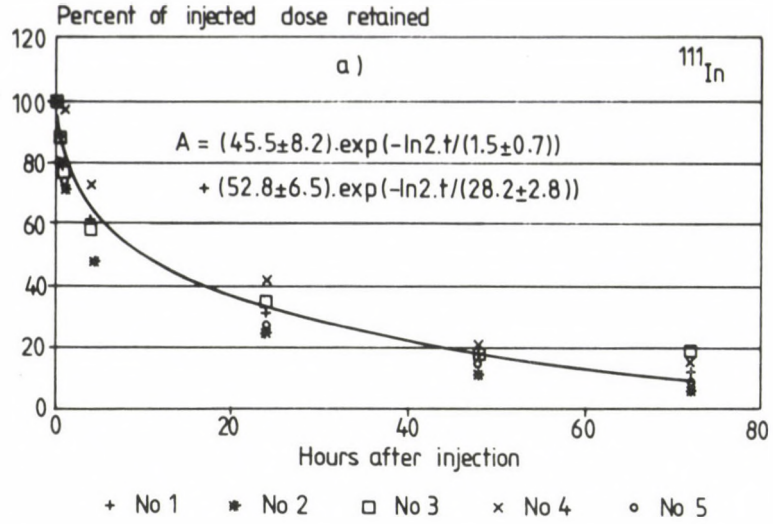


Fig. 3. Blood clearance and tumour uptake of 99mTc-anti CEA IgG in mice bearing human colon carcinoma of different size (a and b)



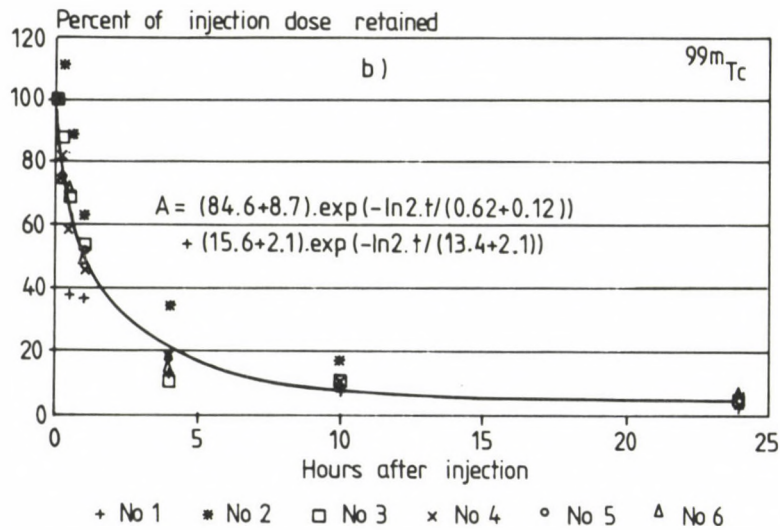


Fig. 4. Blood clearance of 111-In-DTPA labelled (a) and 99mTc (b) anti melanoma monoclonal antibody Fab and $F(ab')_2$ fragments

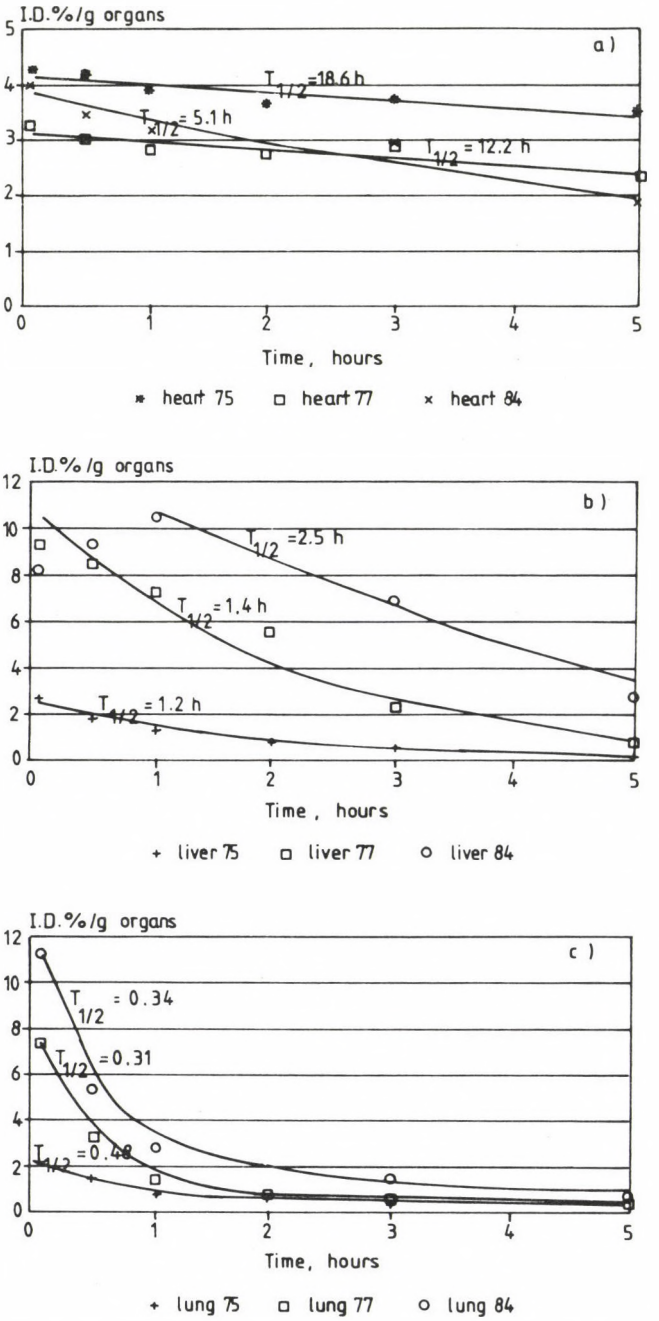


Fig. 5. Heart, liver and lung uptake and retention of the various 99mTc-labelled isonitrile derivatives

Discussion

By means of labelling monoclonal antibodies and their $F(ab')_2$ and Fab fragments of identical specificity with different isotopes one can favourably affect the routes of their biodistribution patterns, routes of elimination and kinetics. The very fast renal excretion of Fab fragment induces a fall in blood radioactivity that contributes to the better imaging of pathological alteration. However, the activity of the kidney and bladder considerably accumulated due to the renal excretion prevents the detection of tumour involvement if any in these organs.

The significantly high accumulation of 111-In-$F(ab')_2$ fragment in the liver and slow elimination from it make it impossible to identify possible liver metastases. Because of the slow elimination of the molecule from the blood, adequate scintigraphy is feasible only at a later point of time (hours 48 and 72). Pharmacokinetics of radioactive antibody molecules may be modified by biological effects as confirmed by experiments with 99mTc-labelled anti CEA IgG in animals with tumour significantly different weights.

In animals with tumours of 2-3 g (that comes up to 7-10% of the whole body weight) the CEA antigens shedding from the tumor and entered the circulation form an immunocomplex with the injected anti CEA antibody and get eliminated from the body in a shorter time ($T_{1/2}$:8.5 hours).

Several laboratories in the world are engaged in searching for isonitrile derivatives suitable for the measurement of myocardial perfusion and imaging of the myocardium.

Pharmacokinetics of the molecule of choice should allow imaging in spite of the disturbing background activity (lung and blood) and activity of accumulating organs, like the liver, in the close vicinity of the heart.

Problems in contrasting explain that the candidate isonitrile derivatives were tested first of all for their accumulation in the myocardium and elimination from the blood, lung and liver. Testing of our isonitrile derivatives has revealed high radiochemical purity and stability. In spite of nearly identical myocardial uptake only the BE 75 agent was effective enough in imaging the myocardium due to the disadvantageous background values and isotope uptake and elimination of the regionally closely situated liver. Figure 5a shows that at all points of time of measurement of BE 75 it was the cardiac muscle that contained the highest activity expressed in per cent of the injected dose/g organ weight. With the other two molecules the trouble is most likely caused by their higher uptake and slower elimination in the liver as confirmed by human studies. In humans, the assessment of cardiac perfusion in multidirectional scintigrams may be disturbed by the significant liver activity.

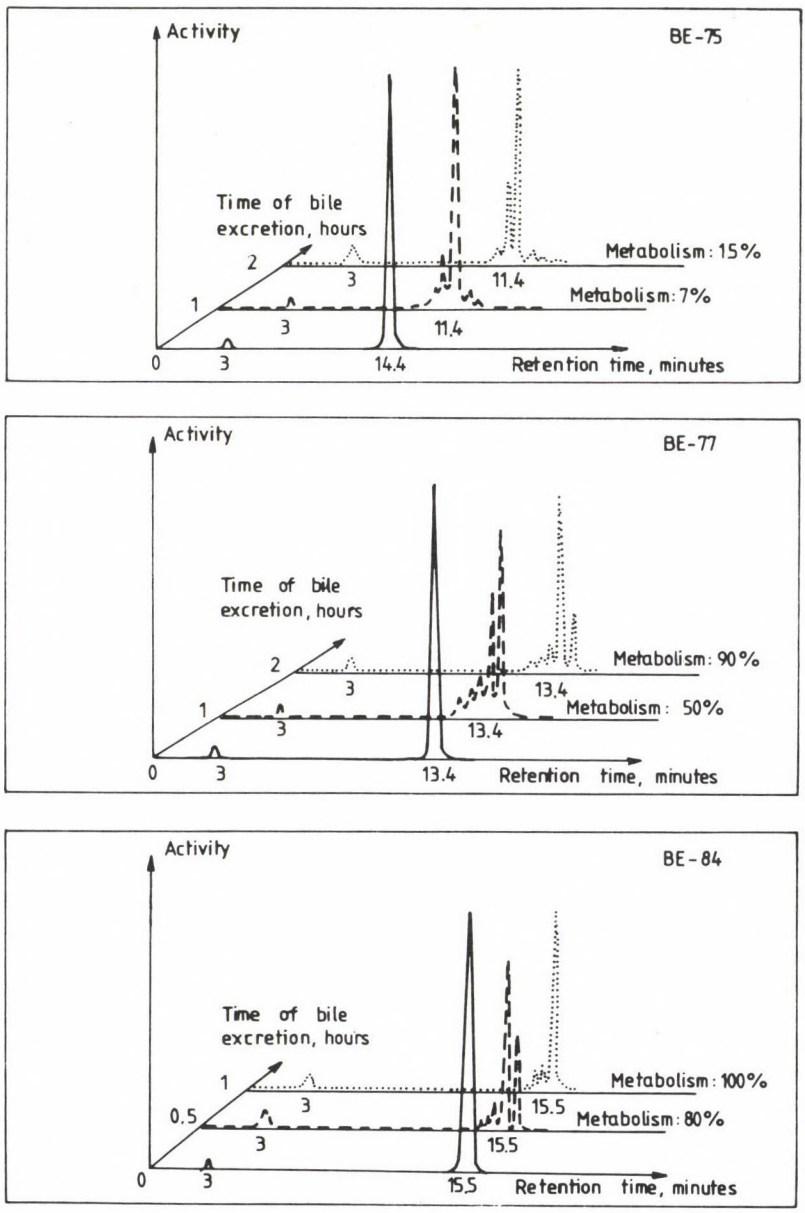


Fig. 6. HPLC chromatographic profiles of the various 99mTc labelled isonitrile derivatives and bile samples collected at various time intervals after i.v. injection of the derivatives

The different biological features of structurally related derivatives appear in varying degrees of metabolization during biliary excretion. Relevant literature data and our own observations confirm that the knowledge of pharmacokinetics of labelled molecules both in research, development and clinical practice is of basic importance. There is an abundance of publications [2, 5, 6, 8, 9] stating that normal distribution and pharmacokinetics of radioactive drugs are subject to several factors e.g. previous medication, accompanying diseases, etc.

Summing up, the knowledge of the pharmacokinetics of radiopharmaceuticals and its modifying factors are essential for their effective diagnostic and therapeutic applications.

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IN EXPERIMENTAL RICIN INTOXICATION THERE IS A DETECTABLE GASTRIC ACID SECRETION IN THE RAT

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According to previous investigations, ricin the toxic protein of castor oil seeds (*Ricinus communis*, Euphorbiaceae) given in subtoxic dose, evokes a parathyroid lesion with a consecutive parathormone mobilization in the rat. The elevated parathormone level rises the plasma calcium-content resulting (among others) in endogenous gastrin mobilization. This latter increases the gastric acid secretion in significant degree.

This, hitherto unknown effect of ricin directs the attention once again to the complexity of its toxic action.

Keywords: ricin, gastric acid secretion, rat

It was shown earlier that RIC the toxic protein of castor oil seeds (*Ricinus communis*, Euphorbiaceae) causes – among others – a parathyroid gland lesion [1] and through a consecutive parathormone mobilization it evokes disturbances in the organism's calcium-magnesium balance, i.e. under RIC effect the plasma Ca^{++}-content rises while the Mg^{++}-level decreases, resulting in an enhanced Ca^{++}:Mg^{++} ratio [2, 3].

Theoretically if parathormone mobilization takes place – due to the elevated plasma Ca^{++}-level – the endogenous gastrin-level should rise and this elevated gastrin-level will cause an enhanced gastric acid secretion [4].

To investigate the above tenet the following experiments were performed.

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Materials and methods*

Adult male Wistar rats weighing 230-250 g were used. Prior to the investigation the animals were fasted in wire-bottomed cages for 24 hours but they were allowed to drink water *ad libitum*.

For the investigation the animals were anesthetized by urethane (1.60 g/kg, i.p. in aqueous solution), and their gastric (acid) secretion has been measured according to Ghosh and Schild and Pissidis and Clark, respectively [5, 6].

After stabilization of the basal secretion of the animals, i.e. about 60 min following the surgical intervention, (insertion of a gastric cannula [5, 6]) a test-dose of 10 ng P was given i.v. ("Peptavlon", I.C.I., U.K.) to establish the reactivity of the rats.

After the completion of P's effect – in about the 180th min of the experimental period – RIC was given i.v. in a sterile, pyrogen-free, aqueous solution in 20 μg/kg dose. ("Ricin nach Kobert", E. Merck, Darmstadt, Germany; Charge No: Br-23-1776-W-42721-722721; LD_{50} = 38.15 ± 0.412 μg/kg, i.p.)

After RIC's effect, a further P-injection was given with the same dose as previously, i.e. 10 ng/animal.

The secreted gastric juice was collected in 10 min fractions and the acid quantities were expressed in mmol HCl/h. The estimations were carried out by routine titration method with 0.1 NaOH solution.

At the beginning of the experimental period (0 min) and in the 20th, 30th, 40th, 50th, 60th, 70th, 80th and 90th min after RIC administration, samples were taken for serum Ca^{++} and Mg^{++} determination.

The ion-estimations were carried out by routine atomic-absorption spectrophotometry and the results were expressed in mmol/l units.

Statistical analysis was performed using Student's *t*-test and significant differences were assumed between values when the probability was less than 5%.

All experimental values in the text and tables are the mean ± SEM of the determinations of *n* animals.

Results

The experimental results are presented in the Tables I and II and in the Figure 1.

It seems that:

1. After RIC administration there is a gradual increase in the serum Ca^{++}-level, which reaches its peak-value in the 40th min and afterwards it starts to decrease but even in the 90th min it does not return to the normal level.

2. The serum Mg^{++}-level shows no significant changes during the first 90 min of RIC's effect and therefore an elevated Ca^{++}:Mg^{++} ratio can be observed.

3. After 40-50 min of RIC administration the gastric acid secretion shows a significant elevation which lasts for about 30-40 min, and later the gastric acid secretion returns to the normal value. (Basal secretion.) To demonstrate this phenomenon we present the experimental graph of one of our experimental animals.

*List of abbrevations used: P: Pentagastrin, RIC: Ricin

Table I

The effect of ricin on serum calcium and magnesium levels of rat

Group Time after ricin administration, min	n	Mg^{++} mmol/l	Ca^{++}	Ca^{++}/Mg^{++} ratio
Control*	15	1.05 ± 0.11	2.32 ± 0.24	2.21
20 min	15	1.05 ± 0.12	2.84 ± 0.29	2.70
30 min	15	1.07 ± 0.10	3.35 ± 0.31	3.13
40 min	15	1.07 ± 0.12	3.79 ± 0.37+	3.54
50 min	15	1.08 ± 0.09	3.45 ± 0.35+	3.19
60 min	15	1.03 ± 0.10	3.24 ± 0.31	3.15
70 min	15	0.96 ± 0.09	3.04 ± 0.32	3.17
80 min	15	0.95 ± 0.09	3.02 ± 0.31	3.18
90 min	15	0.94 ± 0.10	2.89 ± 0.29	3.07

Mean values ± SEM + = $p<0.05$ vs control
*Values at 0 min of experimental period

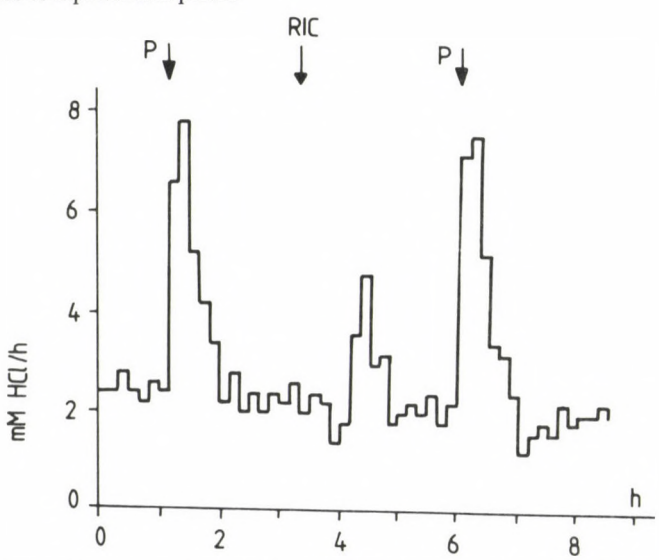

Fig. 1. Experimental graph of a rat. Estimation of gastric acid secretion. P = 10 ng pentagastrin i.v. RIC = 20 μg/kg ricin, i.v. Horizontal axis: time in hours; vertical axis: secreted gastric acid in mmol HCl/hour

Table II

The effect of ricin on the gastric acid secretion of rat

Group	n	Secretion mmol HCl/h	Δ%
Control as BAO[a]	15	2.30 ± 0.6	100.0
Pentagastrin I as PAO	15	7.90 ± 1.6^{+++}	343.5
Ricin as PAO	15	4.85 ± 1.2^{+}	210.9
Pentagastrin II as PAO	15	7.60 ± 1.7^{+++}	330.4

Mean values ± SEM
$^{+}$ = $p<0.05$ vs control
$^{+++}$ = $p<0.001$ vs control

[a]Basal acid output
[b]Peak acid output

Discussion

It was published earlier that after 60 min of RIC administration (20 μg/kg, i.p.) there are well-characterized histological changes in rat parathyroid gland, e.g. increased nuclear volume, increased mitotic activity, etc.

Therefore it was suggested that under the influence of RIC there is a parathormone depletion and the effect of RIC on the serum Ca^{++}-and Mg^{++}-levels – at least partly – is due to its parathormone mobilizing effect [1].

On the other hand, Brodie et al. reported that elevated serum Ca^{++}-level evokes an endogenous gastrin-mobilization resulting in increased gastric acid secretion [4].

Our present data indicate that under RIC's effect most probably both phenomena are present in the organism.

40-50 min after RIC administration there is a significant increase in gastric acid secretion, which compared – as biological titration – to the applied P's effect, corresponds to about 5 ng of P.

It is worth mentioning that according to our unpublished data the RIC's solvent (sterile, pyrogen-free dest. water) causes no detectable changes in the gastric acid secretion.

Considering our previously published data, too [1, 2, 3] – we conclude that this gastric acid secretion increasing effect of RIC is probably based upon a parathormone mobilizing action.

The increased parathormone-level mobilizes Ca^{++} [1, 2] and the excess Ca^{++} evokes an endogenous gastrin depletion [4] resulting in the elevation of gastric acid secretion.

According to our best knowledge this effect of RIC was hitherto unknown but considering its complexity it needs further, detailed investigation.

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BREATHLESSNESS IN HEALTHY SUBJECTS AT PHYSICAL LOAD

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The aim of the study was to ascertain the reasons which lead to discontinuance of exercise on the bicycle ergometer in healthy untrained subjects and to assess the dependence of dyspnea on breathing pattern and on ventilation. The physical load was progressively increased to the maximum in 11 volunteers at the age of 21 ± 1 years. During exercise some cardiovascular and respiratory parameters were measured simultaneously with the degree of dyspnea. Breathlessness was rated by means of a scaling according Borg, where 0 indicates no, 10 maximal dyspnea. Dyspnea was not a reason for termination of maximal exercise, its value being 6 ± 1.9 in men and 4.5 ± 2.3 in women at the end of exercise. The reasons for termination of exercise were the sensations of general fatigue and pain in lower the extremities. The degree of dyspnea correlated with the minute ventilation, with the decrease of end-tidal CO_2 concentration, with the duration of exercise and some other values. The grading varied among subjects. The mathematical dependence of dyspnea was summarised by two regression equations, one without suppression, the other with suppression of interindividual differences in responses.

Keywords: limiting factors of physical exercise, scale for dyspnea assessment, dyspnea during incremental exercise

At maximal physical exercise the *patients* with chronic airway obstruction complain of dyspnea [11, 18]. This sensation is also the principal reason limiting the maximal exercise in these subjects.

Our study has two principal goals. The first one is to tell which are the *limiting factors* for maximal exercise in *healthy* volunteers, and the second one is to provide a mathematical description of breathlessness using cardiovascular and/or respiratory variables measured during exercise in healthy subjects.

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Subjects and methods

The bicycle ergometer exercise was done in 5 young men and 6 young women-medical students. All subjects were healthy untrained nonsmokers. Their forced vital capacity per s (FEV 1) was higher than 0.8 of the forced vital capacity (FVC) and was within ± 0.2 (20%) of predicted normal values of all subjects. Their basic characteristics are in Table I.

Table I

The characteristics of tested subjects

	Men	Women
Number of subjects	5	6
Age (years)	21 ± 1	21 ± 1
Body weight (kg)	78 ± 9	60 ± 6
Body height (cm)	182 ± 5	166 ± 7

means ± SD

The values of FEV 1 and of FVC before the experiment were measured by means of a dry clock spirometer Mijnhardt Bunnik Holland. Other device used is reported in Table II.

The experiment began with the measurement of control resting values. The experimental protocol consisted of 5 steps as follows:

I. All subjects were sitting for 5 mins at rest without both nose-clip and mouth-piece.

II. Other 5 min sitting on a bicycle ergometer and breathing through a standard mouth-piece. The functional values were measured every 30 s and at the end of this period the breathlessness was rated.

III. 5 min of bicycle exercise at the intensity of 1 $W.kg^{-1}$ b.w. The functional values were measured every 30 s, breathlessness every 60 s.

IV. 5 mins of bicycle exercise but at the intensity of 2 $W. kg^{-1}$ b.w.

V. The physical load was increased every 30 s by 25 W up to maximal exercise. The values were registered every 30 s.

The degree of dyspnea [Ψ] was rated simultaneously with the measurement of the cardiovascular and respiratory values by a 10 points vertical scale according to Borg [4], where 0 signifies no, and 10 the maximal breathlessness. The subjects were required to avoid rating non-respiratory sensations such as pain or fatigue in the lower extremities *during* exercise, but they were asked to give their reasons of termination of the maximal exercise - *after* exercise.

The means and S.D. before and at various levels of exercise were calculated and the sexual differences were evaluated by unpaired Student's *t*-test. The probabilities were $p < 0.05$ or < 0.01.

Table II

Measured and calculated cardiovascular and respiratory values in the study of incremental exercise dyspnea and device used

Value		Units	Device
I	Heart rate	$c \cdot min^{-1}$	ECG, Siemens, Elema, Germany
II	Systolic arterial pressure	kPa	auscultation method, tonometer Chirana, Czechoslovakia
III	Diastolic arterial pressure	kPa	auscultation method, tonometer Chirana, Czechoslovakia
IV	Tidal volume	l	Oxycon 4; Mijnhardt, Holland
V	Respiratory rate	$breaths \cdot min^{-1}$	Oxycon 4; Mijnhardt, Holland
VI	Minute ventilation	$l \cdot min^{-1}$	Oxycon 4; Mijnhardt, Holland
VII	Oxygen uptake	$l \cdot min^{-1}$	Oxycon 4; Mijnhardt, Holland
VIII	End-tidal partial pressure of CO_2	kPa	fast CO_2 analyser Normocap, Datex, Finland
IX	Mean inspiratory flow rate	$l \cdot s^{-1}$	Fleisch pneumotachograph + Servomed Hellige, Germany
X	Absolute and relative duration of inspiration	s and fraction	Fleisch pneumotachograph + Servomed Hellige, Germany
XI	Absolute and relative duration of expiration	of respiratory cycle	Fleisch pneumotachograph + Servomed Hellige, Germany
XII	Performance	W	Cycle ergometer Elema Mingocard 3; Siemens, Germany
XIII	Duration of exercise	min	stop-watch

Table III

Cardiovascular and respiratory values before cycling

	Men	Women
Heart rate (min^{-1})	107 ± 22	90 ± 19
Systolic arter.pressure (kPa)(mmHg)(torr)	17.1±0.7(129±5)	15.7±0.5 (118±4)^{xx}
Diastolic arter.pressure (kPa)(mmHg)	10.7±0.7(80±5)	10.3±0.7 (77±5)
Tidal volume (l)	0.93 ± 0.18	0.49 ± 0.13^{xx}
Respiratory rate (min^{-1})	13 ± 3	14 ± 4
Minute ventilation ($l \cdot min^{-1}$)	12.1 ± 1.7	6.9 ± 2.5^{xx}
End-tidal part.pressure CO_2 (kPa)	4.79 ± 0.58	4.58 ± 0.48
Oxygen uptake ($ml \cdot kg^{-1} \cdot min^{-1}$)	5.4 ± 1.2	3.6 ± 1.0
Mean inspiratory flow rate ($1s^{-1}$)	0.59 ± 0.14	0.37 ± 0.14^{x}
Relative duration of inspiration (fraction)	0.35 ± 0.05	0.37 ± 0.04
Dyspnea (Borg)	0	0

Difference between men and women: ^{x} $p<0.05$
^{xx} $p<0.01$

The relationships between functional variables and dyspnea were evaluated at first by plotting the variables from Tables II, III and IV vs breathlessness. Then they were mathematically analysed according to Draper and Smith [8] and Table V.

The technique of Draper and of Smith represents a method of backward elimination where the non-significant variables are progressively eliminated at $p = 0.05$.

This process permits to calculate the regression equations and the values of correlation coefficient *r*, but, usually, it does not eliminate the interindividual differences in the response.

Andel [3] published a further mathematical process rendering possible to eliminate them as we have used it. Its details are in the mathematical appendix.

Statistical methods

The aim of mathematical procedures was to find the most significant variables (from that of Table II) that affect the breathlessness. The standard backward stepwise regression elimination (see e.g. Draper, Smith) was used for two different regression models. The first of them does not take interindividual differences among subjects into account and it is constructed as follows. The values of the j-th measurements of the i-th subject are denoted by v^{z}_{ij}, $i=1,...,11$, $j=1,...,n_i$, where n_i differs from person to person $\Sigma\, n_i = 69$. We suppose that

$$V^{0}_{ij} = c_0 + c_1 V^{1}_{ij} + ... + c_{11} V^{11}_{ij} + e_{ij}, \qquad (1)$$

where on the left hand side of (1) are the measurements of breathlessness (V^{0}_{ij}), on the right hand side are the measurements of variables from Table II (V^{z}_{ij}, $z=1,...,11$), $c_0,...,c_{11}$ are unknown

constants. After excluding the least significant variables using the mentioned backward elimination the final regression model was computed. The results are given in the Table Va.

The results in the Table Vb are computed in the same way, but the constant c_0 may differ for different individuals. That means, we use the initial regression model in the form of

$$V^0_{ij} = c^i_0 + c^1V^1_{ij} + ... + c_{11}V^{11}_{ij} + e_{ij},$$

instead of (1).

Table IV

Cardiovascular and respiratory values at maximum exercise intensity, at the end of exercise

	Men	Women
Heart rate	191 ± 11	195 ± 5
Systolic art.pressure	22.3 ± 0.3(167 ± 2)	21.6 ± 0.1(162 ± 1)^{xx}
Diastolic art.pressure	6.8 ± 0.4(50 ± 3)	7.6 ± 0.4(57 ± 3)^{x}
Tidal volume	2.88 ± 0.34	1.87 ± 0.24^{xx}
Respiratory rate	43 ± 8	46 ± 11
Minute ventilation	123.8 ± 14.2	86.0 ± 12.8^{xx}
End-tidal part.pressure CO_2	4.97 ± 0.34	4.59 ± 0.99
Oxygen uptake	50.5 ± 6.6	41.0 ± 4.1
Mean inspiratory flow rate	4.82 ± 1.5	4.20 ± 1.33
Relative duration of inspiration	0.38 ± 0.08	0.31 ± 0.04
Dyspnea (Borg)	6 ± 1.9	4.5 ± 2.3
Maximum performance ($W.kg^{-1}$)	4.6 ± 1.0	4.7 ± 0.6
Duration of exercise up to maximum(mins)	14.2 ± 1.3	13.5 ± 0.55

For units and remarks see Table III

Table V

Calculated dependence of Ψ on cardiovascular, respiratory and other values during cycling [8]

a) Without correction of interindividual differences

Ψ in the group = 0.02. V_E − 0.52.$P_{ET}CO_2$ + 0.01 systolic art. pressure + c_o

r = 0.74 c_0 = −7.58
number of observations n = 69

b) With correction of these differences

Ψ in a certain subject = 0.06.$\dot{V}_E$ − 0.11 oxygen uptake − 0.04 heart rate + 0.54 duration of exercise + 0.005 systolic art. pressure + c_o^i

r = 0.93 c_o^i = −1.6 to −6.3
n = 69

Remarks:
1. $\dot{V}_E$ = minute ventilation, for units see Tables III and IV,
   $P_{ET}CO_2$ = see VIII in Table II
   c_o and c_o^i see the mathematical appendix
2. In the model the positive correlations in the case of oxygen uptake and of heart rate are "hidden" in the other positive correlations in Table Vb

Results

The results are given in Tables III, IV and V. Table III summarizes the cardiovascular and respiratory values before cycling. The values of systolic arterial pressure, of tidal volume, of minute ventilation and of inspiratory flow rate are significantly higher in men than in women.

Before and at the beginning of the cycling no breathlessness is declared.

During incremental exercise most of measured values, with exception of end-tidal CO_2 and of diastolic art, pressure, increase. At physical load of 1 W. kg^{-1} b.w. the threshold dyspnea appears (equal to 1 point of the Borg's scale) and at physical load of 2 W·kg^{-1} Ψ doubles.

Table IV demonstrates the same parameters as Table III, but obtained at the end of maximal exercise, Ψ being 4.5 in women and 6 in men. At maximal physical

load some of the measured values e.g. of tidal volume, of minute ventilation are significantly different between men and women.

All 11 tested subjects gave their reasons for termination of maximal exercise as the sensations of general fatigue and/or pain in the lower extremities.

By plotting Ψ vs the cardiovascular and respiratory values it can be stated that Ψ increases with increase in heart rate, systolic arterial pressure, tidal volume, respiratory rate, minute ventilation, oxygen uptake, mean inspiratory flow rate, but with decrease of end-tidal CO_2 and of diastolic arterial pressure.

Dyspnea does not change with absolute or relative duration of inspiration and expiration. It becomes more important with the intensity and duration of exercise.

Table V shows the results of the regression analysis. In Table Va the interindividual differences given in Table Vb are not eliminated. Before correcting among the subjects variability we can see that Ψ depends on the values of end-tidal CO_2, on minute ventilation and on systolic arterial pressure. After correction, Ψ correlates with minute ventilation, oxygen uptake, heart rate, duration of exercise and systolic blood pressure. No correlation of Ψ was found with diastolic arterial pressure, tidal volume, respiratory rate, duration of inspiration and of expiration and intensity of physical load.

Discussion

According to our opinion this study might bring some contribution to the better understanding of the exercise dyspnea mechanisms in healthy untrained subjects and perhaps also to the better understanding of the dyspnea as a whole. We have given two aims of our study in the introduction. We suggest that we have answered clearly the first question and relatively well the second one.

The first question concerned the limits of maximum exercise in young healthy subjects. In contrast to cardiovascular and respiratory patients these limits in healthy persons are sensations of general fatigue and pain in the lower extremities. This statement is different from cardiovascular patients whose main limits are cardiac arrhythmias, conduction disturbances and ECG ischemic signs [10, 13]. This limit for respiratory patients is usually dyspnea [11, 18]. It is probably also valid for older "healthy" subjects (unpublished data).

The second problem concerning the mechanisms of dyspnea is far more complicated.

El-Manshawi et al. [9] studied them in healthy exercising subjects and calculated dependence of Ψ on values of tidal volume, on relative duration of inspiration, on respiratory rate and oesophageal pressure, but not on minute ventilation. They found a correlation with all reported values with exception of tidal

volume. Their experimental protocol and mathematical methods were very similar to ours but the authors combined physical exercise with added air-flow resistance.

If we compare our study with that of El-Manshawi et al. [9] we accentuate the calculation of correlations of Ψ with much more factors such as the intensity and duration of physical load and the cardiovascular values. And we arrived to the conclusion that Ψ was dependent not only on the values of minute ventilation reflecting probably closely the work of breathing [7, 15], but also on decrease of end-tidal CO_2, duration of physical load and on changes of systolic arterial pressure (Table V). These relationships are conceivable – the cardiovascular changes can participate in the genesis of exercise dyspnea.

In contrast to [9], the duration of inspiration does not play a major role in our experiments. In our experiments the air-way resistance was not increased, in experiments [9] it was.

We mention the possibility of elimination of among - subjects variability and we show such consequences. Elimination of significant interindividual differences by a mathematical process is somewhat artificial but leads to an increase in the values of correlation coefficient r and changes the influence of some factors. The comparison of Table Va with Table Vb makes the reader drow his own conclusion how important these differences are.

In the discussion we can give our opinion that the exercise dyspnea decreases with training in healthy subjects as well as in asthmatics [6]. It is not without interest that it decreases also after staying for 4 weeks at high altitude of 4000 m above sea level under conditions of hypoxic hypoxia [12].

One contribution of our work is indirect – it appears only from comparison with published papers [5, 16, 17]. Under conditions of increased air-way resistance and/or hypercapnia Ψ was related to the increase of end-tidal or arterial concentrations of CO_2 but during exercise (this study) or during voluntary hyperventilation (unpublished data) it is related to the decrease of CO_2. In this connection we would like to stress the plasticity of the brain processor for transformation of various inputs into sensation of dyspnea.

The study of influence of physical exercise on cardiovascular and respiratory variables and the sexual differences in the response are not new. On the contrary, this impact has been studied for many years in the Department of Sports Medicine, in Prague [14]. Results obtained on many hundreds of untrained and trained subjects and on patients of both sexes were published in the monography [14].

Most results of our study, summarised in Tables III and IV, correspond to the previous observations on healthy untrained subjects. Just the measurement of dyspnea in our laboratories has relatively recently been introduced, but in agreement with other studies [1, 2, 4, 5, 6, 9, 12, 16 and 17] it appears as a useful tool in research.

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IMPACT OF SERUM CONCENTRATION OF THE MEDIUM AND FASTING ON THE IMPRINTABILITY OF THE INSULIN RECEPTORS OF CHANG LIVER CELLS

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When the cells of the Chang cell line came into interaction with a hormone (insulin) an imprinting-like phenomenon took place. The binding capacity of the receptors strengthened and this feature was transmitted to the descendant generations. The quality of the nutrient medium influenced the development of imprinting, when the cells were maintained in a medium containing 2% serum it was more difficult to evoke imprinting than in case the cells were kept in a medium containing 10% serum. If the cells were cultured kept in Tyrode (physiological) solution for 24 hours the possibility to evoke imprinting was lost. Difference could be observed between the behaviour of receptors in nuclear membrane and that of receptors in the plasma membrane; i.e. changes were more dynamic in the plasma membrane.

Keywords: hormonal imprinting, insulin receptors, Chang liver, tissue culture, starvation

Hormone receptors of the organisms at the higher ranks of phylogeny develop on the basis of a genetically determined program; nevertheless, in the perinatal period, the receptor should be adapted to the quality and quantity of the hormone actually existing in the organism. This adaptation is called hormonal imprinting [2]. As an effect of this imprinting, the receptor reaches the binding capacity characteristic to adulthood and the cell develops the full value response capability [3, 6].

The phenomenon of imprinting can be modelled in cell and tissue cultures [4]. In the presence of the hormone, imprinting takes place *in vitro* in the cell taken from the newborn as well as *in vivo* in the newborn animal. In the case of cell lines originating from already mature organisms, however, it is the reinforcement of the previous interaction with the hormone – i.e. the interaction in the living organisms – which occurs; nevertheless, this second interaction is also imprinting-like in its

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character [7, 9]. The *in vitro* methods, however, render possible the artificial modification of the environmental conditions and, thus, allow us to gain information on the mechanism of imprinting, too.

Materials and methods

Chang liver cell line was used for the experiments. The cells were maintained in a medium containing 90% Parker 199 solution and 2% calf serum obtained from colostrum-deprived calfs. Into each H tube containing one coverslip 300000 cells were placed. Twenty-four hours later the cells in some tubes were maintained further in the above-mentioned medium, the cells of other tubes were transferred into a medium containing 10% serum and Parker's solution, while the cells of a third group of tubes were transferred into Tyrode solution. This phase of the experiment lasted for 24 hours. Then a control group was left untreated in a medium containing Parker's solution + 2% serum, while the tubes containing 2% and 10% serum or Tyrode solution were given also insulin (Insulin Semilente MC, Novo, Copenhagen, Denmark) in a final concentration of 10^{-6}M. The cells were kept in the insulin-containing medium for one hour, washed in two changes of Parker's solution and maintained for further 24 hours in a medium containing Parker 199 solution and 2% calf serum. The cell cultures were fixed with 4% formaldehyde solution dissolved in PBS, washed once with PBS and once with distilled water and incubated with FITC – labelled insulin (FITC-fluoresceine isothiocyanate isomer I, BDH, England; FITC/protein ratio, 0.43; protein concentration 0.15 mg/ml) for one hour. The intensity of fluorescence was measured by a Zeiss Fluoval cytofluorimeter attached to a Hewlett Packard 41 CX microcomputer. The microcomputer converted the analogous signals into digital ones and, on the basis of a preprogramed mathematical algorhythm, gave the mean values and the significance values calculated according to Student's *t*-test. The nucleus and the cytoplasm could be separated on the basis of the intensity of the fluorescence and, thus, could be evaluated separately.

The actual condition of the cells was evaluated following Giemsa staining of the preparates.

The experiments were repeated four times, the intensity of the fluorescence was detected in 20 cells at each occasion. So each column on the Figure represents the mean value of 80 cells.

The data obtained were evaluated with the help of the Quatro computer program, which – on the basis of the actual data constructed the Figure, too.

Results and discussion

No differences were observed as far as the morphology of the cells, the adhesion to the surface and the staining were concerned. This phenomenon is understandable in the case of the cells kept in 2% or 10% serum, since they were maintained in the same normal medium for the 24 hours prior the end of the investigation. On the other hand, the above-mentioned finding indicated that maintaining the cells for 24 hours in Tyrode solution did not lead to any irreversible alteration, since – when detected after 24 hours spent in normal medium containing 2% serum – the above-listed cell parameters were normal.

As it has been demonstrated in previous experiments [7], in the culture of Chang cells the extent of the binding in the plasma membrane was enhanced at the second interaction with insulin after previous insulin imprinting. This finding has been corroborated in the present experiments, too, since imprinting took place in the

nutrient media containing either 2 or 10% serum, meanwhile no similar change could be detected when the cells had been kept previously for 24 hours in a medium containing Tyrode solution alone (Fig. 1).

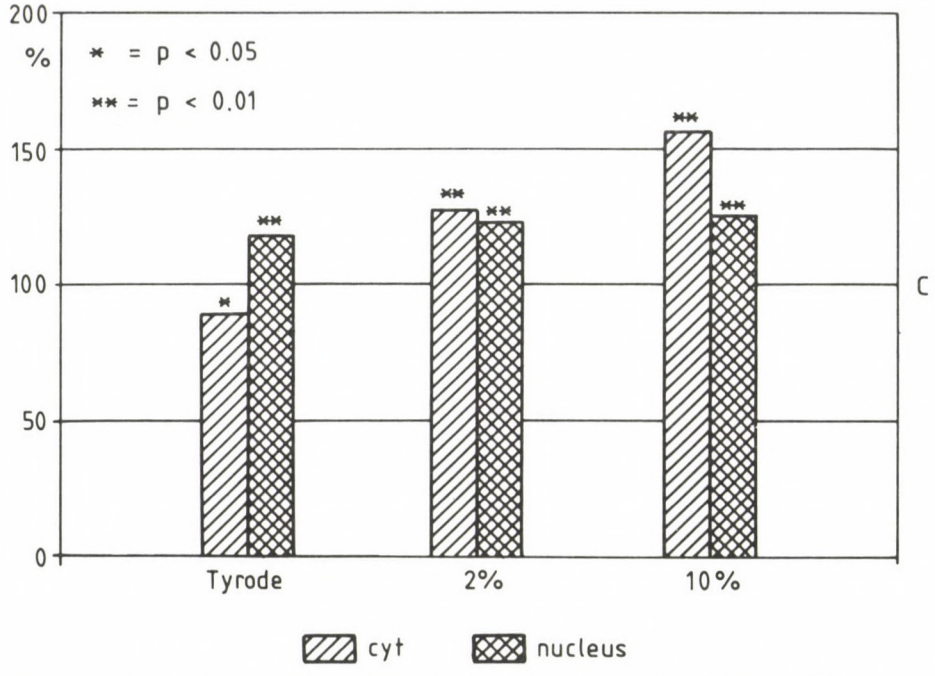

Fig. 1. FITC-insulin binding of Chang liver cells after being maintained for 24 hours in Tyrode solution, 2% and 10% serum; comparison to the controls (C)

The membrane of the cells contains proteins embedded into lipids, and among these receptors (glycoproteins) can also be found. In the fasting state the cell utilizes its own nutrient sources including the proteins. On the other hand, when the molecules serving as stimuli for the receptors are removed from the environment, then no reinforcing influence reaches the receptor; thus, the number of receptors will decrease similarly to that observed when the receptors are being used up as simple nutrient sources of the host cell. This mechanism may presumably account for the observation that no imprinting of the plasma membrane could be evoked immediately after the removal from the Tyrode solution. Nevertheless, the possibility cannot be excluded either that fasting led to damages of postreceptor mechanisms, and the maintenance of these mechanisms might probably be a prerequisite of the development of imprinting. The absence, development of intensification of imprinting in the presence of the Tyrode solution, 2% and 10% serum clearly indicated the importance of the presence of the serum as well as the important role

played by the serum concentration in the development of imprinting. Certainly, it would be all too easy to believe that only nutritive factors may play a role in the above-discussed phenomena, since – due to the uniform composition of Parker's solution – the nutritionally necessary amino acids were equally present in the medium containing 2 or 10% serum. Thus, it seems to be probable that the growth factors which are present in the serum [12], and secure the "state of wellbeing" for the cell, are also necessary for the development of *in vitro* imprinting. Nevertheless, the development of imprinting is secured even by the availability of small amounts of these factors and the nutrient.

Receptors are to be found not only in the plasma membrane but in the nuclear membrane as well [1, 10, 11, 13, 14]. Considering imprinting it is worthy to compare the behaviour of the nuclear receptors and the receptors of the plasma membrane. While the extent of the alterations of the binding capacity of the receptors of the plasma membrane depended upon serum concentrations, the binding by the receptors of the nuclear membrane remained steadily on the same level. Due to the imprinting, however, this steady level was significantly higher than that of the unimprinted controls. Thus, the effect of imprinting was manifested in the nuclear membrane as well as in the plasma membrane; nevertheless, due to environmental factors, the amount – binding capacity – of the receptors of the plasma membrane changed more dynamically than that of the nuclear membrane, which latter could be regarded as being stable.

The phenomenon of imprinting can be observed not only in organisms at the higher ranks of phylogeny but in Protozoa as well [5]. In this case the first interaction with the hormone represents the critical point when imprinting takes place. As a result of this imprinting, the receptors strengthen and this strengthening is inherited also to the progeny generations. Thus, the simple binding sites develop into specific receptors which react similarly to the receptors of the organisms at the higher ranks of phylogeny. If Protozoa are kept in physiological saline solution, the possibility to develop imprinting disappears, and it reappears only long after being transferred into normal medium [8], or to put it in another way, the imprinting takes place similarly in unicellular animals and in isolated cells of the organisms at the higher ranks of phylogeny, and not only the maintenance of the plasma membrane but appropriate nutritive conditions are prerequisites for the development of imprinting.

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THE INFLUENCE OF PERIPHERAL NERVE GRAFT'S PREDEGENERATION STAGE ON THE REGROWTH OF HIPPOCAMPAL INJURED NEURITES AND CONCOMITANT CHANGES IN SUBMICROSOMAL FRACTION PROTEINS OF GRAFTS

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The present work has a twofold aim: 1. to ascertain whether the stimulative influence of peripheral nerve grafts on injured hippocampal neurons depends on the time lapse after transection and 2. to examine whether the mentioned effect runs parallel to the time-dependent changes of proteins contents and composition in the submicrosomal fraction from transected rat sciatic nerves. Fluorescence microscope examination revealed that FITC-HRP labeled cells extending their neurites into the implanted peripheral nerve segments were particulary numerous among the hippocampal neurons when 7- and 35-day-old predegenegated distal stumps were used as grafts.

Discontinous SDS-slab polyacrylamide gel electrohporesis of submicrosomal fraction proteins obtained from distal stumps of rat sciatic nerves was performed at the 7, 14, 21 and 35 days after transection. Among the obtained protein fractions the most interesting seem to be the ones of 47 and 54 kDa, which reached maximal levels at the 7th day and the 50 kDa fraction with a maximum at the 35th experimental day. It is possible that the growth promoting power of the employed grafts depends on the presence of proper proteins.

Keywords: sciatic nerve isografts, neurite outgrowth, submicrosomal fraction's proteins, hippocampus

Neurons in the peripheral nervous system of adult mammals are able to regenerate after nerve injury and to elongate their axons over long distances. In contrast, the great majority of neurons in the central nervous system (CNS) does not regenerate after injury. Their axons do not elongate although they exhibit initial sprouting [10]. Recently successful regeneration over considerable distances was

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obtained by means of grafts consisting of segments of peripheral nerve, implanted into different regions of the CNS [1, 8, 17]. The role played by non-neuronal cells in the guidance or promotion of axonal outgrowth is poorly understood [1, 4]. Recent evidence shows, that the distal stump of transected mammalian peripheral nerve secretes factors which are able to support axonal regeneration *in vivo* [20]. All known neuronotrophic factors are proteins [16] therefore one can expect that the stimulation of neurite outgrowth depends on proteins contained in the peripheral nerve grafts. The putative source of such factors seem to be Schwann cells [1]. In the transected peripheral nerve Schwann cells divide frequently and they are the predominant cell type in the sciatic nerve grafts [13]. The intensity of cellular divisions and metabolic activity in non-neuronal components of transected peripheral nerve are not constant, but depends on the time lapse [20]. Our previous studies revealed, that predegenerated peripheral nerve grafts facilitate neurite outgrowth of damaged hippocampal neurons as compared with non-predegenerated ones [8].

The aim of the present study was to investigate whether the outgrowth of damaged central neurites depends on the time lapse after injury of the peripheral nerve used for grafting and to identify possible protein fractions in the submicrosomal fraction of rat sciatic nerve involved in these processes.

Materials and methods

Experiments were carried out on 313 adult male Wistar C rats (25 animals *in vivo* experiments and 288 rats in submicrosomal fraction analysis). During the experiment the animals received standard diet and water *ad libitum*.

In vivo experiments

In animals of the first experimental group (D7) the sciatic nerve was cut near the hip joint under intraperitoneal chloral hydrate anaesthesia (420 mg per kg b.w.). Seven days later the animals were reanaesthetized and a 10-mm long fragment of the distal stump of the sciatic nerve was dissected. Subsequently it was inserted into a glass cannula (1 mm internal and 2 mm external diameter) containing Ringer's solution for mammals. In other groups fragments of predegenerated sciatis nerves, prepared in the same way, were taken at the 14th, 21st and 35th days after injury (groups D14, D21, D35, respectively). In the control group (N) the peripheral nerve segment was dissected immediately after transection.

The peripheral nerve segments were implanted into the hippocampus according to the method described by Lewin-Kowalik [8]. Prior to the implantation an injury of 3mm depth in the brain tissue was made. The caudal tip of the graft was laid over the the skull bone, tied with 4-0 surgical silk, and fastened to the skull by means of Tissucoll Kit (Immuno AG Wien).

Six weeks after surgery all animals were anaesthetized as above and 10μl of 0,5% FITC-HRP solution, which is retrogradely transported into the cell body, was injected into the free end of the graft. 24 h later rats were perfused transcardially with 200 ml 5% sucrose in phosphate buffer pH 7.2 and subsequently with fixative solution 2,4% formaldehyde buffered as above.

Whole grafted brains were dissected from the skull and frontal frozen sections were made (Frigocut Mod. 2700 Reichert-Jung). Slices of 10 μm thickness were stained with hematoxylin-eosin (H-E), examined in fluorescence (Unival Reichert-Jung) and light microscope and photographed.

Submicrosomal fraction electrophoresis

The submicrosomal fractions were isolated from the distal stumps of adult male Wistar C rats sciatic nerves (160-200 g b.w.) 7, 14, 21 and 35 days following transection. The left sciatic nerve was cut under intraperitoneal chloral hydrate anaesthesia (420 mg/kg b.w.) near the hip joint. The right sciatic nerve remained intact and served as controls. 7, 14, 21 and 35 days after transection the animals were sacrificed by decapitation and both the intact nerves and distal stumps of transected nerves were dissected. Nerves were washed with cold 0.9% NaCl and later on in 2[N-morpholino]ethanesulfonic acid (MES) homogenizing buffer of pH 6.4, prepared according to the method of the Neuchaud et al. [3]. Subsequently the nerves were homogenized in the same buffer (3 ml per 1g nerve tissue) for 1 min (10 000 rpm) by means of a teflon-steel homogenizer. Homogenates were filtered through cotton gauze and filtrates were sedimented by centrifugation at 700 g for 10 min. Supernatants were centrifugated at 20 000 *g* for 10 min and then ultracentrifugated at 105 000 *g* for 120 min to obtain submicrosomal fractions. All preparation procedures were carried out at 0±4 C in the presence of 1mM phenyl-methyl-sulfonate-fluoride (PMSF) as protease inhibitor. The total protein concentration in the final extracts was determined by the method of Lowry [9].

Electrophoresis of submicrosomal fraction proteins were carried out on polyacrylamide slab gels following the discontinous SDS-buffer Laemmli system [7]. Each sample well was loaded with 20-40 μg protein mixed with standard sample buffer. Following electrophoresis, the gels were fixed and stained at room temperature in 0.1% (w/v) Coomassie brilliant blue R-250 (Serva) solution. Later on the gels were destained and photographed. Molecular weights of proteins in each band were estimated by the Southern method [18].

Quantification of proteins along each lane was achieved by scanning and continous plotting of the absorbance at 595 nm on a Beckman CDS-200 densitometer. Individual peaks were expressed as percentages of total area. The mean molecular weights and percentages of total area for each peak were calculated. Statistical analysis was performed by means of the nonparametric Wilcoxon's *t*-test and one-way variance analysis.

Results

Flurescence microscope examination showed that FITC-HRP labeled cells were present among the hippocampal neurons in examined brains derived from N, D7 and D35 groups (Figs 1, 2, 5). The number of these cells was however different in each group. Particulary numerous horseradish peroxidase labeled neurons were visualized in slices derived from brains treated with grafts obtained from 7 days predegenerated distal stump (Fig. 2a). Cell bodies containing marker were regularly dispersed around the tip of the graft. Number of labeled cells was 5 times higher than in slices obtained from nerves treated with freshly transected nerves (Figs 1a, 2a).

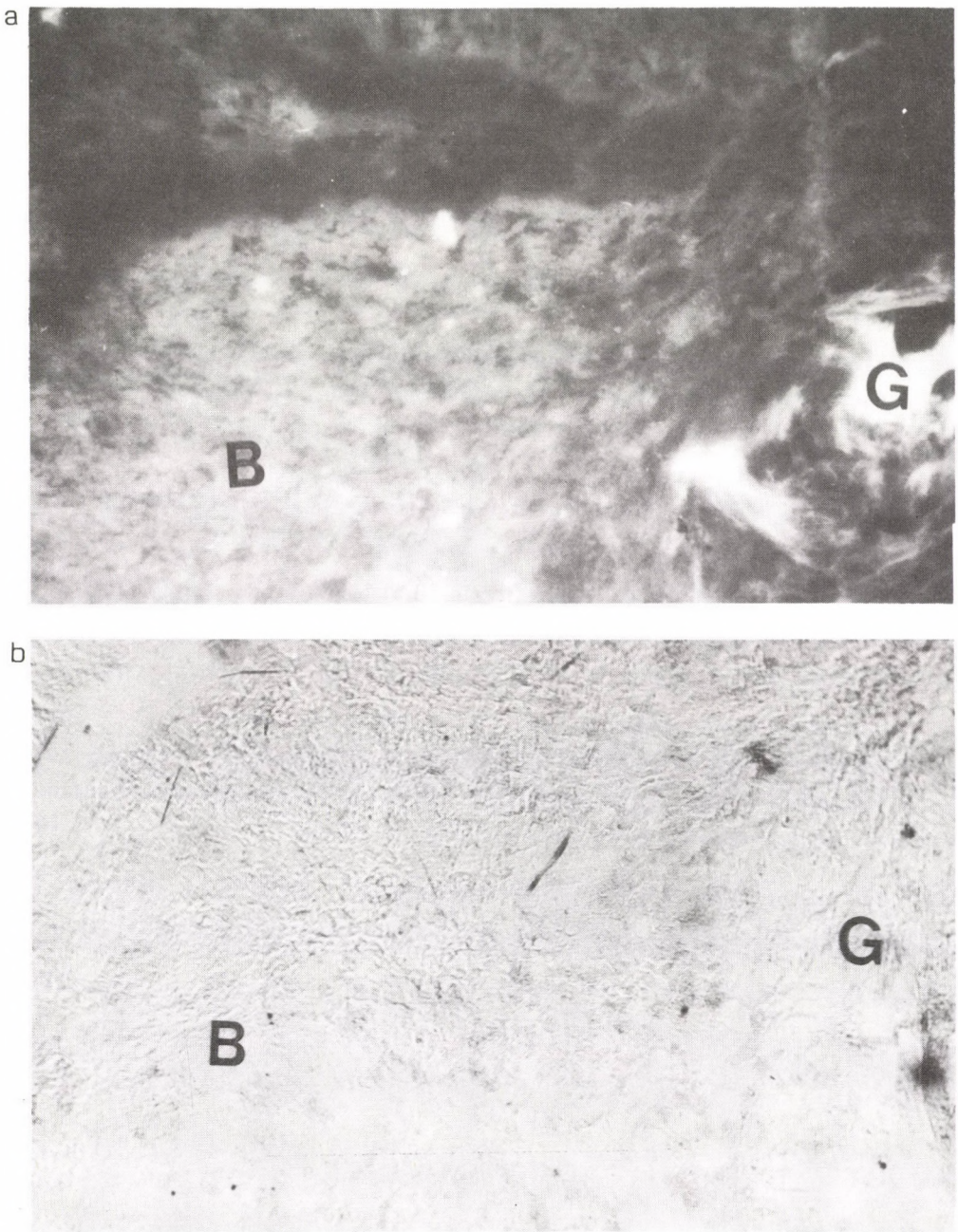

Fig. 1. The site of implantation of control non-predegenerated PN-graft 6 weeks after grafting. a) Fluorescence microscope dark field picture (×250). Only single FITC-HRP labeled cells are seen in the brain tissue (B) near the tip of the graft (G). b) The same site in the hematoxylin-eosin stained slice in light microscopic picture (×250)

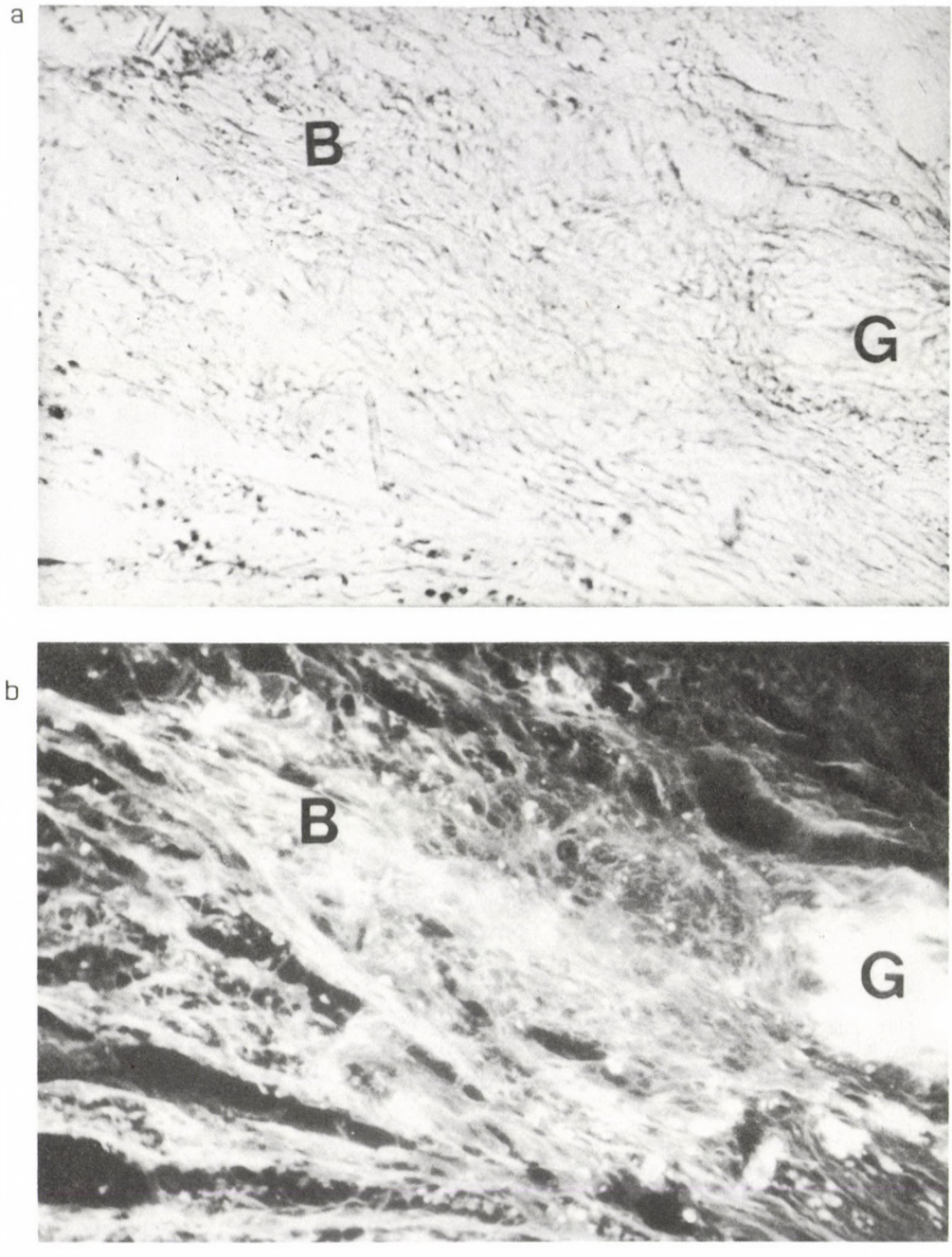

Fig. 2. The site of implantation of 7 days predegenerated PN-graft 6 weeks after grafting. a) Fluorescence microscope dark field picture (×250). Numerous FITC-HRP labeled cells are visualized in the brain tissue (B) around the tip of the graft (G). b) The same hematoxylin-eosin stained preparation in the light microscopic picture (×250)

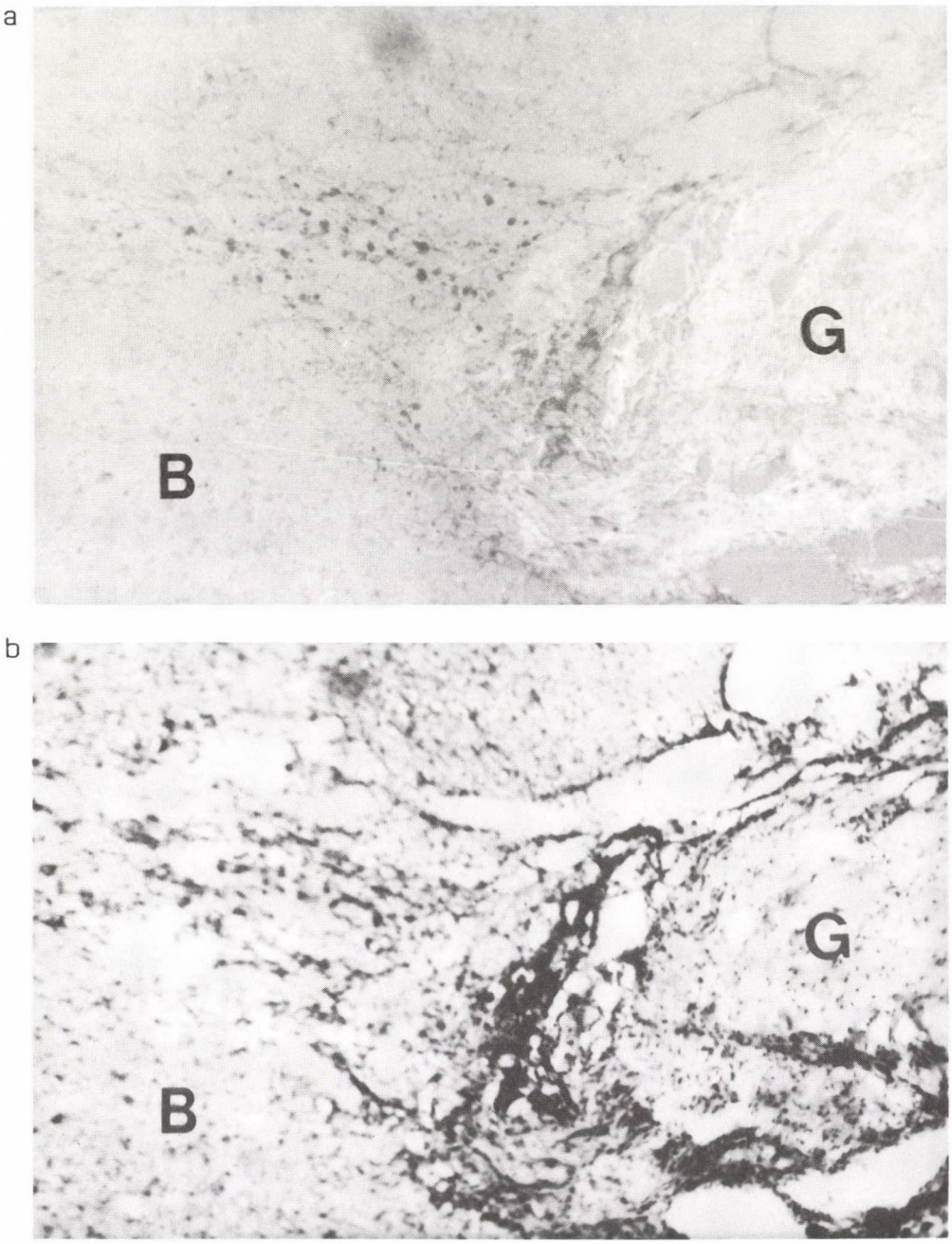

Fig. 3. The site of implantation of 14 days predegenerated PN-graft 6 weeks after grafting. a) Fluorescence microscope dark field picture (×250). FITC-HRP labeled cells are absent in the brain tissue (B) near the tip of the graft (G). b) The same site in the hematoxylin-eosin stained slice in light microscopic picture (×250)

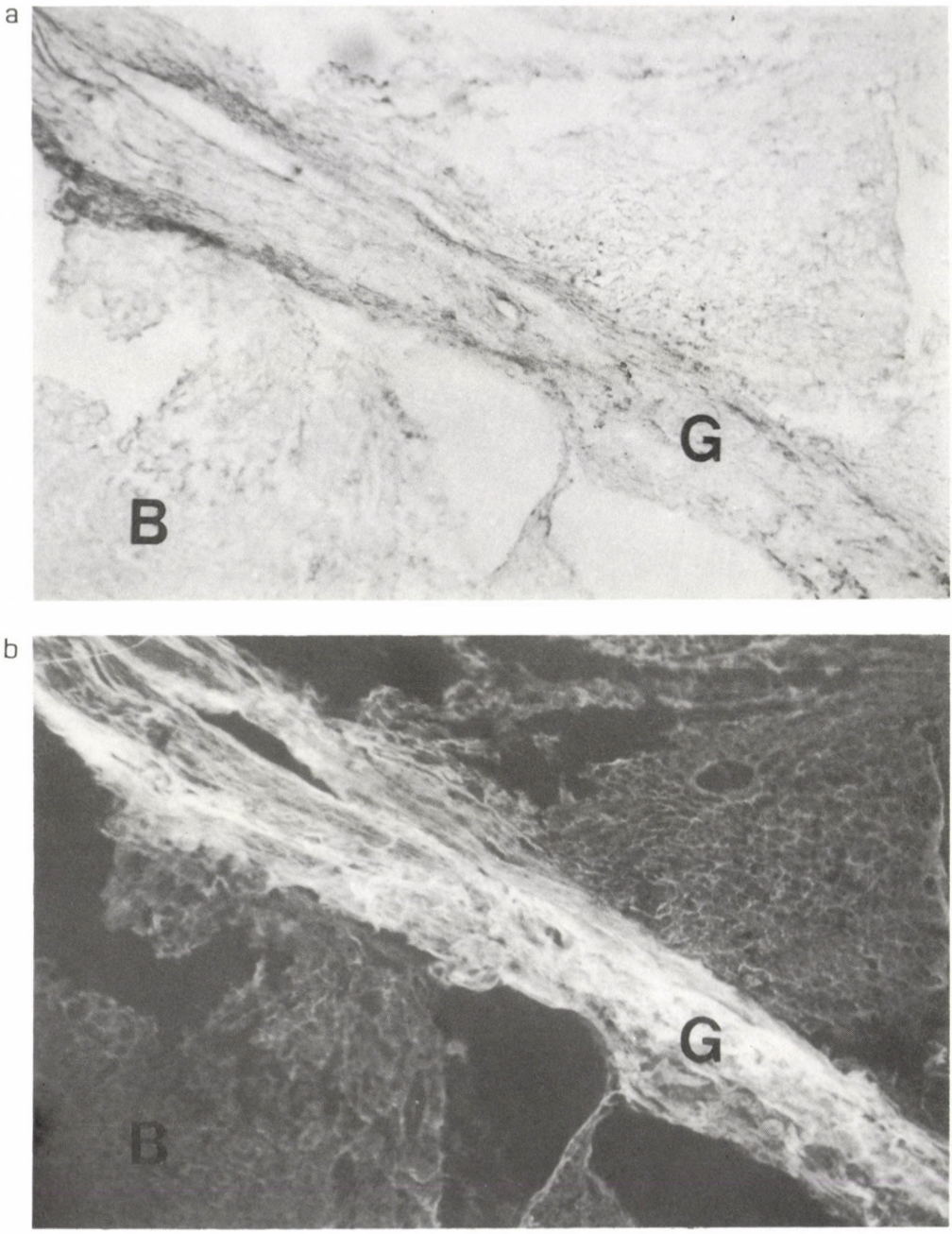

Fig. 4. The site of implantation of 21 days predegenerated PN-graft 6 weeks after grafting. a) Fluorescence microscope dark field picture (×250). FITC-HRP labeled cells are absent in the brain tissue (B) around the tip of the graft (G). b) The same hematoxylin-eosin stained preparation in the light microscopic picture (×250)

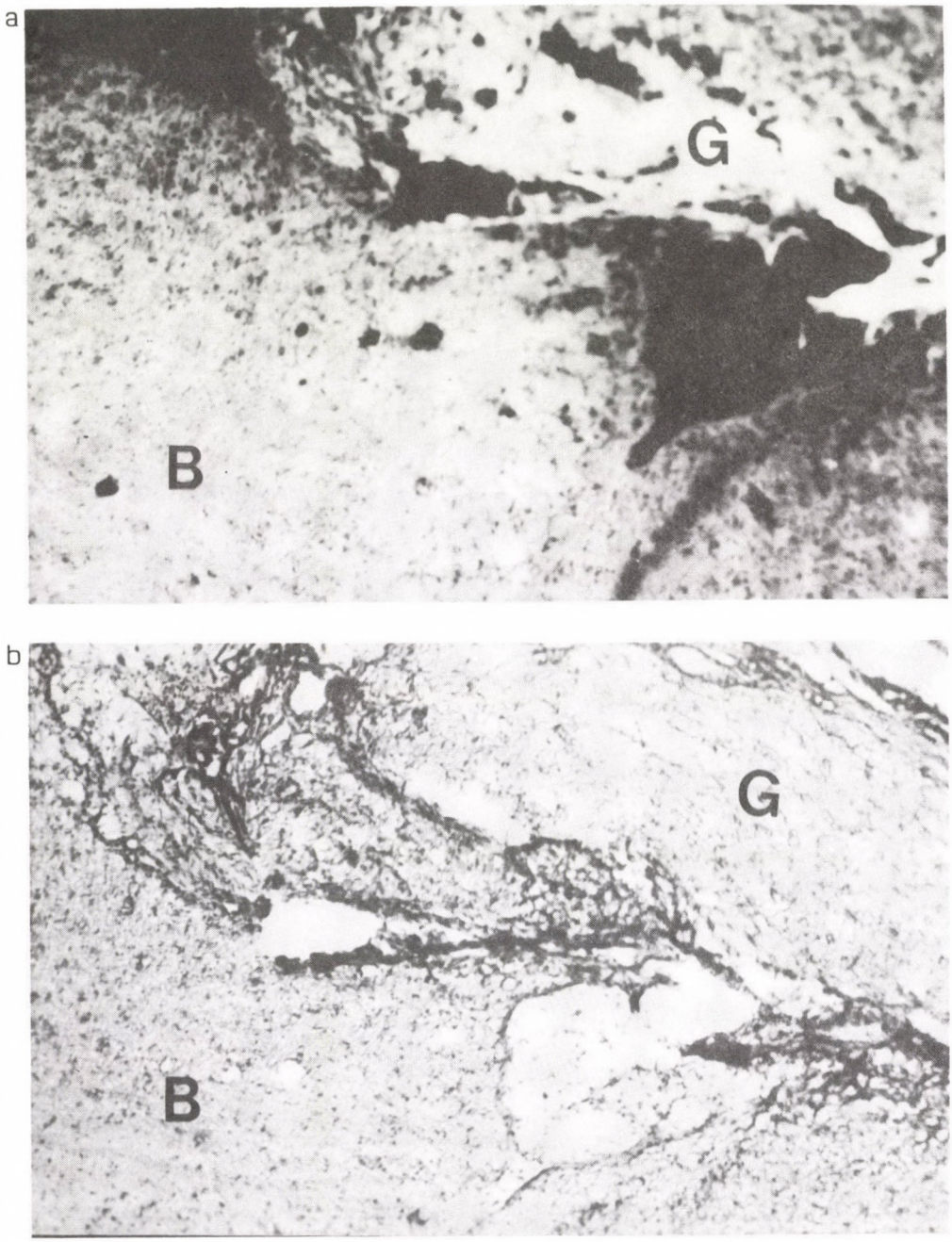

Fig. 5. The site of implantation of 35-day predegenerated PN-graft 6 weeks after grafting. a) Fluorescence microscope dark field picture (×250). FITC-HRP labeled cells are visualized in the brain tissue (B) around the tip of the graft (G). b) The same hematoxylin-eosin stained preparation in the light microscopic picture (×250)

In the brains slices from D14 and D21 groups there were no FITC-HRP labeled cells in the neighbourhood of the graft's tip (Figs 3, 4).

Horseradish peroxidase labeled cells appeared again in D 35 slices, but their number was lower than in the control group (Fig. 5).

The total protein concentrations in submicrosomal fractions in the distal stumps were higher than in controls (Table I). Maximal concentration of protein per 1g nerve tissue was observed in the distal stumps at the 14th day after transection. Proteins from intact and transected nerves were resolved into 28 and 27 bands respectively whose apparent molecular weights ranged between 16-335 kDa. In the distal stump the 34 kDa fractions were undetectable (Table II, Fig. 6).

Table I

Total protein concentration in submicrosomal fractions from transected rat sciatic nerves

Segment of the nerve	Days after transection			
	7	14	21	35
intact	1.31 ± 0.81	1.31 ± 0.81	1.31 ± 0.81	1.31 ± 0.81
distal	3.21 ± 2.98	4.80 ± 2.17	3.26 ± 2.55	3.66 ± 1.64

Numbers denote concentrations of proteins in mg of protein (± SD) per 1g of nerve tissue

In the distal stump at the seventh day following section, four protein fractions were significantly changed: 47, 54, 65, and 69 kDa. Only the concentration of the last fraction was lower than the control. The remaining fractions were higher. Fraction 59 kDa was at this period undetectable (Table II).

At the fourteenth day after transection fraction 69 kDa diminished, fraction 59 kDa appeared again, but at a lower level than in the control. Protein fractions with molecular weight of 36, 50 and 65 kDa increased and the last one reached its maximum (Table II).

Fraction 59 kDa was again undetectable at the 21st postoperative day. Fractions 65 and 50 kDa were higher than in the control, whereas 23 kDa protein was present in a small amount only (Table II).

At the 35th day following sciatic nerve injury the fractions 80, 20, 19, 17, and 16 kDa were undetectable. In higher concentrations than in the control there were 65 and 50 kDa proteins, moreover the last fraction reached at this time its maximum. The amount of the 23 kDa protein was significantly lower as compared to the control (Table II).

Table II

Percentages of protein content in electrophoretograms of submicrosomal fractions from distal stumps of rat sciatic nerves at 7th, 14th, 21st and 35th day following transection

No.	M.W. [kDa]	%				
		Days after transection				
		control	7	14	21	35
1	335 ±16	1.47±0.72	2.42±2.50	1.73±1.33	0.47±0.56	1.68±1.13
2	236 ±10	0.38±0.56	0.23±0.38	0.43±0.40	1.10±1.27	0.40±0.63
3	183 ±6	0.38±0.66	0.40±0.45	0.50±0.84	0.22±0.45	0.32±0.50
4	161 ±5	0.20±0.32	0.45±0.37	0.47±0.44	0.52±0.61	1.02±1.22
5	139 ±6	0.57±0.78	0.77±0.39	1.35±1.39	0.40±0.47	0.65±0.51
6	120 ±5	0.83±1.53	1.38±0.49	0.57±0.91	0.85±0.63	0.27±0.31
7	99 ±4	0.87±0.56	0.70±0.76	1.97±1.27	0.22±0.45	0.43±0.39
8	80 ±2	0.72±1.11	0.58±0.79	0.60±0.94	5.80±6.20	–
9	75 ±2	1.62±1.68	1.62±1.68	1.97±0.84	3.75±1.22	0.42±0.84
10	69 ±2	8.93±4.00	3.78±4.00*	1.72±0.92*	10.00±7.05	8.93±5.34
11	65 ±1	2.37±1.25	12.70±2.55*	15.12±4.38*	7.85±4.30*	9.15±3.63*
12	59 ±2	2.53±1.17	–	0.80±0.90*	–	4.93±4.30
13	54 ±1	2.52±1.39	4.72±1.09*	3.65±1.32	5.47±3.78	8.28±6.48
14	50 ±1	2.13±0.76	3.93±2.34	5.47±3.35*	4.20±1.53*	7.80±4.55*
15	47 ±1	2.90±2.49	6.52±1.88*	6.50±3.66	6.62±4.77	7.90±5.51
16	42 ±1	4.03±3.45	5.73±1.44	4.60±3.20	5.35±4.65	8.60±4.01
17	38 ±1	2.15±1.19	3.40±1.40	5.23±4.86	1.47±2.56	2.93±1.34
18	36 ±0.5	3.07±2.23	4.73±1.38	5.95±1.36*	6.05±4.91	4.53±1.12
19	32 ±1	5.63±4.44	4.18±1.97	8.08±4.29	4.47±2.20	11.83±5.06
20	25 ±0.5	11.43±7.17	8.48±2.48	8.35±6.08	8.20±7.37	5.13±5.57
21	24 ±0.5	2.83±1.90	5.85±3.11	4.18±2.13	6.32±3.38	1.88±0.77
22	23 ±0.5	3.80±1.47	7.05±4.04	2.30±0.93	0.77±0.90*	1.20±1.05*
23	21 ±0.5	1.63±1.17	3.78±2.02	2.18±1.90	1.07±0.62	0.42±0.45
24	20 ±0.5	2.08±1.74	2.40±2.21	2.43±1.39	1.47±1.13	–
25	19 ±0.5	1.64±1.74	1.25±0.89	1.65±0.61	0.45±0.33	–
26	17 ±0.5	1.30±1.64	1.40±1.55	0.93±1.62	0.25±0.30	–
27	16 ±0.5	1.93±1.73	1.02±1.66	1.53±1.07	1.07±0.99	–

Significant changes ($p<0.05$), as compared to control, are marked by *.

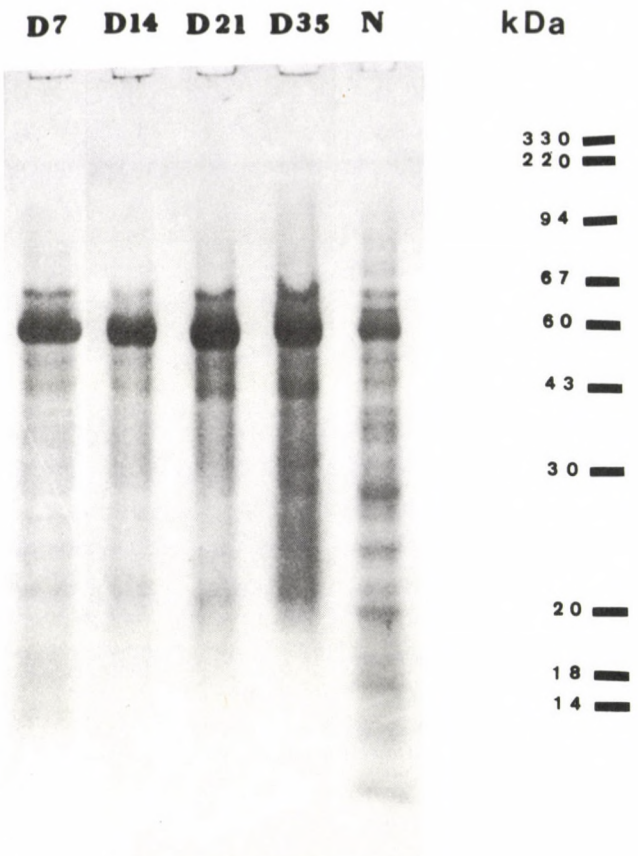

Fig. 6. Electrophoretograms of submicrosomal fractions from distal stumps of rats sciatic nerves 7, 14, 21 and 35 days following transection. Track N contains submicrosomal fraction from control intact nerves

Discussion

The presented results indicate, that despite of continous division of Schwann cells in the distal stump, this part of the nerve exhibit neuronotrophic activity towards CNS neurites. The mechanism of the influence of peripheral nerve grafts on the central fibres regeneration is still undefined. Two of the most prominent components of peripheral nerve trunks, the extracellular matrix (ECM) and the Schwann cells, have been implicated as agents in promoting this regeneration [5, 6, 14]. The neuronotrophic nature of our grafts is not constant, but changes during degeneration and regeneration. Particularly effective influence of grafting was observed when 7

and 35 days predegenerated grafts were implanted. Our observations obtained *in vivo* corroborate with the results reported by Windebank and Poduslo i.e. that extracts isolated from distal stumps immediatly after crush and on the 28–35th days following injury stimulate axon growth in tissue culture [20]. In our experiments similar biphasic effect has been observed, however the early (7-day) phase seems to be the more effective.

The question arrises, what changes of proteins fractions in the peripheral nerve segments used for implantation are responsible for the observed regrowth of injured hippocampal neurites. We are unable to answer this question completely, because of the relatively simple methods used for protein analysis. It is however temptating to look for substances with putative neuronotrophic activity within the groups of proteins which augment particulary at the 7th and also at 35th day following transection (Table II and Figs 3 and 4). At the 7th and 35th days after section only the amounts of the 47, 54 and 50 kDa protein fractions were significantly higher than in control nerves.

Valentini et al. showed that regeneration is impeded if molecules greater than 50 kDa are prevented from reaching the peripheral nerve [19]. Our observations agree with these results because fractions of molecular weight higher than 50 kDa exhibited the greatest changes at the 7th and 35 days after transection.

For the mentioned reasons we suppose that neuronotrophic activity observed in our experiments depends on the specified protein fractions. On the basis of the facts presented here, in the future we intend to investigate the efficacy of individual protein fractions obtained from the 7- and 35-day predegenerated peripheral nerves for regrowth stimulation of CNS injured axons. The identification of the molecular mechanisms underlying these cellular events seems to include the main key to the development of strategies for the repair of the damaged central nervous system.

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THE EFFECT OF DIFFERENT PROSTAGLANDINS ON GASTRIC MUCOSAL INTRACELLULAR SECOND MESSENGER SYSTEM IN THE RAT

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After an oral administration of 100 μg/kg dose, the investigated prostaglandins: – $PGF_{2\alpha}$, PGE_2 and a synthetic PGE_2 derivative: FCE-20700, – exerted a significant effect on cAMP and cGMP content of both parts (antral and fundic) of gastric mucosa, resulting in an elevated cAMP/cGMP ratio, while 6-keto-$PGF_{1\alpha}$, the stable break-down product of prostacyclin, was inactive.

Since the above-mentioned phenomenon seems to be proportionate to the cytoprotective (anti-ulcerogenic) property of the investigated prostaglandins, this cAMP/cGMP ratio "shift" is interpreted as a probable (molecular) sign of the reparative, (anti-ulcerogenic) processes.

Keywords: gastric mucosa, prostaglandins, cAMP, cGMP, cytoprotection, rat

It was reported earlier that prostacyclin (PGI_2) exerting its cytoprotective (anti-ulcerogenic) property in rat gastric (antral and fundic) mucosa, acts via the intracellular second messenger system [2, 3].

Considering that other PG-analogues are also cytoprotective, it seemed to be worth to study whether other PGs activate the same system?

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Materials and methods*

Twenty-four groups of adult female Wistar rats (n = 5/group) weighing 215-235 g were used. Prior to experiments the animals were fasted for 24 h but water was available *ad libitum*.

PGs were given orally (by a gastric tube) in a single dose of 100 μg/kg, freshly dissolved in 0.05 M Tris-buffer, pH 9.6.

The following PGs were investigated:

$PGF_{2\alpha}$, PGE_2, 6-keto-$PGF_{1\alpha}$ and FCE-20700, a PGE_2-analogue, (11-deoxy-13,14-didehydro-16(S)-methyl-PGE_2-methylester,) manufactured by Farmitalia Carlo Erba (Milan, Italy) [1, 7].

The control animals received Tris-buffer only, in appropriate amounts by gastric tube.

1, 5, 15, 30 and 60 min following treatment the animals were killed and their stomach was removed. The fundic (oxyntic cell area) and antral mucosae were separately scraped down and homogenized in ice-cold Tris-EDTA-buffer.

Emphasis was put on rapid processing and the whole procedure was carried out in an ice-bath.

The cAMP and cGMP contents of the homogenizate have been determined by radioimmunoassay, according to the manufacturer's instructions, using TRK-432 (Lot 52A) and TRK-500 (Lot 19C) kits of Amersham International (Amersham, U.K.) respectively.

The reproducibility and intraassay variation for cAMP and cGMP measurements showed a coefficient of variation less than 11% over the range 0.2-16.0 pmol/50 μl sample of cAMP, and 100 μl cGMP, respectively. These data are in accordance with the manufacturer's data.

The cyclic nucleotide content was expressed as pmol/mg protein. The protein content of the samples has been estimated with Lowry's method [8].

Within each animal group mean ± SEM was calculated and analyzed statistically using Student's *t*-test. Significant differences (vs the control) were assumed when the probability was less than 5%.

Results

The experimental results are presented in the Tables I, II and III.

On the basis of the investigations performed it seems that an effect of different PGs on the gastric (antral and fundic) mucosal cyclic nucleotide turnover is a general property of all (biologically effective) PGs.

$PGF_{2\alpha}$ exerted a slowly developing decrease of cAMP in both parts of gastric mucosa – which after 60 min turned over in an increase of the cAMP level. The influence on the cGMP levels – although less impressive – showed a similar pattern, resulting in a significant elevation of the cAMP/cGMP ratios. (15^{th} and 30^{th} min, respectively.)

**List of abbreviations:*
cAMP: Adenosine-3'-5'-cyclic Monophosphate
cGMP: Guanosine-3'-5'-cyclic Monophosphate
PG: Prostaglandin

Table I

The effect of different prostaglandins on gastric mucosal cAMP-content in the rat

Group	cAMP pmol/mg protein	
	Antrum	Fundus
$PGF_{2\alpha}$		
Control	7.918 ± 1.271	4.948 ± 0.526
1 min	6.071 ± 1.290	6.012 ± 1.958
5 min	6.489 ± 1.721	4.732 ± 1.416
15 min	1.540 ± 0.440^{++}	2.716 ± 0.398^{+}
30 min	9.998 ± 2.366	3.642 ± 1.286
60 min	13.906 ± 3.505^{+}	7.654 ± 0.988^{+}
PGE_2		
Control	8.630 ± 0.492	5.563 ± 0.442
1 min	30.156 ± 3.019^{+++}	15.326 ± 1.430^{++}
5 min	14.004 ± 1.778^{+}	12.690 ± 1.322^{+}
15 min	15.934 ± 2.003^{+}	22.314 ± 2.817^{++}
30 min	20.632 ± 2.116^{+}	16.778 ± 1.870^{+}
60 min	12.311 ± 1.625^{+}	11.070 ± 0.932^{+}
FCE-20700		
Control	8.834 ± 0.493	5.980 ± 0.366
1 min	34.106 ± 4.319^{+++}	22.071 ± 1.835^{++}
5 min	15.724 ± 1.988^{+}	14.159 ± 1.488^{+}
15 min	21.931 ± 2.457^{++}	35.758 ± 5.369^{+++}
30 min	31.511 ± 4.673^{+++}	13.640 ± 1.641^{+}
60 min	15.093 ± 1.776^{+}	24.982 ± 3.080^{++}
6-keto-$PGF_{1\alpha}$		
Control	8.123 ± 1.426	5.038 ± 0.319
1 min	7.952 ± 2.060	4.945 ± 0.687
5 min	8.374 ± 2.806	6.045 ± 2.143
15 min	6.334 ± 1.311	6.786 ± 1.370
30 min	13.496 ± 2.015^{+}	7.298 ± 0.944
60 min	8.515 ± 2.548	4.749 ± 0.923

$+ = P < 0.05$, $++ = P < 0.01$, $+++ = P < 0.001$

Table II

The effect of different prostaglandins on gastric mucosal cGMP-content in the rat

Group	cGMP pmol/mg protein	
	Antrum	Fundus
$PGF_{2\alpha}$		
Control	1.386 ± 0.084	0.665 ± 0.060
1 min	0.999 ± 0.190	0.853 ± 0.192
5 min	1.421 ± 0.154	0.782 ± 0.217
15 min	0.608 ± 0.192	0.148 ± 0.048^{++}
30 min	0.922 ± 0.309	1.044 ± 0.326
60 min	3.506 ± 1.307^{+}	2.250 ± 0.647^{+}
PGE_2		
Control	1.553 ± 0.146	0.679 ± 0.058
1 min	0.830 ± 0.209	0.406 ± 0.097
5 min	0.774 ± 0.172^{+}	0.384 ± 0.092^{+}
15 min	1.324 ± 0.214	0.566 ± 0.113
30 min	1.032 ± 0.074^{+}	0.356 ± 0.083^{+}
60 min	1.426 ± 0.116	0.429 ± 0.095
FCE-20700		
Control	1.638 ± 0.321	0.718 ± 0.177
1 min	0.692 ± 0.172^{+}	0.358 ± 0.082^{+}
5 min	0.696 ± 0.202^{+}	0.363 ± 0.094^{+}
15 min	2.258 ± 0.317	0.621 ± 0.153
30 min	0.351 ± 0.004^{+++}	0.196 ± 0.061^{++}
60 min	1.616 ± 0.336	0.256 ± 0.063^{++}
6-keto-$PGF_{1\alpha}$		
Control	1.432 ± 0.281	0.716 ± 0.173
1 min	1.604 ± 0.223	1.228 ± 0.253
5 min	1.206 ± 0.326	1.212 ± 0.288
15 min	1.438 ± 0.357	1.464 ± 0.226
30 min	2.380 ± 1.106	0.820 ± 0.051
60 min	1.990 ± 0.414	0.868 ± 0.134

$+ = P < 0.05$, $++ = P < 0.01$, $+++ = P < 0.001$

Table III

The effect of different prostaglandins on gastric mucosal cAMP/cGMP ratio in the rat

Group	cAMP/cGMP	
	Antrum	Fundus
$PGF_{2\alpha}$		
Control	5.71	7.44
1 min	6.08	7.05
5 min	4.57	6.05
15 min	2.53	18.35
30 min	10.84	3.49
60 min	3.97	3.40
PGE_2		
Control	5.57	8.19
1 min	36.33	37.75
5 min	18.09	33.05
15 min	12.03	39.42
30 min	19.99	47.13
60 min	8.63	25.80
FCE-20700		
Control	5.39	8.33
1 min	49.29	61.65
5 min	25.59	39.01
15 min	9.71	57.59
30 min	89.78	69.59
60 min	9.34	97.59
6-keto-$PGF_{1\alpha}$		
Control	5.67	7.04
1 min	4.96	4.03
5 min	6.65	4.99
15 min	4.40	4.64
30 min	5.67	8.90
60 min	4.28	5.47

PGE_2 evoked a significant cAMP elevating effect in both parts of gastric mucosa, – while its effect on cGMP levels were an opposite one. The result was an elevation of the cAMP/cGMP ratio in the antral mucosa, which returned to normal after 60 minutes. At the same time PGE_2 caused a long-lasting and more pronounced elevation in the fundus.

FCE-20700, the synthetic PGE_2-analogue, showed similar properties as its mother-derivative but its effect was more significant than that of PGE_2.

6-keto-$PGF_{1\alpha}$ the stable break-down product of prostacyclin, practically showed no effect on the gastric mucosal cyclic nucleotide turnover.

Discussion

It is widely accepted that prostacyclin is one of the most active PGs so far, as its cytoprotective (anti-ulcerogenic) effect is concerned [5, 9, 10].

It has been shown [2, 3, 4] that prostacyclin significantly elevates the cAMP/cGMP ratios of rat's gastric mucosa.

Moreover, the H_2-receptor blocker cimetidine, the potent anti-ulcer drug, also exerts the same effect, as prostacyclin [6].

Therefore it was concluded that a rise in the cAMP/cGMP ratio might be one of the indicators of the anti-ulcerogenic, reparative processes in the gastric mucosa.

According to our present investigations, the different PGs exerted a cAMP/cGMP ratio elevating ("shifting") effect in the order of their anti-ulcerogenic, cytoprotective property.

It is known that PGE_2 is a very potent cytoprotective substance, – and among the presently investigated PGs this material (together with its synthetic derivative, FCE-20700) was the most effective drug also.

$PGF_{2\alpha}$ showed only a transient shifting of the cAMP/cGMP ratios, reflecting that this PG-derivative has only a slight anti-ulcerogenic effect [11, 12].

6-keto-$PGF_{1\alpha}$ was virtually ineffective. As it has been described [13] this PG is biologically inactive.

The physiological and/or pathological relevance of the changes in the above mentioned cAMP/cGMP ratio is not yet fully understood. Evidences accumulated in the literature show that this "shift" seems to be in close connection with cytoprotection and anti-ulcerogenic drug effect. Further investigations are needed to elucidate all the details of the mechanisms involved in this phenomenon.

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EFFECT OF INTRAVENOUS CIMETIDINE, RANITIDINE AND PENTAGASTRIN AND INTRAGASTRIC PROSTAGLANDIN E_1 TREATMENTS ON GASTRIC TRANSMUCOSAL POTENTIAL DIFFERENCE IN THE RAT

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Effects of intravenous cimetidine, ranitidine and intragastric prostaglandin E_1 (alprostadil) treatments on the transmucosal potential difference (PD) of the stomach were compared. It was also investigated whether the above-mentioned drugs influenced the decrease of PD which followed both intragastric administration of 30% alcohol or Ca^{++} solution in 5 Mm final concentration and intravenous administration of pentagastrin. Both cimetidine and ranitidine treatments led to significant ($p < 0.05$) increase of PD, the effect of ranitidine was dose dependent. Prostaglandin E_1 in a dose of 40 μg/kg led to significant decrease of PD (< 0.05). Both intragastric administration of prostaglandin E_1 in a dose of 40 μg/kg and intravenous administration of ranitidine in a dose of 10 mg/kg significantly diminish the effect of Ca^{++} and alcohol to decrease PD. Neither prostaglandin E_1, nor ranitidine pretreatment had any effect on the rapid and highly significant ($p < 0.01$) decrease of PD following i.v. pentagastrin administration. It is hypothesized that transmucosal PD of the stomach provides information not only on the actual condition of the mucosal barrier but on the electrophysiology of gastric secretion as well.

Keywords: cimetidine, ranitidine, pentagastrin, PGE_1, gastric, transmucosal potential difference, Ca, alcohol

Transmucosal PD of the gastric mucous membrane represents a sensitive numerical index and is accepted as a marker characteristic [2, 13, 28]. When the barrier function is intact, the PD is stable and the luminal surface of the mucosa is electronegative compared to the serosal surface [5, 13, 23]. However, under

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influences which functionally or structurally damage the mucosa PD decreases [8, 25, 26]. Unequivocal data of both animal and human studies indicate that H_2 receptor inhibitors enhance transmucosal PD [1, 9, 10, 11, 12, 18, 31]. The effect of topically administered prostaglandins on the electrophysiology of the gastroduodenal mucous membranes has not been clarified in detail so far. Intravenous administration of pentagastrin leads to rapid and marked decrease of PD both in humans and in animal experiments [29, 30, 31]. The exact mechanism of this marked electrophysiological alteration of the gastric mucous membrane is still unknown.

The aim of the present animal experiment was to compare the effects of the intravenous administration of the H_2 receptor inhibitor cimetidine and ranitidine and the intragastric administration of prostaglandin E_1 (PGE_1) on transmucosal PD. It was also investigated whether the above-mentioned drugs influenced the decrease of PD following intragastric administration of such mucosa-damaging agents like alcohol and Ca^{++}.

Material and methods

The experiments were carried out on CFY male rats (LATI, Gödöllő, Hungary), weighing 150 to 250 g. The animals were kept fasting for 24 h but water was given *ad libitum*. The abdomen was opened under intraperitoneal inactin anaesthesia (0.1g/kg), the cardia was ligated and proximal duodenostomy was performed 0.2 cm below the pyloric ring. The stomach was filled up via the duodenostomy with 3 ml of physiological saline solution. The recording electrode was inserted into the duodenum. The reference electrode was placed into the peritoneal cavity filled up with physiological NaCl solution. Polyethylene cannulae (PP 30) filled up without air contamination with 3% agar gel containing 4 M KCl saturated with AgCl served for recording and reference electrodes. Both the recording and the reference electrodes led to one-one glass vessel containing 4 M KCl. Ag-AgCl calomel electrodes (Radelkis, OP 833) submerged into the vessels. The calomel electrodes were connected to a mV-detector equipment (Radelkis, OP 208). The PD values were recorded digitally with the help of an MTA-Kutesz type recorder. Calibration of the deflections appearing on the paper was carried out both before and after the experiments, with the help of a device which was submerged into the KCl solution and provided standardized direct current [6].

Transmucosal PD was measured continuously throughout the investigation.

Intravenous and/or intragastric administration of the drugs and chemical substances was started 30 minutes after the beginning of the experiment, i.e. after the stabilization of the basal PD value. The intravenous injections were given into the femoral vein.

The experiments were carried out according to the following set-up, with the bellow-listed substances.

Investigation

- on the mucosal electrophysiological (PD) effect of intravenously administered H_2 receptor inhibitors: transmucosal PD was measured in 8–8 animals after intravenous administration of Ranitidine (Glaxo) in doses of 1 mg/kg and cimetidine (Richter Gedeon) in a dose of 100 mg/kg;
- on the effect of intragastric prostaglandin E_1 (Alprostadil, Upjohn): PGE_1 was given to 7–7 animals in doses of 0.4, 4 and 40 μg/kg intragastrically;

- on the effect of intravenous pentagastrin: 100 μg/kg pentagastrin (GERMED) was given i.v. to 7 animals;
- on the effect of 30% alcohol given intragastrically either alone or after pretreatment with 40 μg/kg i.g. PGE_1 (7 animals/group);
- on the effect on PD of Ca^{++} solution given in an intragastric final concentration of 5 Mm either alone or after i.g. pretreatment with PGE_1in a dose of 40 μg/kg, or after pretreatment with ranitidine in a dose of 10 mg/kg (7 animals/group);
- on the effect on PD of i.v. pentagastrin administered in a dose of 100 μg/kg either alone or after i.v. pretreatment with ranitidine in a dose of 10 mg/kg, or after intragastric pretreatment with PGE_1 in a dose of 40 μg/kg (7 animals/group).

The animals were studied in the control (untreated) group.

The mean values (± SEM) of PD are given at the evaluation of the data.

For the statistical analysis the paired and unpaired *t*-test were used.

Results

Increase of transmucosal PD was detected after the i.v. administration of cimetidine. In the 10th minute following the drug exposure, the mean of the PD values was significantly higher than the mean of the baseline values. Thereafter, by the end of the 35-minute-long follow-up period, PD values remained practically unchanged when it was compared to the mean PD value of the untreated control group, however, the difference was still significant.

Only one dose of cimetidine was investigated, since the aim of the present experiments was the comparison to the electrophysiological effect of the other well-known H_2 receptor inhibitor, i.e. ranitidine. The dose of cimetidine was chosen according to the data of Scarpignato et al. [17]; namely, as to the observation that in Shay rats it was the 100 mg/kg dose of cimetidine which led to 50% reduction of acid secretion.

Both the 1 mg/kg and the 10 mg/kg doses of ranitidine led to significant increase of PD. As early as 5 minutes after the administration of the higher, the mean PD value was significantly higher, than the mean of the baseline values.

The enhancement of PD following ranitidine administration proved to be dose-dependent. In the 35th minute of the experiment, the mean PD value was significantly higher in the animals treated with the 10 mg/kg dose of ranitidine than in those receiving either ranitidine in a dose of 1 mg/kg or cimetidine therapy (Fig. 1).

Both the 0.4 and the 4 μg/kg doses of prostaglandin E_1 can be regarded as being cytoprotective, in contrast to the effect of the 40 μg/kg dose to decrease acid secretion.

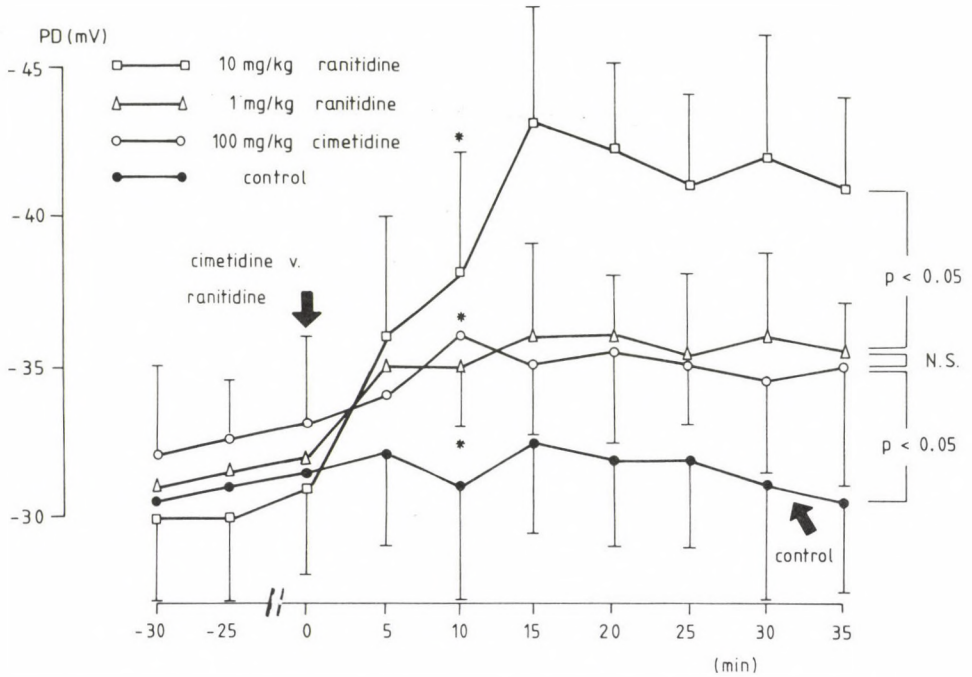

Fig. 1. Effect of intravenous cimetidine and ranitidine on the transmucosal potential difference (PD) of the gastric mucus membrane in the rat. *$p < 0.05$ when compared to the mean ($\bar{x} \pm$ SEM) of PD values measured before the drug exposure ($n = 8$, in all groups)

When the effect of three doses of intragastrically administered PGE_1 was investigated, significant alteration of PD was observed but after the administration of the highest dose (40 μg/kg), when compared either to the baseline value or the values measured in the controls. Following the topical administration of PGE_1 in a dose of 40 μg/kg, significant decrease of PD was observed during a period of 20 minutes beginning at the 5th minute after the treatment. No significant alteration of PD could be evoked by the intragastric administration of lower does of PGE_1 (Fig. 2). In the 20th second, on the average, following intravenous administration of pentagastrin (100 μg/kg) rapid and significant decrease of PD began. The maximal decrease was seen during the first minute following the exposure to pentagastrin, and, after gradual regeneration, it was in the 10th minute when PD values returned to the baseline level (Fig. 3).

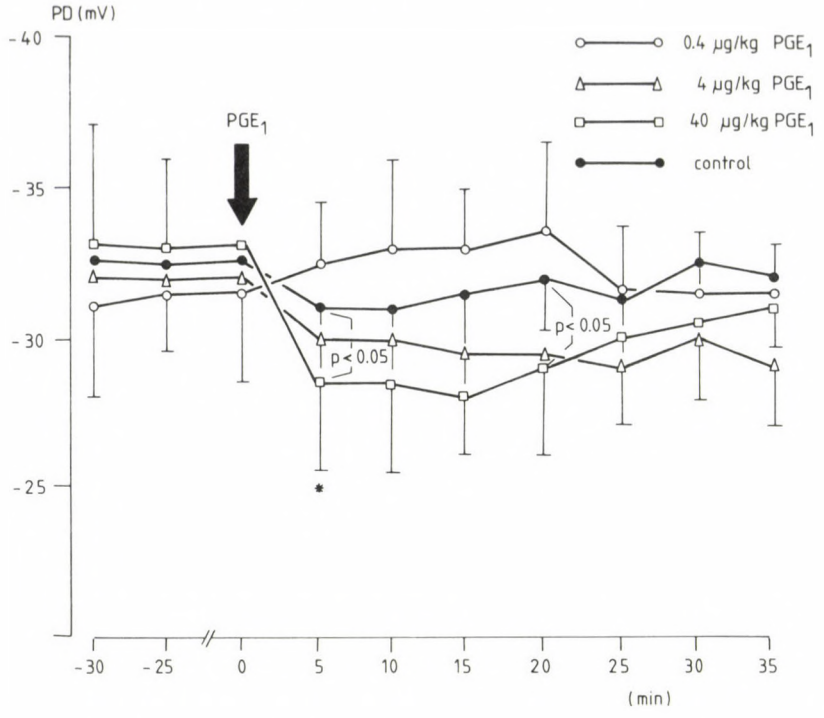

Fig. 2. Effects of different doses of intragastrically administered prostaglandin E_1 on the transmucosal potential difference (PD) of the gastric mucous membrane in the rat. * $p < 0.05$ when compared to the mean ($\bar{x} \pm$ SEM) of PD values measured before the drug exposure ($n = 7$, in all groups)

PGE_1 pretreatment in the acid secretion reducing dose (40 µg/kg) ameliorated the effect of intragastrically administered 30% alcohol solution to decrease transmucosal PD. In the 5th minute following alcohol exposure, significant difference could be observed between the mean PD values of the groups pretreated with PGE_1 or left untreated. In the time course of PD regeneration, however, no difference could be observed between the two groups investigated (Fig. 4).

Both intragastric pretreatment with PGE_1 in the acid secretion reducing dose (40 µg/kg) and i.v. pretreatment with ranitidine in a dose of 10 mg/kg ameliorated significantly the effect of intragastrically administered Ca^{++} solution (final concentration 5 Mm) to decrease PD. Pretreatment with either of the drugs enhanced the rate of PD regeneration; in the pretreated animals PD returned to the baseline level at the 35th minute following Ca^{++} administration, meanwhile in those animals who didn't receive pretreatment the decrease of PD remained significant even after 45 minutes (Fig. 5).

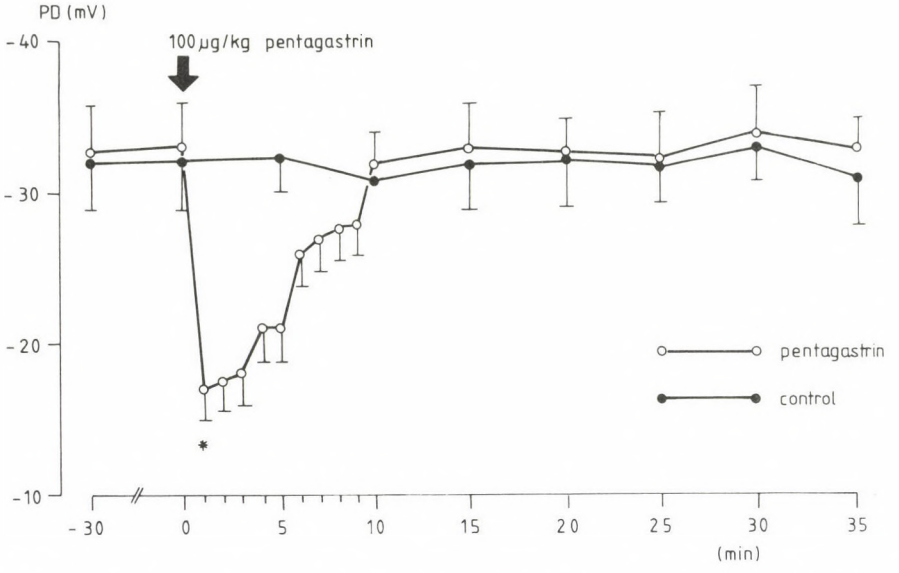

Fig. 3. Effect of intravenous pentagastrin (100 µg/kg) on the transmucosal potential difference (PD) of the gastric mucous membrane in the rat. * $p < 0.01$ when compared to the mean ($\bar{x} \pm$ SEM) of the PD values measured before the exposure to the drugs (n = 7)

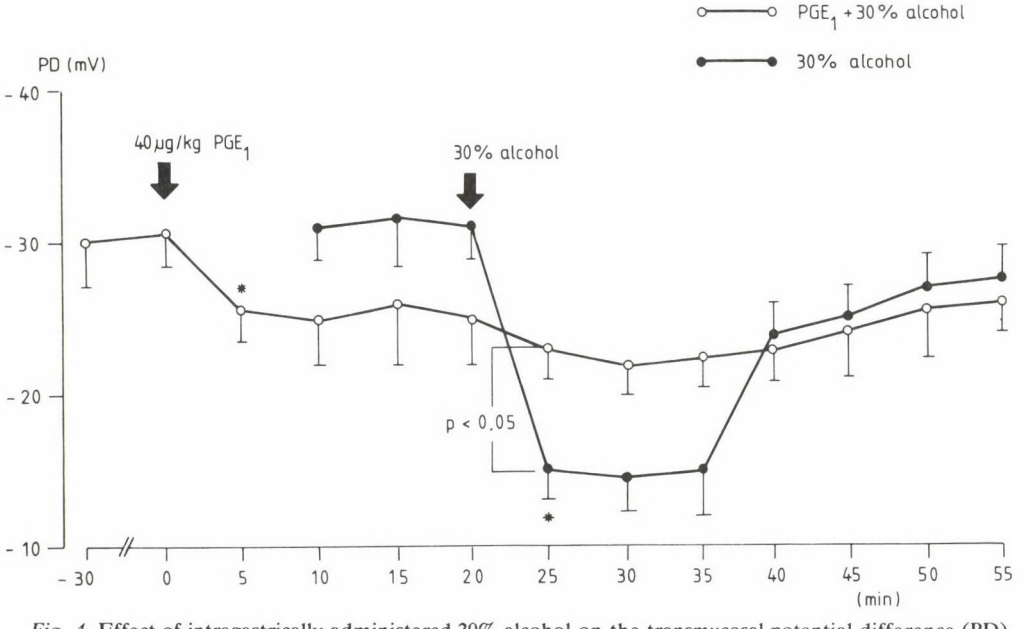

Fig. 4. Effect of intragastrically administered 30% alcohol on the transmucosal potential difference (PD) of the gastric mucous membrane in the rat, with and without pretreatment with prostaglandin E_1 in a dose of 40 µg/kg. * $p < 0.05$ when compared to the mean ($\bar{x} \pm$ SEM) of the PD values measured before the exposure to the drugs (n = 7, in all groups)

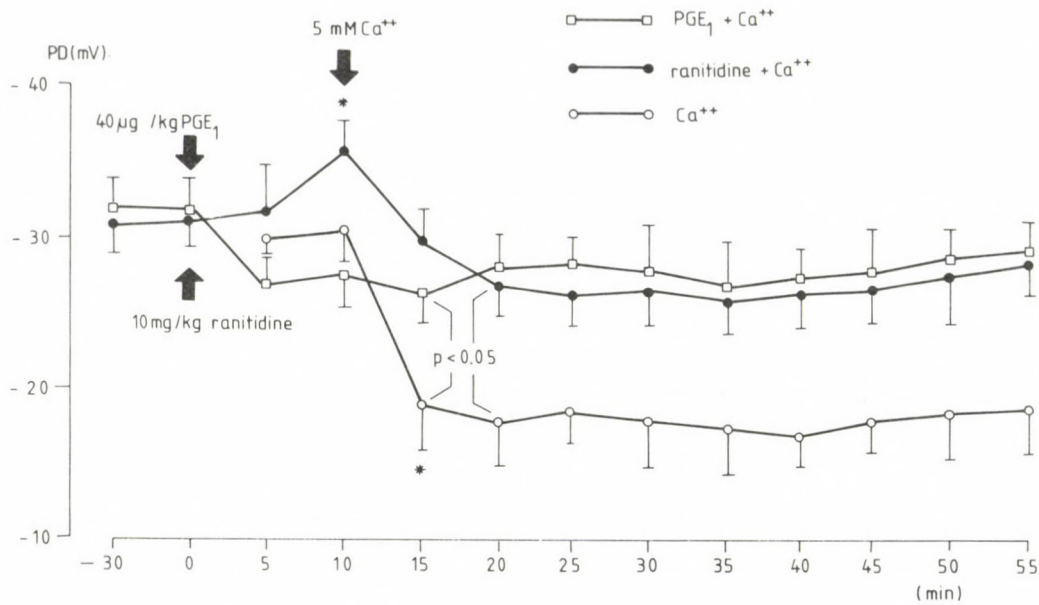

Fig. 5. Effect of intragastric 5 Mm Ca^{++} on the transmucosal potential difference (PD) of the gastric mucous membrane in the rat, with and without pretreatment with prostaglandin E_1 in a dose of 40 µg/kg or ranitidine in a dose of 10 mg/kg. * $p < 0.05$ when compared to the mean ($\bar{x} \pm$ SEM) of the PD values measured before the exposure to the drugs ($n = 7$, in all groups)

Pretreatment neither with ranitidine in high dose (10 mg/kg), nor with PGE_1 in the acid secretion decreasing dose (40 µg/kg) was able to alter the rapid and highly significant decrease of PD following i.v. pentagastrin administration.

In the 10th minute following pentagastrin administration, however, the gradually regenerating PD reached the baseline level but in the animals pretreated with PGE_1, meanwhile in those animals who received ranitidine pretreatment PD was lower by 20 per cent than the baseline level even in the 20th min (Fig. 6).

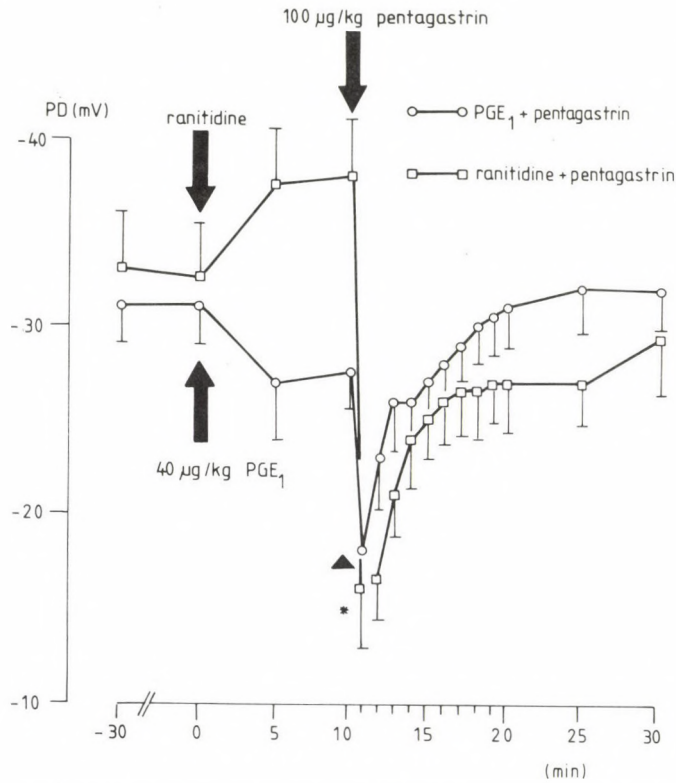

Fig. 6. Effect of intravenous pentagastrin treatment (100 µg/kg) on the transmucosal potential difference (PD) of the gastric mucous membrane in the rat, with and without pretreatment with prostaglandin E_1 in a dose of 40 µg/kg and/or ranitidine in a dose of 10 mg/kg. * $p < 0.01$, $p < 0.05$ when compared to the mean ($\bar{x} \pm$ SEM) of the PD values measured before the exposure to the drugs ($n = 7$, in both groups)

Discussion

It is the active chloride ion transport against concentration and electrochemical gradient towards the lumen which primarily maintains the negative electrical polarization of the lumen of the gastric mucous membrane; nevertheless, the active Na^+ transport also plays a species-dependent role [5, 20, 23].

The gastric mucosal barrier prevents both the passive rediffusion of hydrogen ions into the mucosa and the flow of sodium ions into the lumen [6].

Transmucosal PD is known as a sensitive numerical index of this barrier function and widely used not only in basic research on the function of gastric mucosa but, recently, in clinical research as well [25, 26, 27].

The influences which damage the mucosal barrier lead to the decrease of PD. More recently an increasing number of publications provide data on the protective mucosal effects exerted on PD [1, 11, 18].

It is well-known that H_2 receptor inhibitors, like cimetidine and ranitidine, enhance PD [1, 12, 18, 31].

The above-mentioned data were confirmed in the first phase of the present experiments. Intravenous ranitidine treatment increased transmucosal PD in a dose-dependent manner, the higher dose led to more rapid (within 5 minutes) and higher increase of PD than the lower dose of ranitidine or the intravenously administered cimetidine.

In their human investigations Müller et al. [18] also observed dose-dependent increase of PD after the i.v. administration of ranitidine. On the basis of our present knowledge, the effect of H_2 receptor inhibitors to increase PD is based on the separation of the H^+ ion and Cl^- ion secretion of the parietal cells and the inhibition of the selective H^+ secretion the electronegativity of the uninterruptedly active Cl^- secretion strengthens, and the negative polarization of the mucosal surface increases without any change of the netto transmucosal transport of chloride ions [1, 9, 19].

Similar mechanism accounts for the omeprazol-induced increase of PD [14].

That is to say, the degree of PD alterations which can be observed after the administration of H_2 receptor inhibitors is parallel with and proportional to the inhibition of the H^+ secretion of the parietal cells.

When administered in low or medium doses, prostaglandin E_1 did not alter transmucosal PD. Intragastric administration of PGE_1 in a dose inhibiting secretion, however, significantly decreased PD. Data on the electrophysiology of the gastric mucosa have been provided mainly with respect to the effect of prostaglandin E_2, less data have been published on the effect of PGE_1.

In their *ex vivo* rat model Morris et al. [16] could not evoke any alteration of PD with the topical administration of PGE_2.

On the other hand, following the administration of PGE_2 McGreevy et al. [14] observed oedema of the subepithelial cells of the gastric mucous membrane and widening of the lateral intercellular spaces, together with the decrease of PD. The basis of the cytoprotective effect of prostaglandins is still questionable.

In our previous investigations [28] it has been demonstrated that Ca^{++} solution in an intragastric concentration of 5 Mm evokes significant and permanent decrease of the transmucosal PD: moreover, in accordance with the data of other authors, similar effect of 30% alcohol could also be demonstrated.

In the present experiments i.g. administration of high doses of prostaglandin E_1 ameliorated the effect of intragastric alcohol or Ca^{++} administration to decrease PD, which finding indicates the protection of the mucosal barrier against harmful influences. Following PGE_1 pretreatment, however, not only the degree of the fall of

PD after intragastric Ca^{++} exposure decreased but also the rate of PD regeneration increased.

Morris et al. observed quicker regeneration of the salicylate-induced decrease of PD if prostaglandin E_2 pretreatment was administered: topical administration of PGE_2 in the same dose, however, did not evoke significant alteration of PD.

Dajani et al. [4] administered PGE_1-methylester in cytoprotective dose and observed reduction of the decrease of PD following the administration of drugs damaging the mucosal barrier: nevertheless, similar effect of PGE_2 could not be demonstrated. It is known form the experiments of Robert [21] and Morris [15] that topical administration of PGE_2 does not protect the surface epithelial cells from the structural damages induced by salicylate, and also PD decreases. In the present experiments neither PGE_1, nor ranitidine pretreatment could prevent the effect of barrier-damaging drugs to decrease PD. Nevertheless, the lesser degree of PD alterations and, further, the shortening of PD regeneration time, which is indicative of the functional restitution of the epithelium, were both suggestive to the assumed cytoprotective effect. It is known primarily from the human and animal studies of Tarnawski [29, 30] that intravenous treatment with pentagastrin or human gastrin leads to "dramatic extent" of PD decrease and the maximal decrease of PD can be observed at the 25th second, on the average, following the drug administration. In experiments in rats, however, the decrease of PD was demonstrated both in the corpus and in the forestomach covered with squamous epithelium, and the decrease of PD proved to be independent of the alterations of acid secretion.

The basis of the mucosal electrophysiological effect of pentagastrin has not been clarified so far. Tarnawski [30] explained the change of PD by the increase of canalicular membrane area of the parietal cells.

In the present experiments the maximal decrease of PD following pentagastrin administration was observed in the 30th second, on the average. Neither ranitidine, nor prostaglandin E_1 pretreatment influenced the rapid and highly significant alteration of PD. Both high-dose H_2 receptor inhibitor and prostaglandin E_1 pretreatments ameliorated the effect of drugs damaging locally the mucosa the decrease PD; in the present study, however, similar effect as not seen following pentagastrin administration. Thus, the conclusion may be drawn that pentagastrin alters PD but not by influencing the mucosal barrier. Further, it may be also concluded that transmucosal PD reflects not only the actual condition of the gastric mucous membrane barrier but it indicates the physiological alterations of other functions as well.

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POLYMERASE CHAIN REACTION AND OTHER GENE TECHNIQUES IN PHARMACOGENETICS: AN INTRODUCTION AND REVIEW

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The author gives a short description of the polymerase chain reaction technique and restriction fragment length polymorphism which revolutionized molecular biology and have entered pharmacogenetics, too.

Examples of the use of these techniques are given in the polymorphism of oxidative (phase I) and conjugative (phase II) reactions.

Keywords: PCR, RFLP, pharmacogenetics

Introduction and brief description of the methods

1. Polymerase chain reaction

The polymerase chain reaction (PCR) is an *in vitro* DNA amplification technique. The method is rapid and very powerful. Within few hours one can isolate and amplify a specific DNA segment in such an order that the target segment can be visualized after electrophoretic gel run.

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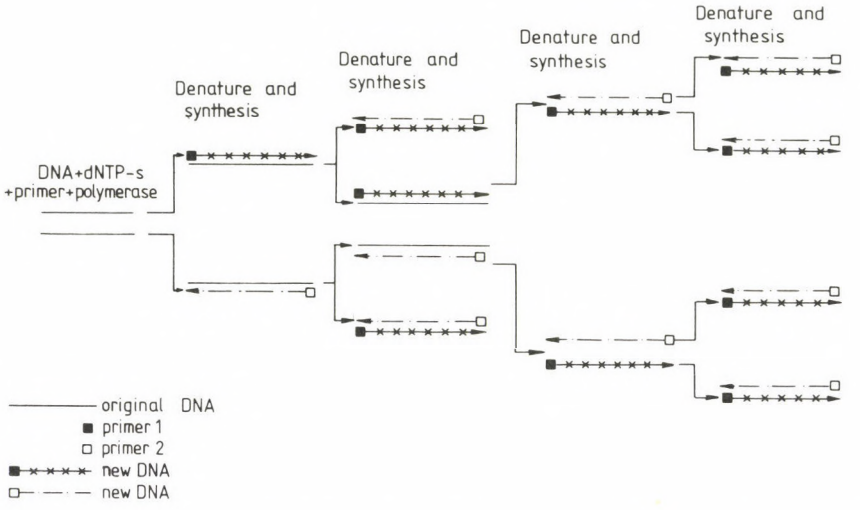

Fig. 1. Schematic view of PCR. The short, primer terminated DNA copies first appear only in the third cycle but double with each cycle and become dominant to the long primer extensions

The scheme of the procedure is demonstrated on Fig. 1. The essence of the method is programmed heating and cooling of the DNA sample. The reaction mixture basically contains original DNA (extracted from leucocytes e.g.), deoxyribonucleotides (each four of them), so called primers (purpose designed) and polymerase enzyme in buffer. The specially designed and synthesized primers are small (usually not more than 20 bases) DNA strands which hybridize to target DNA sequences and mark the DNA segment to be copied. They function as triggers for the polymerase enzyme. There are several types of polymerases isolated from bacteria but the most frequently used one is the Taq-polymerase. It is isolated from the bacterium *Thermus aquaticus* and is very thermostabile. The polymerase needs a free 3' end and produces a complementary DNA segment in direction 5' to 3' from the deoxyribonucleotides present.

The PCR reaction itself is carried out in programmable heating blocks. The three main steps are as follows:

Step 1. A rapid heating of the DNA containing mixture. The original DNA denatures, the double strand opens.

Step 2. A rapid cooling. The primers anneal to the target template single DNA strands.

Step 3. A rapid heating. The polymerase makes the synthesis of the template to the complementary strand at its optimal temperature.

Duration and temperature of the steps depend on the given system. These steps are repeated several times. Usually 25 to 30 cycles are carried out. Theoretically the result is an exponential accumulation of the target DNA segment according to the formula 2^n, where n is the number of cycles. Practically a 10^6-fold amplification can be achieved.

The usual temperature for step 1 is 95°C, for step 2 it varies between 40 to 60°C and for step 3 70 to 75°C. The usual duration of step 1 is one minute, of step 2 one to three minutes and for step 3 one minute. PCR takes three to four hours in all.

PCR is followed then by Southern blotting or simple electrophoretic gel run in order to identify the multiplied DNA segment.

Applications of PCR

PCR is becoming widely used in basic molecular biology, in forensic medicine and clinical medicine. It is used for detecting and screening of various hereditary metabolic disorders (such as cystic fibrosis) and in the diagnosis of tuberculosis as well [3].

The technique reached pharmacogenetics, too. Pharmacogenetics deals with monogenically determined differences in the metabolism of xenobiotics (drugs, environmental agents toxic or even carcinogenic ones, etc.). Before PCR only phenotyping was available. Routine phenotyping means measuring the parent drug and its metabolite in collected urine samples after test drug exposure *in vivo* and calculating metabolic ratios. Subjects can be classified as extensive, intermediate or poor metabolizers. A large number of factors (concommitant drug treatment, complience with the procedure etc.) are however known to interfere with phenotyping procedures leading to false results (phenocopy).

PCR enables us to look directly at the gene (wild type and mutant(s) make classifications to homozygous effective, heterozygous effective and homozygous poor metabolizers and make comparisons between genotype and phenotype in the individual as well as in the population.

1.1 Concrete applications

1.1.1 Polymorphism of oxidative (phase I) reactions. Debrisoquine

The antihypertensive agent debrisoquine is mainly metabolized to the pharmacologically inactive 4-OH form by the hepatic cytochrome P450IID6 and its expression is regulated by the CYP2D6 gene located on chromosome 22 [10].

Extensive (EM) metabolizers are prone to lung cancer [2], poor metabolizers (MP) may experience profound hypotensive effect at usual dosing. The frequency of PMs in Caucasian population is between 4 and 11.5% [1,9].

Quite a few clinically important and widely used drugs (beta blockers, antidepressants, antiarrhythmic agents, etc.) share the same metabolic route showing polymorphism of their metabolism cosegregating with debrisoquine 4-hydroxylation [4].

PM phenotype is inherited as an autosomal recessive trait. The basis of PM phenotype is mutation in the wild type CYP2D6 gene which results in lack of enzyme activity.

Using restriction fragment length polymorphism (RFLP) analysis with XbaI enzyme [14] four allelic variants are to be detected and allels of 44 kilobase (kb) and 11.5 kb are common in PMs but the molecular basis of the 44 kb allele is unclear. It is however possible to detect further mutations termed 29A and 29B by PCR followed by electrophoretic gel run. Details of the method are described by Daly et al. [7].

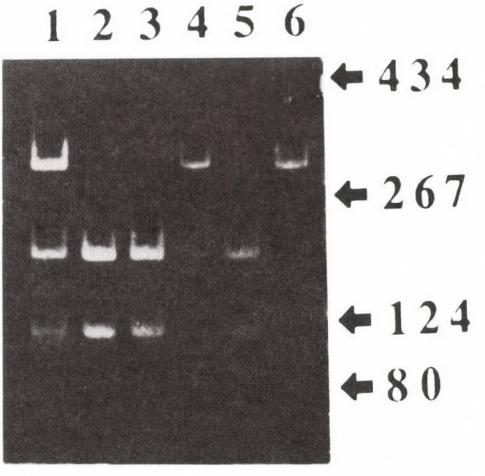

Fig. 2. Debrisoquine genotyping. A typical gel run of DNA amplified by PCR and digested with Bst NI restriction enzyme (29B allele assay). Lane 1 is genotype wt/29B (EM),2 is wt/29A (EM), 3 is wt/wt (EM),4 is 29A/29B (PM), 5 is wt/wt (EM) and 6 is 29B/29B (PM) (reprinted from Daly et al., Ref. 7. by kind permission of Chapman and Hall, England)

Figure 2 demonstrates a typical gel run on 29B mutation. The simultaneous detection of genotype and phenotype results in histograms, such as on Fig. 3 where hetero- and homozygous EMs and homozygous PMs can be clearly identified.

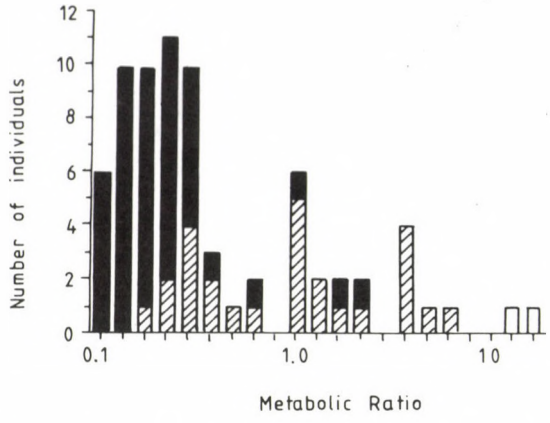

Fig. 3. The relationship between debrisoquine phenotype (metabolic ratio of debrisoquine and its 4-OH metabolite in 0-8 h urine) and genotype (as determined by PCR and RFLP).
■ : homozygous EM ▨ : heterozygous EM □ : homozygous PM (reprinted from Daly et al., Ref 7. by kind permission of Chapman and Hall, England)

Coumarin

Coumarin is a benzopyran derivative found in many plants and essential oils [12]. In man, the main metabolite is 7-hydroxy-coumarin and the site of the metabolism is the liver. Coumarin bears an anti-carcinogenic action and partly inhibits the metabolism of 7,12-dimethylbenz/a/-anthracene to carcinogenic metabolites.

The human liver cytochrome P-450 responsible for coumarin 7-hydroxylase activity was identified as a 49 kDa protein and is the member of the cytochrome P450IIA subfamily [11].

There are 40-fold differences in the coumarin 7-hydroxylase activities of human liver cells [16]. Differences of such a magnitude in metabolic rates strongly support the hypothesis of monogenic determination in the background.

Human population phenotyping results obtained from urine sample analysis with TLC hinted at eventual polymorphism of coumarin 7-hydroxylase activity [6].

The gene encoding coumarin 7-hydroxylase activity is the CYP2A3 gene belonging to the CYP2A gene family [16]. There is only a 3 nucleotide difference between the wild type and the variant gene and hence only a single amino acid difference ($Leu_{160} \rightarrow$ His) between the effective and the defect enzyme produced.

We have tried to identify the wild type and the mutant gene with specially designed primers by PCR and were able to demonstrate heterozygous and homozygous individuals (Fig. 4). We emphasize however, that these are only preliminary results, and the sequencing of the PCR product is not done at present.

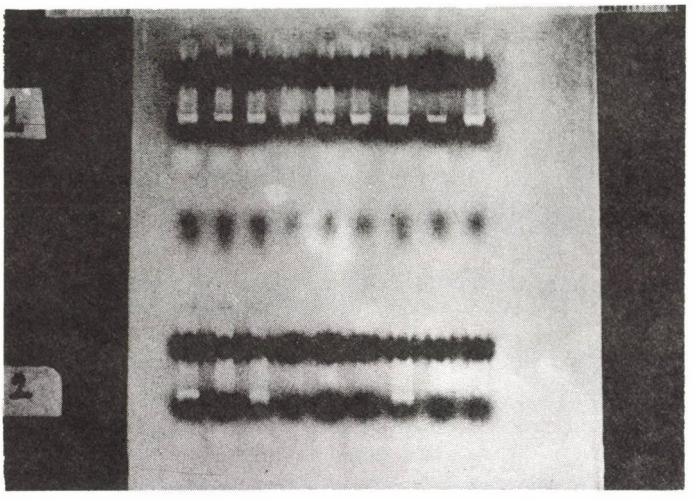

Fig. 4. Coumarin 7-hydroxylase genotyping. The result of PCR with two sets of three different primers. Row 1 represents the wild type and row 2 represents the mutant gene after electrophoretic agarose gel run of th PCR products. Lanes 1,3 and 7 (from left to right) represent wt/mutant genotypes, the other are wt/wt

2. Restriction fragment length polymorphism (RFLP)

The essence of the method is the digestion of DNA with restriction endonucleases. These enzymes are obtained from microorganisms and digest double stranded DNA at specific cleavage sites. Digestion is then followed by electrophoresis on agarose gel and Southern blotting.

2.1 Polymorphism of oxidative (phase I) reactions. Debrisoquine

A cDNA probe for CYP2D6 is labelled with ^{32}P dCTP by random priming. The DNA sample is digested with restriction enzyme and separated by electrophoresis on agarose gel. The DNA is transferred to nylon membranes and the filter probed with the ^{32}P labelled cDNA [7]. A total of five variant bands can be detected by the use of XbaI restriction enzyme and the 11 kb band is most likely to represent the PM associated allelic variant.

2.2 Polymorphism of conjugative (phase II) reactions
N-acetyltransferase

It is known for more than 30 years, that N-acetylation of certain drugs shows polymorphism in man. The defect of N-acetylation is inherited as an autosomal recessive trait. There is a roughly 50 to 50 per cent distribution between the rapid and slow acetylator phenotype in Caucasian populations [8, 15]. Slow acetylators are prone to developing urinary bladder cancer when exposed to arylamines [5].

The prototype drug of this polymorphism is isoniazid, but perhexilene, procainamide, dapsone, certain sulfonamides and dihydralazine share a common pathway in their metabolism.

Two different N-acetyltransferase genes were identified in human genomic DNA libraries: one is encoding the enzyme that shows polymorphism and the other which does not (for review see ref. 13). The two genes occupy two different loci.

There are differences in the restriction enzyme patterns of the monomorphic and polymorphic nat genes.

According to genotype population studies 25.8% has the wild type gene, 45.2% have the S1,27.4% the S2 and 1.6% the S3 mutant gene in Caucasians (slow acetylator phenotype 52-68%). In contrast 68% have the wild type, 25% have the S2,7% have the S3 and none have the S1 mutant gene in Japanese population, where the frequency of slow acetylators is only 10 to 15 per cent.

Acknowledgements

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ELECTROPHYSIOLOGICAL CHANGES INDUCED BY LYSOPHOSPHATIDYLCHOLINE, AN ISCHAEMIC PHOSPHOLIPID CATABOLITE, IN RABBIT ATRIAL AND VENTRICULAR CARDIAC CELLS

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The effects of palmitoyl-lysophosphatidylcholine (LPC) were studied on the cellular electrical activity of rabbit heart preparations. LPC (100 μmol/l) caused a considerable enhancement of the automaticity of the SA nodal and Purkinje fibers and frequently induced irregular firing in both supraventricular (SA node, atrium, AV junction) and ventricular (Purkinje fibers, papillary muscle) myocardial regions. The 'automatotropic' and arrhythmogenic effects of LPC were accompanied by a lengthening of the atrioventricular conduction time. In ventricular muscle fibers LPC (100 μmol/l) decreased the resting potential (RP), the maximum rate of depolarization (V_{max}) and the amplitude (APA) and duration (APD) of the action potential, and often evoked action potentials of 'slow response' type. In atrial muscle cells, 100 μmol/l LPC was capable of inducing hyperpolarization, with concomitant increases in RP, V_{max}, APA and APD; higher concentrations (300 and 600 μmol/l) of LPC resulted in decreases in RP, V_{max}, APA and APD, i.e. phenomena similar to those observed with 100 μmol/l LPC in the ventricular myocardium. The results seem to support the assumption that lysolipids accumulating in the ischaemic myocardium may play a pathogenetic role in the development of both supraventricular and ventricular dysrhythmias accompanying coronary artery occlusion.

Keywords: lysophosphatidylcoline, electrophysiological actions, atrium, AV junction, ventricle, rabbit

There is no longer any doubt that the altered lipid metabolism is an important factor in the genesis of electrophysiological changes and cardiac arrhythmias accompanying acute myocardial ischaemia [8, 20, 24]. The abnormal activation of phospholipases after coronary artery occlusion leads to hydrolysis of phosphoglycerides and to the formation of amphipathic phospholipid catabolites [2,

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8, 21]. Many authors hold the view that the accumulation of lipid intermediates of a detergent nature (long-chain fatty acids, fatty acid esters and lysolipids) in the ischaemic myocardium may play a role in the development of the very early postocclusion [12, 20] and reperfusion [2] arrhythmias, since these compounds are capable of mimicing the electrophysiological changes *in vitro* that may be observed in the ischaemic heart *in vivo* [3, 9, 10, 12, 13, 14].

In response to phospholipase A_1 and A_2, lysophosphoglycerides, primarily lysophosphatidylcholine (LPC) and lysophosphatidylethanolamine (LPE) are formed from the phospholipids of the mammalian myocardium. The intracellular microelectrode technique was first applied to ventricular myocardial preparations isolated from various animals in order to investigate the cardiac electrophysiological effects of the lysophospholipids by Corr et al. (7-11, 30, 35, 36). They established that, even in concentrations of 100-200 μmol/l [7], extracellular LPC gives rise to changes *in vitro* in the characteristic parameters of the transmembrane potentials from the Purkinje fibres and papillary muscle cells [7, 10, 11, 22, 35, 36] that are similar to those caused by superfusion with 'ischaemic blood' [31] or by regional ischaemia *in situ* [13]. They assumed that the lysolipids released in the hypoperfused myocardium, accumulating there and undergoing insertion into the sarcolemmal phospholipid bilayer [22] are the biochemical mediators of the ventricular arrhythmias induced by coronary artery occlusion (*lysolipid hypothesis*) [35].

The working hypothesis and the results by Corr et al. led us to extend the investigation of LPC to the supraventricular regions (SA and AV nodes, atrial working muscle), with an attempt to carry out more complex electrophysiological analysis concerning the effects of the substance. Certain features of our examinations were reported earlier [14–18].

Methods

Myocardial strips were excised from the heart of young, 1000-1500 g rabbits and were maintained in modified Locke's solution. The nutrient solution contained (mmol) NaCl 125, KCl 5.6, $NaHCO_3$ 25, $CaCl_2$ 2.12 and glucose 11, and was gassed with 95% O_2 and 5% CO_2 (pH 7.4). Sinoatrial nodal, atrial, AV-nodal, Purkinje fiber and papillary muscle preparations were isolated from the heart. On examination of the SA-, AV-nodal and Purkinje automaticity, the preparations functioned spontaneously and the frequencies (beats/min) of the surface potentials were counted and registered with the mediation of bipolar platinum electrodes; additionally, an intracellular microelectrode technique was used to record the transmembrane potentials from the SA-nodal cells and Purkinje fibres. On another occasion, the preparations were electrically driven by rectangular stimuli (100/min, 1 ms, at twice electrical threshold strength) originating from a biological stimulator. The effects of LPC on the transmembrane potentials were analyzed in detail in atrial and ventricular (papillary muscle) heart cells and in Purkinje fibres. The following parameters were measured: resting potential (RMP, mV); action potential amplitude (APA, mV); action potential duration at 25, 50, 75 and 90% repolarization times (APD, ms); and maximum rate of depolarization (V_{max}, V/s). An extracellular technique was used to study the atrioventricular conduction time in a separate experimental series, in heart preparations

containing the right atrium, the interventricular septum and the right ventricle. The principles and further details of the microelectrophysiological methods employed were described earlier [5, 6, 32, 37, 38, 39].

Synthetic, protein-free LPC (L-α-palmitolglycerol-3-phosphatidylcholine; Sigma Chemical Co.) was used. Its effects were investigated by applying concentrations (100, 300 and 600 μmol/l) comparable to the ischaemic tissue concentrations. The incubation time was 30 minutes, and the duration of pumping through the control nutrient solution ("washing") was 120-180 minutes. The results were subjected to statistical evaluation with the one- and two-tailed *t* tests.

Results

The increases in the numbers of potentials recorded per minute with the extracellular technique from the sinoatrial and atrioventricular nodes and from the Purkinje fibres indicate that LPC (100 μmol/l) enhances the spontaneous discharge rate of the SA node and the Purkinje fibres; it has a lower effect on the AV junctional automaticity (Fig. 1). This compound frequently gives rise to irregular firing and arrhythmia (Fig. 2). The mechanism of the positive chronotropic effect of LPC is that at unchanged diastolic and threshold potentials, the slope of the slow diastolic depolarization increases, as shown by the action potentials registered from the sinoatrial node (Fig. 3). LPC may induce slow diastolic depolarization and spontaneous activity even in the "resting" Purkinje fibres (Fig. 4). The positive chronotropic, 'automatotropic' and arrhythmogenic features of LPC are accompanied by a negative dromotropic effect in the AV-junction: the compound appreciably lengthens the AV conduction time (Fig. 5).

Table I

Electrophysiological changes induced by lysophosphatidylcholine (LPC) in rabbit Purkinje fibers

Treatment modality	APA (mV)	RMP (mV)	Action potential duration (ms) at repolarization 25%	50%	75%	90%	V_{max} (V/s)
Control n = 30	113.0 ± 1.2	85.9 ± 1.7	111.5 ± 0.9	145.3 ± 1.0	157.3 ± 4.7	190.0 ± 2.3	325.9 ± 5.6
LPC 100μmol/l 30 min n = 30	106.4 ± 2.5*	80.3 ± 2.0*	107.4 ± 2.0	139.8 ± 0.9**	154.2 ± 0.8	172.3 ± 0.7**	154.9 ± 5.5**
Recovery 120 min n = 40	115.1 ± 1.4	82.1 ± 0.9	111.5 ± 0.5	152.9 ± 0.7	176.7 ± 0.5	187.9 ± 0.7	244.4 ± 0.9

Means ± S.E. * $p < 0.05$, ** $p < 0.001$ as compared to control

Abbreviations: APA = action potential amplitude; RMP = resting potential; V_{max} = maximum rate of depolarization

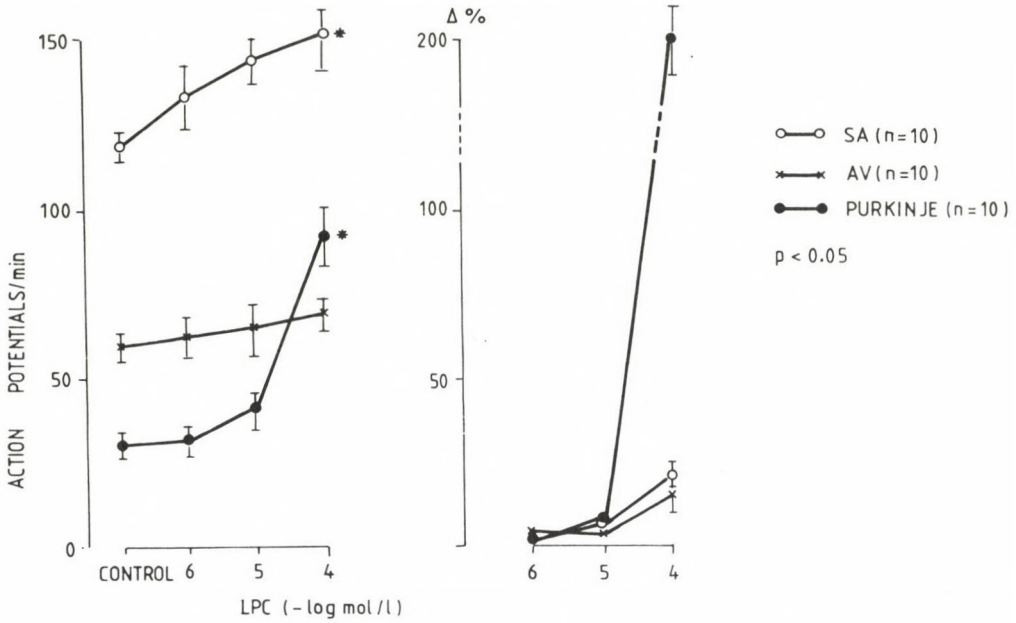

Fig. 1. Effects of lysophosphatidylcholine (LPC) on the impulse formation of SA and AV nodal cells and Purkinje fibres (values measured after 30 min)

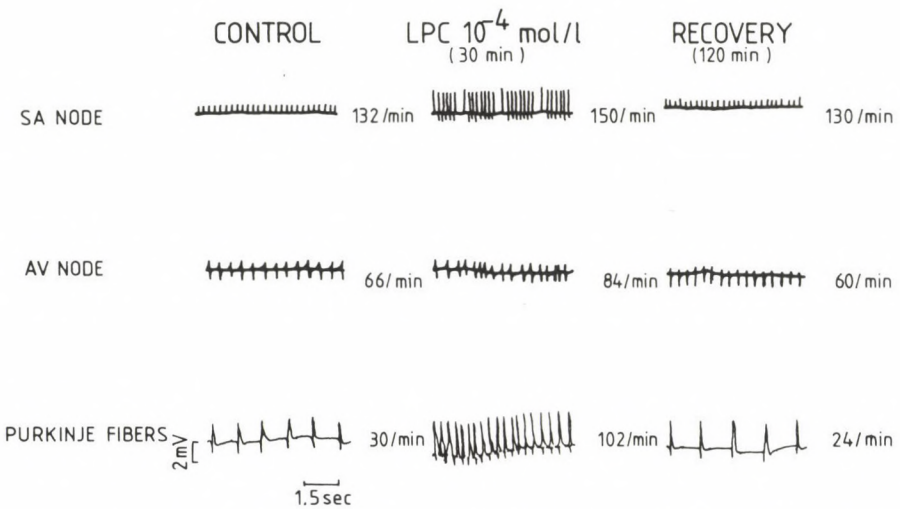

Fig. 2. Effect of lysophosphatidylcholine (LPC) on the automaticity of sinoatrial and AV nodal cells and Purkinje fibres in isolated rabbit heart preparations (extracellularly recorded action potentials). Pronounced sinus and junctional arrhythmia

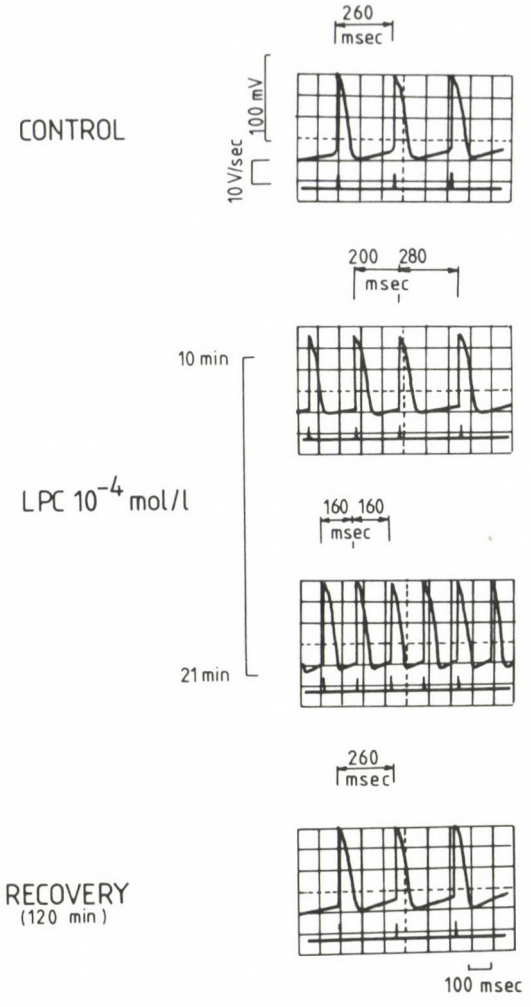

Fig. 3. Effects of lysophosphatidylcholine (LPC) on the action potentials recorded with intracellular microelectrode technique from SA nodal cells. After 10 min, sinus arrhythmia developed; after 21 min, the slope of the slow diastolic depolarization increased. (The short horizontal line on the left represents the zero potential; bottom trace: the differential of intracellular record; the length of the spike indicates the maximum rate of depolarization, i.e. V_{max})

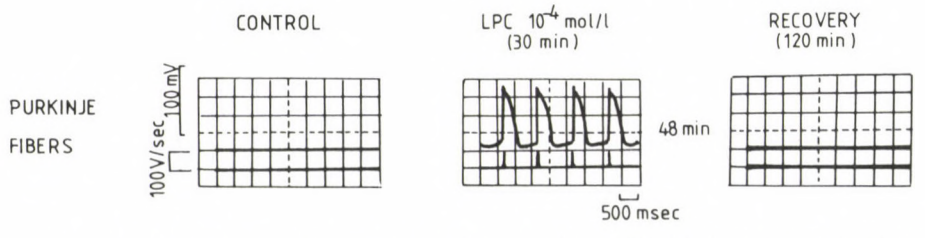

Fig. 4. Lysophosphatidylcholine (LPC) induces spontaneous firing (48/min) in resting rabbit Purkinje fibers (intracellularly recorded action potentials; "0" potential and V_{max} as in Fig. 3)

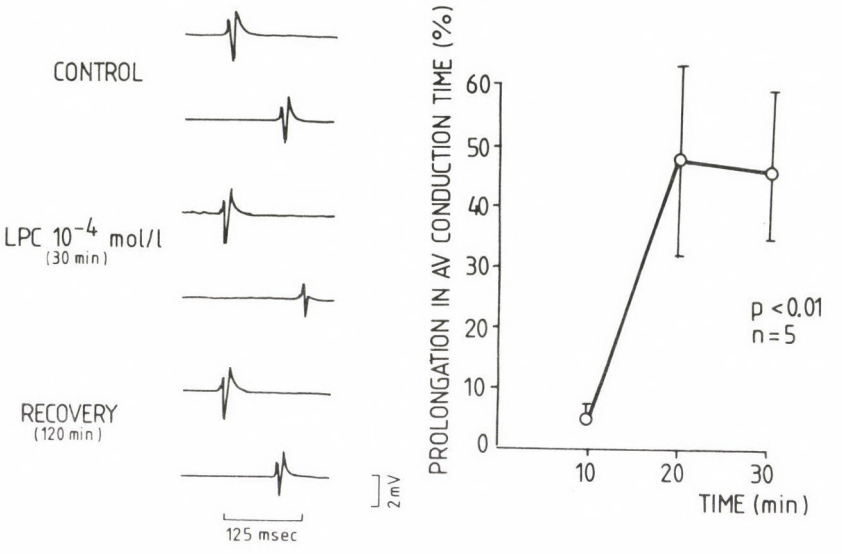

Fig. 5. Effect of lysophosphatidylcholine (LPC) on the AV conduction time. Left panel: prolongation of the interval between the appearance of right atrial (upper traces) and right ventricular (bottom traces) surface potentials. Right panel: percentage increase of the AV conduction time during incubation with LPC

Table II

Electrophysiological changes induced by lysophosphatidylcholine (LPC) in rabbit papillary muscle

Treatment modality	APA (mV)	RMP (mV)	Action potential duration (ms) at repolarization 25%	50%	75%	90%	V_{max} (V/s)
Control n = 45	100.1 ± 1.2	83.0 ± 0.7	124.5 ± 1.4	145.8 ± 1.4	174.7 ± 1.5	187.0 ± 1.8	117.8 ± 3.9
LPC 100 μmol/l 30 min n = 50	84.8 ± 1.6**	70.3 ± 1.3**	72.7 ± 1.2**	108.3 ± 1.9**	132.2 ± 1.7**	148.7 ± 1.5**	38.1 ± 0.4**
Recovery 120 min n = 25	101.7 ± 0.8	84.0 ± 0.5	96.5 ± 1.1	130.0 ± 1.5	150.7 ± 0.5	165.6 ± 0.7	98.6 ± 4.0

Means ± S.E. ** $p < 0.001$ as compared to control

Abbreviations: APA = action potential amplitude; RMP = resting potential; V_{max} = maximum rate of depolarization

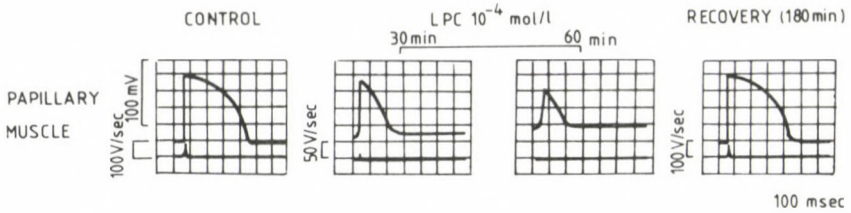

Fig. 6. Action potential of 'slow response' type induced by lysophosphatdylcholine (LPC) in papillary muscle cell (intracellularly recorded action potentials; "0" potential and V_{max} as in Fig. 3)

Table III

Electrophysiological changes induced by lysophosphatidylcholine (LPC) in rabbit atrial myocardium

Treatment modality	APA (mV)	RMP (mV)	Action potential duration (ms) at repolarization 25%	50%	75%	90%	V_{max} (V/s)
Control n = 130	92.77 ± 0.75	75.98 ± 0.43	27.72 ± 0.41	36.06 ± 0.39	50.12 ± 0.5	70.04 ± 0.82	105.63 ± 3.88
LPC 100 μmol/l 30 min, n = 40	103.6 ± 0.9*	83.0 ± 1.0*	28.4 ± 0.2	37.3 ± 0.1*	53.0 ± 0.3*	78.1 ± 1.2*	132.6 ± 2.3*
LPC 300 μmol/l 30 min, n = 46	82.44 ± 0.46*	71.78 ± 0.63*	26.33 ± 0.33**	34.57 ± 0.3*	47.91 ± 0.36*	66.06 ± 0.61*	75.76 ± 2.13*
LPC 600 μmol/l 30 min, n = 26	57.0 ± 1.62*	54.99 ± 2.58*	20.64 ± 0.47*	27.36 ± 0.53*	39.45 ± 0.54*	55.15 ± 1.02*	48.21 ± 2.74*
Recovery 120 min, n = 73	90.46 ± 0.55	75.57 ± 0.79	26.75 ± 0.49	35.73 ± 0.53	48.61 ± 0.62	74.78 ± 0.79	87.27 ± 1.91

Means ± S.E. * $p < 0.05$ ** $p < 0.001$ as compared to control

Abbreviations: APA = action potential amplitude; RMP = resting potential; V_{max} = maximum rate of depolarization.

Tables I and II reveal the changes caused by LPC (100 μmol/l) in the characteristic parameters of the transmembrane potentials registered from the Purkinje fibres and the papillary muscle cells. LPC decreases the resting potential, the amplitude and duration of the action potential, and the maximum rate of depolarization. This depressent effect is more marked in the papillary muscle cells; it was sometimes so strong that merely 'slow response' action potentials resulted (Fig. 6). In a concentration of 100 μmol/l LPC causes opposite changes in the atrial working muscle cells: it increases the resting potential, the amplitude and duration of the action potential (25, 50, 75 and 90% repolarization times), and the maximum rate

of depolarization (Table III); a cardiodepressant effect in the atrial muscle can be observed only if the nutrient solution contains a higher concentration of LPC (300 or 600 μmol/l). The latter concentration often induced 'slow response' action potentials in the atrial cells too, in the same way as the concentration of 100 μmol/l did in the papillary muscle.

The electrophysiological changes induced by LPC were reversible: the starting values were generally measured again after superfusion for 120-180 minutes with the control nutrient solution.

Discussion

It was first demonstrated by Sobel et al. that potentially arrhythmogenic amphipathic moieties, the lysophosphoglycerides, accumulate within a few minutes in the ischaemic myocardium and in the venous blood flowing away from it [9, 35, 36]. As a consequence of the dissolution of the membrane phospholipids, and the incorporation of the resulting lysolipids in the sarcolemma, the electrophysiological properties of the cardiac cell membrane clearly change, and may form the basis of the pathological electrical activity and arrhythmias [15, 18, 22, 24, 27, 29, 35, 40]. The estimated average concentration of the two most prominent myocardial lysolipids, LPC and LPE in ischaemic tissue, is LPC+LPE = 1.2 mmol/l [8]. In well oxygenated heart preparations *in vitro* [7], much lower LPC and LPE concentrations than this (100-300 μmol/l) cause electrophysiological changes analogous to those observed *in vivo* in the ischaemic heart [13]. If it is considered that the myocardial distribution of the amphiphiles is probably inhomogeneous, and that the local concentrations of the lysolipids are certainly even higher in the cellular and subcellular membranes or close to them, the assumption appears justified that lysolipids may contribute to ischaemic arrhythmogenesis [8, 10,. 12, 16, 20, 26, 34, 35]. Even in very low concentration (20 μmol/l), protein-free LPC is able to induce cardiac arrhythmia (ventricular tachycardia and fibrillation) in the heart of rat [27, 29], hamster [25] and rabbit [28] perfused with Krebs-Henseleit solution according to Langendorff. If this ability of LPC is born in mind, it is noteworthy that, by activating adenyl-cyclase, this compound increases the cyclic AMP content of the cells even in the catecholamine-depleted heart [1], while it inhibits the sarcolemmal Na^+-K^+-ATPase [23]. These biochemical effects presumably play a role in the occurrence of LPC-induced arrhythmias.

Corr et al. carried out studies with the intracellular technique on Purkinje fiber and papillary muscle preparations isolated from various species (dog, cat and rabbit), and found that LPC (75-300 μmol/l) decreases the resting potential, the APA and APD, and the maximum upstroke velocity of phase O (V_{max}) in a concentration-dependent manner; similar effects are displayed by LPE and palmitoyl-carnitine [7,

10, 35, 36]. In response to LPC (100-200 μmol/l) in canine Purkinje fibres, Corr et al. observed the formation of 'slow response' action potentials; these can be abolished with verapamil and manganese, which block the slow transmembrane Ca^{++} influx, but not with tetrodotoxin, which blocks the fast Na^{+} channel [11]. These experimental results suggest that action potentials of 'slow response' type in the presence of LPC are mediated by the slow inward Ca^{++} current and not by depressed fast Na^{+} channels [11]. In identical preparations, LPC (75-100 μmol/l) induced delayed after-depolarizations (DAD), and triggered activity was also observed [30].

The results of our experiments with 100 μmol/l LPC on rabbit Purkinje fibres and papillary muscle are similar to those described by Corr et al [14, 15, 16, 19]. This concentration of LPC also increased the resting potential, V_{max}, and the amplitude and duration of the action potential in the atrial cells; only when higher concentrations were applied (300 and 600 μmol/l) did the depressant changes observed in the ventricle come into prominence [17, 18]. A similar phenomenon was found earlier in the case of the prostanoid precursor arachidonic acid (100 μmol/l), which exerted 'excitatory' actions on the guinea pig atrium, and cardiodepressant effects on canine Purkinje fibres [37]. The higher concentrations of LPC required to produce membrane depolarization in the atrial cells compared to the ventricular muscle cells may be due to a result of a difference in membrane incorporation. On the other hand, the differences between atrial and ventricular muscle may relate to the level of catabolic enzymes in the atria compared to the ventricle.

A major difference in lysophospholipase activity exists in different tissues and it is highly probable that atrial myocardium may have a much greater capacity to catabolize LPC incorporated, thereby requiring much higher levels to produce the same membrane effect (Peter B Corr, personal communication). Further studies are needed to clarify the mechanism of the two-directional atrial electrophysiological effects of LPC, depending on its concentration.

On the other hand, our data indicate that the automaticity-enhancing, 'automatotropic' and arrhythmogenic properties of LPC are accompanied by a negative dromotropic effect: this compound considerably lengthens the AV conduction [15, 16]. A similar depressant effect on the intraventricular conduction has been reported by Nakaya et al [28].

As concerns the *mechanism* of the arrhythmogenic effect of LPC at a cellular level, LPC may act as a non-specific depressant of membrane channels, since it decreases the rapid inward current carried by sodium, the outward current carried by potassium, the slow inward current carried by calcium, and the time-dependent background current [8]. Depression of the rapid inward Na^{+} current appears likely, and although the decrease in V_{max} may in part be a consequence of the decrease in the resting potential, LPC seems to exert direct depressant effects on gNa^{+},

independent of alterations in RMP [8]. In low concentrations (10-20 μmol/l), LPC acts biphasically on the excitability in the Purkinje fibres: an initial increase reflecting the alteration of passive membrane properties is followed by a subsequent decrease of excitability because of changes in active membrane properties [4]; *in vivo*, the excitability varies similarly in the ischaemic myocardium, too. During the initial phase of enhanced cardiac excitability due to LPC, lidocaine in a concentration equivalent to the clinically effective antiarrhythmic plasma level (4 μg/ml) decreases excitability [33].

To summarize, it may be underlined that the electrophysiological changes induced by LPC *in vitro* are extremely variable, for this reason the supposed *in vivo* effects are presumably depending on the local lysolipid concentrations of the given myocardial area. It was demonstrated by Sedlis et al. that LPC release from the myocardium could be detected also in patients with pacing-induced ischaemia [34]. They found significant coronary sinus LPC concentration increase within 4 minutes of atrial pacing and LPC accumulation preceded clinical indicators of ischaemia. Therefore, we assume that lysolipids may play a partial pathogenetic role in the development of both atrial and ventricular arrhythmias of ischaemic origin. In the future, the therapy must be directed to preventing the formation and/or accumulation of these toxic ischaemic metabolites [2, 25, 40].

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INTACT KIDNEY FUNCTION DURING CONTRALATERAL RENAL ARTERY CLAMPING IN DOGS

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Experiments were carried out on 32 Nembutal anaesthetized mongrel dogs from both sexes. After 45 min control period unilateral renal ischemia was achieved by clamping the left renal artery for 90 min. In part of the experiments (n = 8) after clamp removal 3 consecutive 45 min periods were performed. The function of the intact right kidney was investigated. Mean artieral pressure (MAP), heart rate (HR), glomerular filtration rate (GFR), urine flow rate (V), fractional excretions of sodium (FENa), potassium (FEK) and chloride (FECl) and plasma levels of atrial natriuretic peptide, dopamine and antidiuretic hormone were evaluated. During ischemia MAP was elevated from 122.5 ± 3.1 to $140.2 \pm 2,7$ mmHg ($p < 0.001$), HR decreased from 119 ± 4 to 102.5 ± 3.9 beats/min ($p < 0.01$) as compared to the control period. GFR did not change significantly, while all excretory parameters increased: V from 8.7 ± 1.2 to 14.5 ± 1.7 µl/min/gr kidney tissue ($p < 0.05$); FENa from 2.3 ± 0.2 to $3.6 \pm 0.3\%$ ($p < 0.01$); FEK from $40.0 < 3.5$ to $51.2 < 2.8\%$ ($p < 0.05$); FECl from $1.8 < 0.3$ to $2.6 < 0.3\%$ ($p < 0.05$). MAP remained elevated in the first and the second postischemic periods and was paralleled by the sustained increase in FENa and FECl, while FEK remained higher to the end of the experiment. ANP was significantly elevated during ischemia: on 75 min – $p < 0.01$ and on 105 min. – $p < 0.05$. AVP and dopamine showed no statistically significant changes during the investigated periods. We conclude, that the enhanced function of the intact kidney compensates to a considerable extent the clamped left kidney function and is due to the elevated MAP, as well as to the increased ANP plasma levels.

Keywords: renal artery clamping, contralateral kidney function, atrial natriuretic peptide, vasopressin, dopamine

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A great amount of information concerning the progress of postischemic acute renal failure has been obtained from studies in which ischemic damage to the kidney has been produced by various periods of complete unilateral renal artery occlusion. However, the function of the contralateral normal kidney is also of considerable physiological and clinical interest as its presence ensures the survival of the animal.

Renal ischemia is known to be a stimulus that alters the discharge of mechano- and/or chemoreceptor afferents [6] thus affecting contralateral renal function through neurohormonal mechanisms, which differ strongly among species [13]. On the other side, clamping of one renal artery decreases vascular capacity and increases peripheral resistance followed by subsequent changes in systemic hemodynamics and hormonal status. Considering the works of Harris et al. and Freeman et al., where an elevated plasma renin activity (PRA) is established, in this study the participation of vasopressin, as a part of an integrated system for the control of blood pressure [10] and dopamine – a precursor of epinephrine and norepinephrine and a regulator of sodium excretion [3] are tested.

The present study was designed to evaluate the renal excretory function of contralateral kidney during 90 min unilateral renal ischemia in anaesthetized dogs. In addition plasma levels of atrial natriuretic peptide (ANP), vasopressin (AVP) and dopamine were monitored.

Methods

Animal preparation: Studies were performed on 32 Nembutal (CEVA) anaesthetized (30 mg/kg BW) mongrel dogs (15.5±0.79 kg) of both sexes. Surgical preparation included: tracheostomy, cannulation of both ureters for urine collections, both femoral arteries for arterial blood pressure monitoring and arterial blood sampling, and one femoral vein for infusion of isotonic saline at a rate 0.1 ml/kg BW/min during the whole experiment. A 60-min recovery period elapsed before starting the experiments.

Ischemic renal failure was induced by clamping the left renal artery for 90 min. Urine and blood samples were collected every 15 min. Blood samples were taken an 30, 75, 105, 135, 150, 180, 220 and 270 min, as dopamine plasma levels were monitored in the samples from the 30 and 75 min only. The experimental protocol consisted of three 45-min periods: 1 control and 2 ischemic periods. In 8 of the animals 3 additional 45-min postischemic periods followed.

Analytical procedures: Arterial blood pressure was recorded by an electronic pressure transducer (Gould/Statham P23ID) and heart rate (HR) was estimated by the peaks of systolic blood pressure. Urine flow rate (V) was measured gravimetrically. Glomerular filtration rate (GFR) was estimated using the clearance of endogenous creatinine (Ccr). Plasma and urine creatinine concentrations were determined by the Jaffe reaction (Boehringer tests). Plasma and urine concentrations of sodium and potassium were measured by ion selective electrodes (Corning Na/K analyzer 902), plasma and urine concentrations of chloride – by culonometry using a chloride titrator (Radiometer CMT 10). From these data fractional excretions of sodium (FENa), potassium (FEK) and chloride (FECl) were calculated. Plasma levels of dopamine was monitored spectrofluorimetrically [1, 2]. Plasma concentrations of ANP and vasopressin were determined by radioimmunoassays, as previously described [12], using

commercially available 125I-ANP and 125I-AVP (Amersham) and polyclonal antibodies from rabbits (Amersham). Arterial blood samples were collected in prechilled EDTA-tubes. Plasma was separated by immediate centrifugation at 0°C (3000 rpm, 10 min) and stored at -70°C until assayed.

Statistical analysis was performed by Student's *t*-test for paired and unpaired data as required. Results are presented as means ± SEM; p values less than 0.05 were accepted as significant.

Results

Table I

Effect of unilateral 90 min renal ischemia on systemic hemodynamics and intact kidney function

	Control period	Ischemic periods		Postischemic periods		
		1	2	1	2	3
	n=32	n=32	n=32	n=8	n=8	n=8
MAP	122.5	134.9 **	140.2 ***	143.9 **	137.3 *	134.0
mm Hg	±3.1	±2.5	±2.7	±4.0	±3.6	±4.2
HR	119.0	107.6 *	102.5 **	115.9	112.1	110.6
beats/min	±4.0	±3.8	±3.9	±11.8	±12.9	±13.4
GFR	0.337	0.389	0.405	0.381	0.409	0.392
ml/min/g	±0.025	±0.023	±0.024	±0.037	±0.041	±0.052
V	8.7	13.6 *	14.5 *	13.6	13.5	10.6
µl/min/g	±1.2	±2.0	±1.7	±2.2	±2.3	±1.7
FENa	2.29	2.25	3.56 **	4.42 ***	4.24 **	3.25
%	±0.24	±0.30	±0.32	±0.66	±0.78	±0.32
FEK	39.97	46.87	51.21 *	59.78 *	56.31 *	56.59
%	±3.5	±3.2	±2.82	±4.37	±5.72	±8.11
FECl	1.77	2.06	2.63 *	3.03 *	3.36 *	2.80
%	±0.27	±0.24	±0.31	±0.46	±0.69	±0.45

Values are given as means ± SEM. Abbreviations: MAP – mean arterial pressure, HR – heart rate, GFR – glomerular filtration rate, V – urine flow rate, FENa, FEK, FECl – fractional excretions of sodium, potassium and chloride; n – number of animals. The value for GFR and V are expressed per gram kidney weight.

* – $p<0.05$, ** – $p<0.01$, *** – $p<0.001$, comparison to control period

As shown on Table I during 90 min renal ischemia MAP was significantly elevated ($p < 0.001$), while HR decreased ($p < 0.01$), as compared to the control period. MAP remained increased in postischemic periods ($p < 0.01$ for the first and $p < 0.05$ for the second period), but HR returned to control levels. Hematocrit (Hct) was constant throughout the study (Table I). GFR did not change in the course of the

experiment. Urine flow rate paralleled the dynamics of MAP. It rose during the time of clipping of the left renal artery by 67% ($p < 0.05$), as compared to the control period, and remained elevated after its cessation. Similarly, excretory fractions of all measured electrolytes increased progressively during ischemia, as compared to their control values: FENa% – by 55%, ($p < 0.01$); FEK% – by 21% ($p < 0.05$) and FECl% – by 49% ($p < 0.05$) and sustained their high levels in postischemic periods (for the second postischemic period FENa% increased by 85% ($p < 0.01$), FEK – by 41% ($p < 0.05$) and FECl% – by 90% ($p < 0.05$). On Fig. 1 the data of MAP, V, FENa and FECl for the 8 animals followed during postischemic periods are presented. The function of the left clamped kidney was considerably diminished during postischemic periods: GFR decreased by 77.2% (from (.30±0.01 to 0.069±0.02 ml/min/g), $p < 0.001$; V – by 56.6% (from 7.7±1.65 to 3.3±1.1 μl/min/g), $p < 0.05$; sodium tubular reabsorption – by 77.9% (from 44.3±3.2 to 9.8±2.9 μmol/min/g), $p < 0.001$; potassium tubular reabsorption – by 92.1% (from 0.69±0.13 to 0.05±0.03 μmol/min/g), $p < 0.001$ and chloride tubular reabsorption – by 76.2% (from 30.4±2.4 to 7.2±2.2 μmol/min/g), $p < 0.001$), as compared to their control values (data not shown). The data for the hormones investigated are presented an Table II. However, when Student's *t*-test for paired data was performed ANP plasma concentrations were significantly elevated on 75 min ($P < 0.01$) and 105

Table II

Effect of unilateral 90 min renal ischemia on hematocrit, ANP, AVP and Dopamine plasma levels

	Control period	Ischemic period			Postischemic period			
	30 min	75 min	105 min	135 min	150 min	195 min	225 min	270 min
Hct	42.69	42.56	42.78	43.28	44.00	43.94	43.69	44.50
%	±0.99	±0.92	±0.97	±0.95	±2.50	±2.42	±2.46	±2.44
	n=32	n=32	n=32	n=32	n=8	n=8	n=8	n=8
ANP	21.50	24.94	26.33	30.23	42.32	22.99	25.93	21.30
pg/ml	±2.58	±3.12	±3.73	±4.90	±12.11	±5.26	±5.05	±3.44
	n=17	n=15	n=16	n=15	n=8	n=7	n=8	n=8
AVP	0.860	0.416	0.748	0.809	0.391	0.508	0.442	0.680
pg/ml	±0.218	±0.084	±0.220	±0.207	±0.081	±0.147	±0.107	±0.283
	n=18	n=16	n=19	n=20	n=8	n=8	n=8	n=8
D	0.516	0.542						
μg/ml	±0.073	±0.080						
	n=14	n=12						

Values are given as means ± SEM. Abbreviations: Hct – hematocrit, ANP – atrial natriuretic peptide, AVP – vasopressin, D – dopamine; n – number of animals

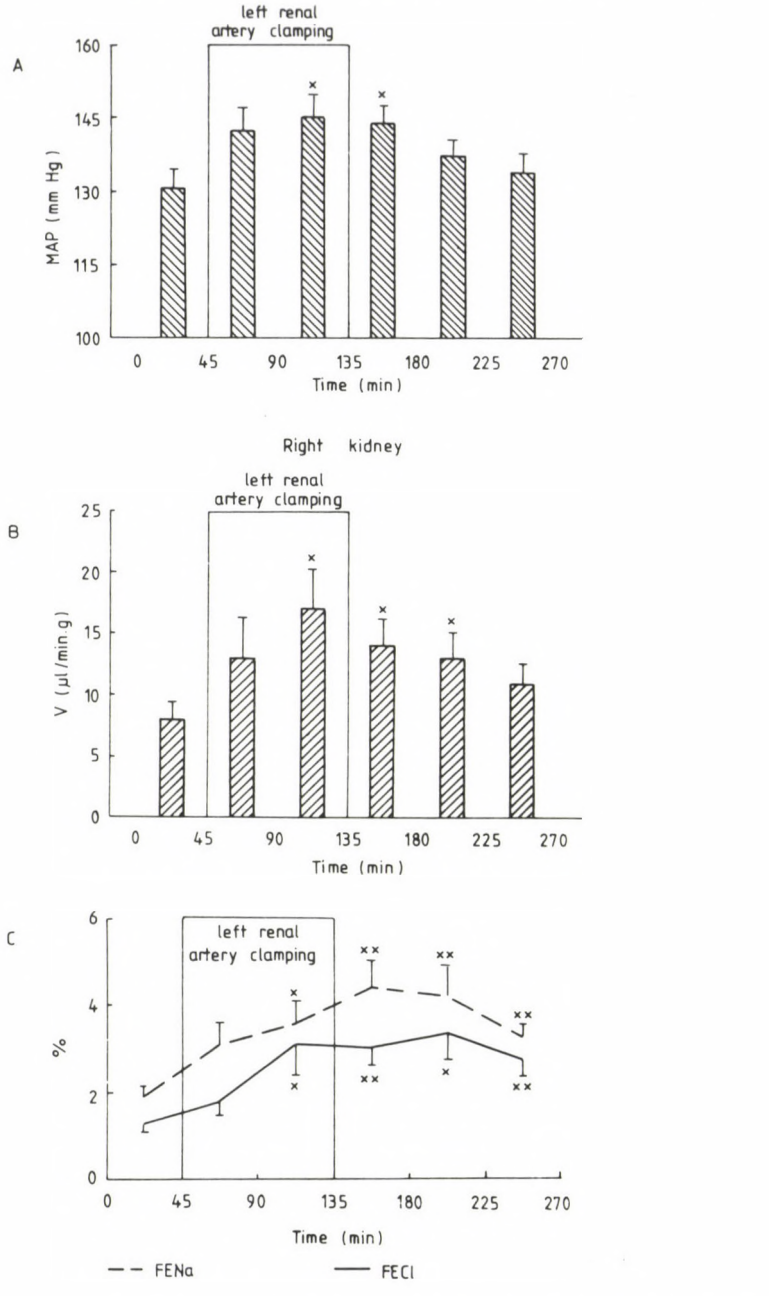

Fig. 1. Mean arterial pressure (MAP) and right kidney urine flow rate (V), fractional excretion of sodium (FENa) and potassium (FEK). The experiments were carried out on 8 animals. The data from 1 control, 2 ischemic and 3 postischemic periods are presented. The values for V are expressed per gram kidney weight. * – $p < 0.05$; ** m- $p < 0.01$, comparison to control period

min ($p < 0.05$). The changes of the excretory parameters were not concomitant with any variations of plasma levels of AVP and dopamine (Table II), which did not show any statistically significant alterations during the periods investigated.

Discussion

The results of the present study demonstrate that in anaesthetized dogs the excretion of water and electrolytes of the intact kidney is enhanced during 90-min. contralateral renal artery clamping, parallel to the elevation of MAP. This hypertensive response is consistent with findings in other reports [4, 7, 8, 14]. According to Hermansson et al. it is due, besides to the activation of renin-angiotensin system (RAS) during ischemia, also to a stimulation of an afferent renal nerve activity [9]. Since AVP and dopamine do not change significantly during ischemia their influence on MAP in anaesthetized dogs with unilateral renal artery clamping can be eliminated. However, for the elevation of MAP an additional role of the redistribution of blood volume and the increase of the peripherial resistance after the prompt exclusion of the left kidney from circulation should be considered, confirmed by the reflex decrease of HR at the time of ischemia. Further, in agreement with the already mentioned works of Harris et al. and Freeman et al., in our investigation MAP maintained its high values in postischemic periods, possibly due to the sustained elevation of PRA, established in their studies [7, 8].

Our results demonstrate an augmented excretion of water and electrolytes of the intact kidney, which to a considerable extent compensates the clamped left kidney function during ischemic as well as during postischemic periods, thus indicating kidney ability for a quick adaptation to the new demands of the organism. As it was pointed out already, in our study the enhancement of diuresis and electrolyte excretion is concomitant with the increase of MAP, which occurred to be one of the reasons for the results.

Recent experimental findings show that ANP is involved in volume and pressure homeostasis [11]. On the other hand, it is recently reviewed by K. Goetz, that concentrations of ANP within or slightly above the normal range, as in our case, do not cause potent or rapidly developed natriuretic responses in experimental animals [5]. However, our data demonstrate a significant elevation of ANP plasma concentrations on 75 and 105 min, despite the increase of diuresis and natriuresis to the end of the experiment. Hence, it appears that during the ischemic period ANP acts together with MAP to enhance right renal excretory function. Furthermore, AVP plasma levels do not change significantly either indicating that neither the 90-min unilateral ischemia, nor any hemodynamic changes during the experiment (15) are stimuli capable to alter its concentrations in anaesthetized dogs. However, the

influence of dopamine on excretory function of the intact kidney appears also unlikely since its plasma levels do not change at the time of ischemia.

Moreover, our results demonstrate that GFR does not change throughout the whole experiment, which indicates that the enhancement of excretory function of contralateral kidney during unilateral ischemia and in postischemic periods are connected mainly with changes of tubular transport processes. Other influences of intrarenal factors as redistribution of renal blood flow, actions of locally produced hormones, etc. can also be considered.

In summary, the results of the present study demonstrate that during 90-min unilateral renal artery clamping the renal excretory function is compensated by the enhanced function of the opposite kidney. This is due to the elevated MAP, as well as to the increased ANP plasma levels.

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REGIONAL DIFFERENCES IN THE CONTRACTILE ACTIVITY OF NEUROPEPTIDE Y, ENDOTHELIN, OXYTOCIN AND VASOPRESSIN: COMPARISON WITH NON-PEPTIDERGIC CONSTRICTORS

AN *IN VITRO* STUDY IN THE BASILAR AND MESENTERIC ARTERIES OF THE RAT

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The vasoconstrictor effect of the peptides neuropeptide Y (NPY), endothelin (ENDO), vasopressin (VPR) and oxytocin (OXY) ($10^{-11} - 10^{-7}$ M) was compared in the isolated basilar (BAS) and mesenteric (MES) arteries of rat. The contractile activity of these peptides was compared to that of three nonpeptidergic constrictors: noradrenaline (NA), serotonin (5-hydroxytryptamine, 5-HT) and prostaglandin $F_2\alpha$ ($PGF_2\alpha$) ($10^{-8} - 10^{-4}$ M). As regards EC_{50} values, $PGF_2\alpha$ was equally potent in both vessels studied, 5-HT was more potent in BAS and NA was without contractile effect in BAS. Pronounced regional differences were found for the peptides studied. BAS was more sensitive in EC_{50} values to the peptides in the order ENDO ≥ VRP > OXY > NPY. In MES, OXY and NPY caused no and VPR caused weak contraction, whereas the effect of ENDO was pronounced, with a similar EC_{50} value as in BAS. In conclusion, marked regional differences were found in response to contractile agents in the vascular beds studied. Peptidergic constrictor mechanisms might be of large importance in the regulation of cerebral blood flow during physiological or pathophysiological conditions.

Keywords: neuropeptides, neuropeptide Y, endothelin, vasopressin, oxytocin, basilar artery, mesenteric artery, rat

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It is now well recognized that – in addition to classical transmitters – the nerve fibers supplying the cardiovascular system contain other transmitters as well, including regulatory peptides. To our present knowledge, the most widely distributed peptides are the constrictor neuropeptide Y (NPY), and the dilators calcitonin gene – related peptide, substance P and vasoactive intestinal polypeptide [see 14, 21, 22, 28]. Oxytocin (OXY) was also found in some nerves supplying blood vessels [2, 25]. There is evidence for the co-existence of noradrenaline with a vasopressin- (VPR-) like peptide in some sympathetic nerves [10]. Recent investigations have identified an extremely potent constrictor peptide produced in endothelial cells, called endothelin (ENDO) [29]. Several studies have shown its existence in neural tissue as well [17, 19, 31]. A recent study showed that ENDO is also present in the posterior pituitary system [32]. Therefore, these peptides may influence vascular tone both locally, released from nerves supplying blood vessels, and as circulating hormones, originating from various sources (nerves, hypophysis, endothelium). If they are produced/released locally, they can act differently in various vascular areas, depending on the local concentration. If they act, however, as circulating hormones, a possible differential reactivity of different vascular beds may influence the blood supply of the various organs differently, thus, causing blood redistribution. It is therefore of particular interest to study regional differences in the vascular responses to vasoactive agents.

The aim of the present study was to compare the contractile activity of the peptides NPY, ENDO, VPR and OXY in two vascular beds of the rat: in the basilar (BAS), and mesenteric (MES) artery. Since previously all these peptides have been suggested to play an important role in the development of cerebral vasospasm [1, 2, 3, 5, 7, 8, 17, 20, 21, 25, 26], the present work might help to elucidate the selectivity of these peptides for the cerebral vessels. The action of the peptides was also compared to the effect of the following non-peptide constrictors: noradrenaline (NA) and serotonin (5-hydroxytryptamine, 5-HT) and prostaglandin $F_2\alpha$ ($PGF_2\alpha$).

Methods

Male Sprague-Dawley rats weighing 300–400 g were anaesthetized with diethyl ether. The brains were quickly removed and put into cold Krebs-Henseleit solution. Subsequently, pieces of the mesenterial tissue containing small branches of the mesenteric artery were excised. Care was taken to avoid stretching or injuring the vessels. 2 mm long vessel segments (several segments from each animal) were placed on two L-shaped stainless steel holders, one of which was attached to a Grass FT-03 force transducer for measurement of isometric circular muscle contractions following exposure to various agents. The organ bath contained Krebs-Henseleit solution of the following composition (in mM): NaCl 118; KCl 4.5; $CaCl_2x2H_2O$ 2.5; $MgSO_4x7\ H_2O$ 1.2; $NaHCO_3$ 25; KH_2PO_4 1.0; glucose 6.0 and aerated with a gas mixture containing 88.5% O_2 and 11.5% CO_2. The temperature of the solution was kept at 37°C, the pH was 7.35 [11]. In a maximally depolarizing solution all NaCl was replaced with KCl to achieve a final K^+ concentration of 123.5 mM. After mounting, the vessels were incubated for 40–60 min

at a tension of 2.5 and 4-5 mN in BAS and MES, respectively. The passive load applied was of a magnitude that would give a maximum contractile response of the vessels as tested in separate control experiments. Before starting the experiments, the contractile ability of the vessels was tested with the maximally depolarizing solution. The following drugs were used: L-noradrenaline, prostaglandin F_2 (Sigma), 5-HT creatinine sulphate (Alcon), neuropeptide Y, endothelin, arginine-vasopressin, oxytocin (all Peninsula). All drugs were dissolved in saline. The concentracion of the agonist inducing half maximum contraction (EC_{50}) was assessed graphically for each dose-response curve. Student's *t*-test for unpaired samples was used for statistical analysis of the data, and a difference was considered as significant if $p < 0.05$. Data are shown as means ± standard error of the mean. The number of different vessel segments studied is shown by *n*.

Results

The E_{max} values for the maximally depolarizing potassium solution were different in the two vascular beds tested, showing a stronger contractile ability of the vascular smooth muscle in MES, which has approximately double the number of cell layers compared to BAS. Therefore, to compare the effect of an agonist in various vascular beds, its contractile action was expressed as per cent tension of the maximal contraction observed in the maximal depolarizing solution (Figs 1–7). To compare the effect of the different constrictors in the same vascular region, the absolute values of the achieved tension (mN) are shown (Figs 8–9). In the Tables I-II the E_{max} (mN) and EC_{50} (M) values for the various agonists are also indicated.

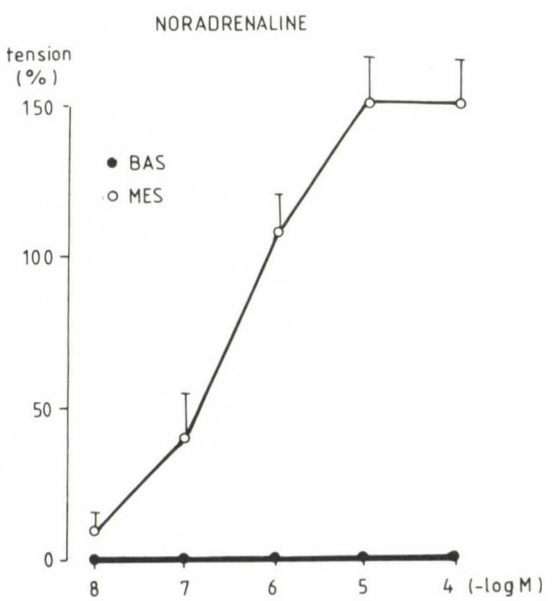

Fig. 1. Contractile effect of noradrenaline (10^{-8}–10^{-4} M) in the basilar (BAS, n = 11) and mesenteric (MES, n = 11) arteries of rat. Contraction is expressed as per cent of the contraction induced by the maximal depolarizing solution

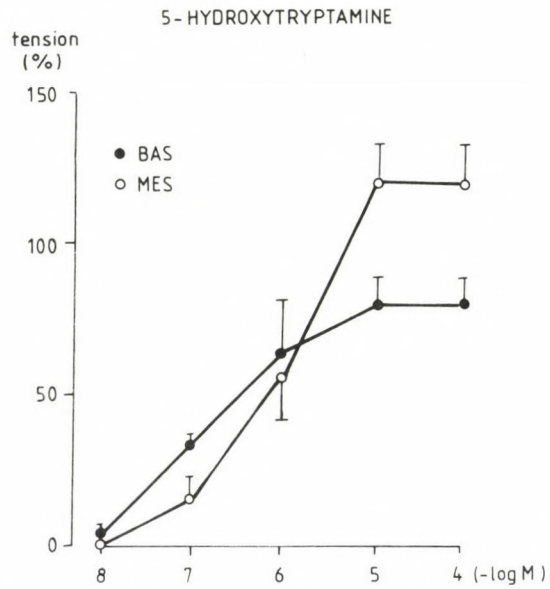

Fig. 2. Contractile effect of 5-hydroxytryptamine ($10^{-8}-10^{-4}$ M) in the basilar (BAS, n = 10) and mesenteric (MES, n = 17) arteries of rat. Contraction is expressed as per cent of the contraction induced by the maximal depolarizing solution

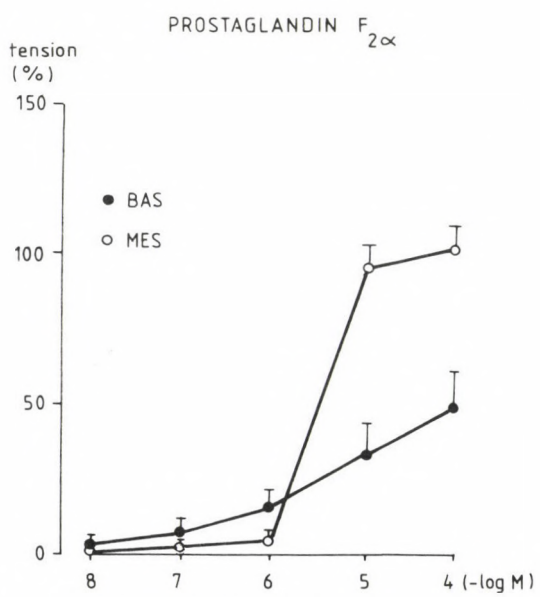

Fig. 3. Contractile effect of prostaglandin $F_2\alpha$ (10^{-8} – 10^{-4} M) in the basilar (BAS, n = 7) and mesenteric (MES, n = 9) arteries of rat. Contraction is experessed as per cent of the contraction induced by the maximal depolarizig solution

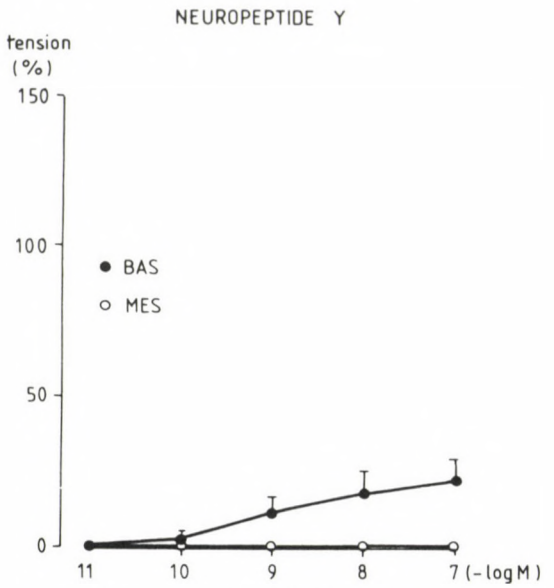

Fig. 4. Contractile effect of neuropeptide Y ($10^{-11}-10^{-7}$) in the basilar (BAS, n = 10) and mesenteric (MES, n = 17) arteries of rat. Contraction is expressed as per cent of the contraction induced by the maximal depolarizing solution

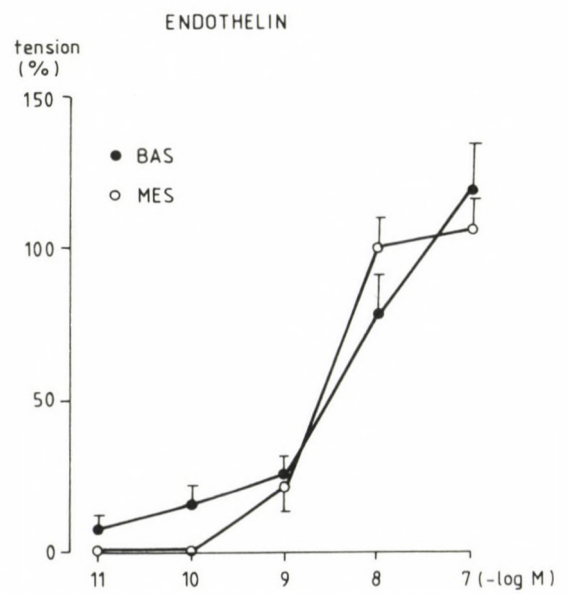

Fig. 5. Contractile effect of endothelin ($10^{-11}-10^{-7}$) in the basilar (BAS, n = 7) and mesenteric (MES, n = 9) arteries of rat. Contraction is expressed as per cent of the contraction induced by the maximal depolarizing solution

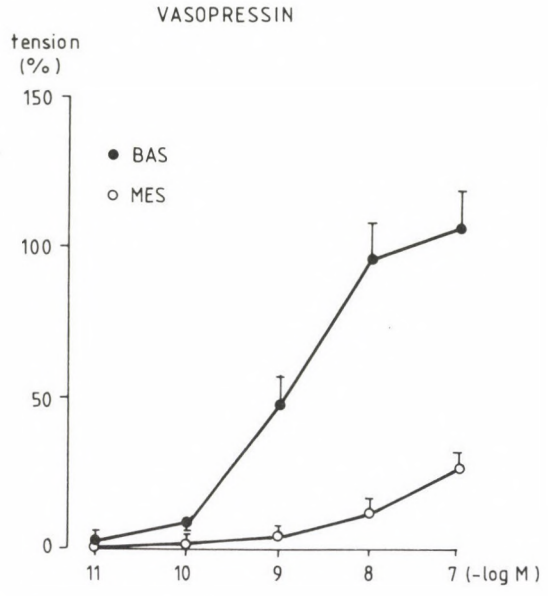

Fig. 6. Contractile effect of vasopressin ($10^{-11}-10^{-7}$ M) in the basilar (BAS, n = 11) and mesenteric (MES, n = 9) arteries of rat. Contraction is expressed as per cent of the contraction induced by the maximal depolarizing solution

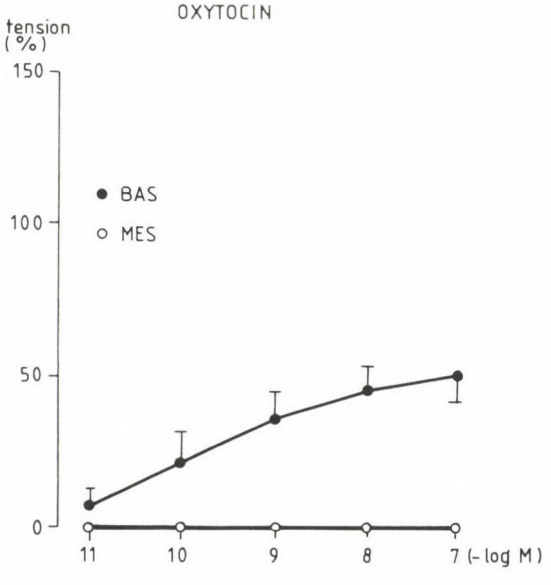

Fig. 7. Contractile effect of oxytocin ($10^{-11}-10^{-7}$ M) in the basilar (BAS, n = 12) and mesenteric (MES, n = 11) arteries of rat. Contraction is expressed as per cent of the contraction induced by the maximal depolarizing solution

Non-peptidergic constrictors

NA, 5-HT and $PGF_2\alpha$ contracted the BAS, and MES in the concentration range of $10^{-8}-10^{-4}$ M (Figs 1−3). With the exception of NA in BAS, all agents caused substantial contractions (Figs 1−2). The EC_{50} value for 5-HT in BAS was significantly lower than in MES (Tables I−II). The maximal contraction in per cent of the contraction induced by maximal depolarization was higher in MES than in BAS (Figs 1−3).

Peptidergic constrictors

NPY, ENDO, VPR and OXY contracted the arteries in the concentration range of $10^{-11}-10^{-7}$ M (Figs 4−7). NPY and OXY caused relatively weak contractions in BAS, and NPY was without contractile action in MES (Fig. 4). Endothelin, however, caused strong contractions both in BAS and MES. A fairly weak contractile action of VPR was found in MES, in comparison to the pronounced response in BAS (Figs 6−7). The EC_{50} value for vasopressin was significantly lower in BAS than in MES (Tables I−II).

Table I

Contractile effect (E_{max} and EC_{50} values) of various agonists in the basilar artery of the rat

Agonists	n	E_{max}(mN)	EC_{50}(M)
Noradrenaline	11	0	–
5-hydroxytryptamine	8	2.47±0.43	$3.21\pm1.04\cdot10^{-7}$**
Prostaglandin $F_{2\alpha}$	9	1.62±0.43	$1.21\pm0.63\cdot10^{-5}$
Neuropeptide Y	10	0.83±0.24	$2.54\pm0.93\cdot10^{-8}$
Endothelin	7	4.07±0.98	$9.21\pm4.39\cdot10^{-9}$
Vasopressin	11	3.35±0.51	$2.68\pm0.94\cdot10^{-9}$***
Oxytocin	12	1.48±0.22	$2.33\pm0.94\cdot10^{-9}$

The contractions induced by 123.5 mM K^+ varied between 3.09±0.21 and 3.41±0.34 mN in the different groups. **,*** indicates significant differences between the EC_{50} value of the agonist in BAS and in MES. ($p<0.01$ and 0.001, respectively)

Table II

Contractile effect (E_{max} and EC_{50} values) of various agonists in the mesenteric artery of the rat

Agonists	n	E_{max}(mN)	EC_{50}(M)
Noradrenaline	11	15.82 ± 1.45	$5.63 \pm 0.93 \times 10^{-7}$
5-hydroxytryptamine	9	13.16 ± 1.28	$2.41 \pm 0.62 \times 10^{-6}$
Prostaglandin $F_{2\alpha}$	9	11.16 ± 1.28	$5.12 \pm 0.22 \times 10^{-6}$
Neuropeptide Y	17	0	–
Endothelin	9	12.22 ± 1.13	$4.58 \pm 0.62 \times 10^{-9}$
Vasopressin	9	3.49 ± 0.83	$4.31 \pm 0.87 \times 10^{-8}$
Oxytocin	11	0	–

The contractions induced by 123.5 mM K^+ varied between 10.08 ± 0.59 and 11.80 ± 0.98 mN in the different groups

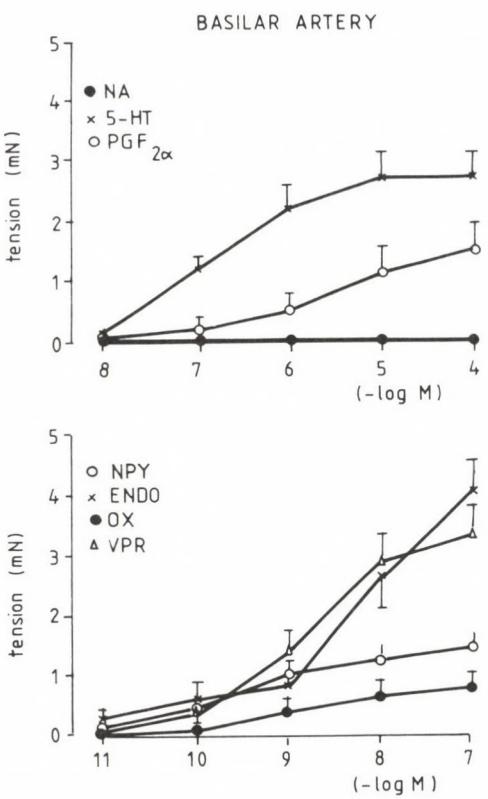

Fig. 8. Contractile effect of noradrenaline (NA), 5-hydroxytryptamine (5-HT), prostaglandin $F_{2\alpha}$($PGF_{2\alpha}$) (10^{-8} – 10^{-4} M) and neuropeptide Y (NPY), endothelin (ENDO), vasopressin (VPR) and oxytocin (OXY) (10^{-11} – 10^{-7} M) in the basilar artery of rat. (Absolute values of the achieved tensions are presented (mN). The number of vessels studied are presented on the legends to Figs 1-7)

Comparison of the responses of the various arteries

In Figs 8–9 the absolute values of the contractions induced by non-peptidergic (upper panel) and peptidergic (lower panel) agents are shown. In BAS, the most potent constrictors were 5-HT, ENDO and VPR, whereas in MES NA, 5-HT, $PGF_2\alpha$ and ENDO were strongest. No contractions were observed in response to NA in BAS and to NPY and OXY in MES.

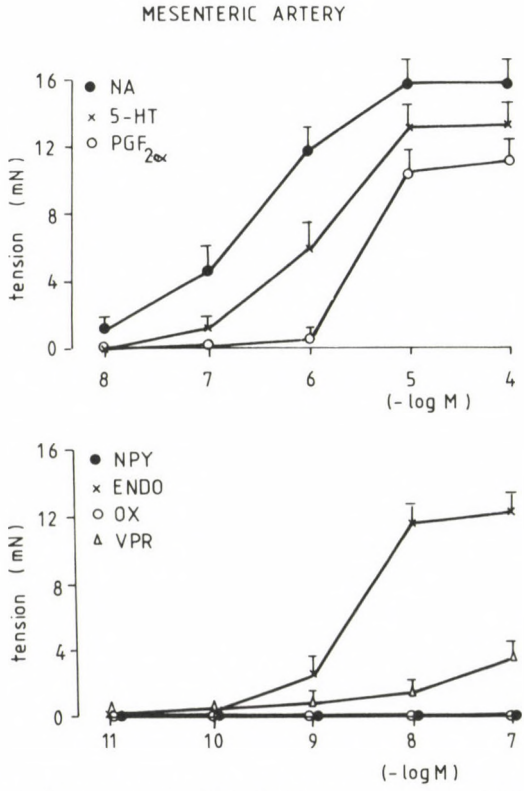

Fig. 9. Contractile effect of noradrenaline (NA), 5-hydroxytryptamine (5-HT), prostaglandin F_2(PGF_2) (10^{-8}–10^{-4} M) and neuropeptide Y (NPY), endothelin (ENDO), vasopressin (VPR) and oxytocin (OXY) (10^{-11}–10^{-7} M) in the mesenteric artery of rat. (Absolute values of the achieved tensions are presented (mN). The number of vessels studied are presented on the legends to Figs 1-7)

Discussion

Non-peptidergic constrictors

NA failed to induce any contraction in BAS, consistently with previous findings in this vessel [13]. This is due to the absence of postsynaptic alpha-adrenoceptors mediating contraction in this artery. In MES, however, NA induced marked contractions. In general, alpha-adrenergic contraction is weak in large pial arteries of various species, in comparison to the response in large non-cerebral arteries like the mesenteric artery [18]. It is noteworthy that in the mesenteric artery the noradrenaline-induced maximal contraction was higher than the contraction in response to the maximal depolarizing solution. Similar phenomenon was observed in the mesenteric artery of the cat as well [18]. The dense sympathetic innervation of the vessels of the splanchnic region contributes as a regulating mechanism for blood redistribution [6]. 5-HT caused equal maximal contractions, but at significantly lower concentrations in BAS. The high constrictory sensitivity to 5-HT in cerebral vessels might implicate a role for 5-HT in the pathophysiology of subarachnoid hemorrhage and migraine headache [4].

Neuropeptide Y

Previous studies have demonstrated a potent contractile action of NPY in various vascular beds, with threshold effects in the nanomolar concentration range. NPY has been suggested to play a role in the modulation of sympathetic neurotransmission. [21, 22, 28]. We found relatively weak contractions in response to NPY in BAS and no response in MES. In BAS, the contraction induced by 10^{-7} M NPY did not reach 20% of the contraction induced by the depolarizing potassium solution. Similarly to results of the present study, pial arteries have been found to be more sensitive to NPY than peripheral ones in man and rabbit [9].

Endothelin

The peptide was isolated in 1988 from the supernatant of an endothelial cell culture [29]. It has been suggested that increased release of ENDO increases vascular resistance and contributes to chronic hypertension or vasospasm [24, 30]. In human cranial blood vessels (including pial arteries and veins, the middle meningeal artery and the superficial temporal artery), ENDO has been shown to cause pronounced contractions, with higher sensitivity and higher maximal contractile tension in the pial artery [12]. The potent contraction in cerebral (especially pial) arteries (see also [3, 8,

12, 20]) supports the hypothesis that ENDO may play a role in the development of cerebral vasospasm.

Oxytocin, vasopressin

Both VPR and OXY contracted BAS whereas MES responded with a weak contraction to only VPR. For VPR the EC_{50} value in BAS was significantly lower than in MES. The EC_{50} values in BAS were similar for VPR and OXY, whereas the E_{max} was considerably higher for VPR. Both E_{max} and EC_{50} values for VPR and ENDO were similar in the pial artery. This finding (in accordance with recent *in vivo* results by Faraci [8]) suggests that VPR might act as a sensitive regulator of cerebrovascular tone. In 1984 Katusic et al. reported that VPR causes potent endothelium dependent relaxations in the basilar artery of the cat [15]. Since the effect of VPR was less pronounced in the coronary artery, and a constriction was observed in systemic resistance vessels, these authors speculated that during hemorrhage and shock VPR may cause redistribution of blood to the brain. In 1987, Katusic and Krstic [16] seemed to confirm this hypotesis in rat vessels, since they found that VPR causes pronounced (endothelium-independent) contractions in the aorta and renal artery, but not in the common carotid artery. The present results in rat are against this hypothesis, since BAS was very sensitive to VPR, while no endothelium-dependent (or-independent) relaxant action of the peptide was observed (unpublished data). The strong contraction of rat cerebral vessels to VPR and OXY supports the hypothesis of Svendgaard et al. [5, 27] that these substances may be involved in the cerebral vasospasm following subarachnoid hemorrhage.

Less is known about the vascular effects of the other neurohypophyseal hormone, oxytocin. In human BAS its potent contractile action has been observed [1]. The present results show that OXY causes contraction also in the rat BAS. Although OXY has many peripheral and central cardiovascular actions (see [23]) it is worthwhile to notice the similarities in the vascular action of both neurohypophyseal hormones, which may indicate a similar function.

Acknowledgement

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EFFECT OF SMALL CHANGES IN EXTRACELLULAR MAGNESIUM CONCENTRATION ON THE TONE OF FELINE MESENTERIC ARTERIES: INVOLVEMENT OF ENDOTHELIUM

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The aim of this study was to investigate the effect of small alterations in extracellular magnesium concentration on the tone of feline mesenteric arteries and to examine the role of endothelium in these responses. We measured isometrical tension of isolated arterial rings, placed between two stainless steel wires in a tissue chamber containing Krebs-Henseleit solution, aerated with a gas mixture containing 95% O_2 and 5% CO_2 at 37°C. After precontraction with noradrenaline, a decrease of extracellularc magnesium concentration from 1.2 mM to 1.0 and 0.8 mM resulted in sustained relaxations, whereas the elevation of extracellular magnesium from 0.8 mM to 1.2 mM caused an increase in vascular tone when endothelium was intact. The magnesium-withdrawal related dilations were absent in endothelium-denuded vessels and were inhibited by oxyhemoglobin ($5 \cdot 10^{-6}$ M) and methylene blue (10^{-5} M), suggesting the involvement of endothelium-derived relaxing factor in this vascular response. Nifedipine ($5 \cdot 10^{-7}$ M) or dichlorobenzamil ($3 \cdot 10^{-5}$ M), however, did not affect the magnesium-deficiency related relaxations. Therefore, in this vascular action of magnesium, nifedipine-sensitive calcium channels or the sodium- calcium antiport system are not involved. We conclude that small alterations in extracellular magnesium concentration, possibly within the physiological range, are able to modify the basal formation and release of EDRF, and thus alter arterial smooth muscle tone in this vascular bed. This endothelium- and magnesium-dependent system appears to be more sensitive than the direct smooth muscle actions of magnesium. The possible physiological and pathophysiological consequences of these observations are discussed.

Keywords: extracellular magnesium, endothelium-dependent relaxation, basal EDRF release, mesenteric artery, vascular smooth muscle

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Recent observations have shown, that small changes in the extracellular magnesium concentration modify noradrenaline-induced smooth muscle contractions and acetylcholine-induced endothelium-dependent relaxations in the cerebral and mesenteric arteries of the cat [6, 24, 25]. Marked regional differences were found in the modulatory action of magnesium on the contractions; the cerebral vessel was more sensitive. A regional difference, however, was not observed in the modulatory action of magnesium on acetylcholine-induced relaxations.

Extracellular magnesium modulates the basal release of endothelium-derived relaxing factor (EDRF) as well [8, 13, 26, 27, 28, 31]. Recent results in cerebral arteries and in systemic veins showed that this action is pronounced even in the concentration range between 0.8 and 1.2 mM [26, 27, 28]. The present experiments have been designed to elucidate whether the feline mesenteric arteries are also responsive to changes in magnesium concentration around the physiological concentration range. The mechanism of magnesium action was also investigated.

Methods

Nine cats of both sexes weighing 1.9–2.8 kg anaesthetized with sodium pentobarbital (Nembutal, 30 mg/kg i.p.), and exsanguinated via a femoral artery catheter were used for this study. Microsurgical methods and a Zeiss operation microscope were used to remove and clean small segments of the mesenteric artery. Care was taken to avoid stretching or injuring the vessel.

One to 3 mm long vessel segments were placed on two L-shaped stainless steel specimen holders (0.1 mm in diameter), one of which was attached to a Grass FT03 (Quincy, Mass, USA) force transducer, for isometrical tension measurements. The position of the other holder could be adjusted by a micromanipulator. The vessel segments were placed into a tissue chamber containing Krebs-Henseleit solution of the following composition (in mM): NaCl 119; KCl 4.6; $CaCl_2$ 1.5; $MgCl_2$ 1.2; $NaHCO_3$ 15; NaH_2PO_4 1.2; glucose 6 and bubbled with a gas mixture containing 95% O_2 and 5% CO_2. The temperature of the solution was kept at 37°C, the pH was 7.4 [10]. The vessels were incubated for 60–90 min at a tension of 600 mg. All measurements were carried out in the presence of $5 \cdot 10^{-6}$ M indomethacin and $5 \cdot 10^{-7}$ M propranolol, to block the production of cyclooxygenase products and β-receptor activation, respectively. Magnesium concentration of the Krebs-Henseleit solution was adjusted to 1.0 and 0.8 mM by altering the NaCl concentration: thus isotonicity of the solution was maintained at normal. First, cumulative dose-responses for noradrenaline (10^{-8}–10^{-5} M) and acetylcholine (10^{-8}–10^{-5} M) were tested. Acetylcholine caused a dose-dependent relaxation, bringing the vascular tone to the level recorded before the application of the precontractile agent. (In some vessel preparations, acetylcholine caused dose-dependent, but partial relaxations, bringing the vascular tone maximally to about 50% of the level of precontraction. Since this response might reflect a partial endothelium damage, these vessels were excluded from the present investigations.)

The vessels were precontracted with $5 \cdot 10^{-6}$ M noradrenaline. After the tone reached a steady level, the incubation medium containing a given concentration of magnesium was replaced with a medium containing the same concentration of noradrenaline, indomethacin and propranonol, but a slightly altered level of magnesium. After the new tone stabilized, similar replacement of the solution was performed again, restoring the original magnesium concentration in the medium. Finally, responses to acetylcholine were tested. After repetitive washouts with the normal solution containing 1.2 mM

magnesium, the vessel tone returned to the baseline. Then, after precontraction, another change in the magnesium concentration was performed. The changes in the extracellular magnesium from 1.2 mM to 1.0 and 0.8 mM, and from 0.8 mM to 1.2 mM were tested (in this latter case the vessels were incubated for 5 min in the solution of 0.8 mM magnesium before the application of noradrenaline). Magnesium withdrawal from 1.2 mM to 0.8 mM was also tested in vessels pretreated with hemoglobin ($5 \cdot 10^{-6}$ M), methylene blue (10^{-5} M), nifedipine ($5 \cdot 10^{-7}$ M) or dichlorobenzamil ($3 \cdot 10^{-5}$ M) for 15 min followed by a contraction by $5 \cdot 10^{-6}$ noradrenaline.

Endothelium was removed by gentle rubbing of the intimal surface of the arterial segments with a stainless steel wire. The effectiveness of the endothelium removal was verified in each ring by absence of relaxation in response to acetylcholine [7].

The following drugs were used: acetylcholine chloride (Sigma, St. Louis, USA), indomethacin (Sigma), noradrenaline (Gedeon Richter Chemical Works, Budapest, Hungary), propranolol hydrochloride (Sigma), nifedipine (Bayer, Germany), methylene blue (Sigma), dichlorobenzamil (Dr. E. J. Cragoe Jr., USA). All drugs were dissolved in saline, except indomethacin and nifedipine (dissolved in 50% ethanol), dichlorobenzamil (dissolved in dimethylsulphoxide) and noradrenaline (dissolved in distilled water containing ascorbic acid 1 mM). Hemoglobin was prepared as previously described [5] from the carefully oxygenated arterial blood of the cat. The blood was collected through a polyethylene cannula from the femoral artery into heparin-containing tubes. The blood was centrifuged at 2000 *g* and washed several times with Krebs-Henseleit solution. Red blood cells were hemolized then with distilled water and diluted in Krebs-Henseleit solution. Oxyhemoglobin concentration of the stock solutions was measured spectrophotometrically.

Student's *t*-test for paired and unpaired samples was used for statistical analysis of the data, and a difference was considered as significant if $p < 0.05$. Data are shown as means ± standard error of the mean.

Results

Lowering of extracellular magnesium concentration from 1.2 to 1.0 mM caused sustained relaxations of arteries precontracted with $5 \cdot 10^{-6}$ M noradrenaline (Fig. 1 and Fig. 2, left panels). The vessel tone stabilized at approximately 95% of the original tone induced by noradrenaline. If the magnesium concentration was lowered to 0.8 mM, the tone decreased to 50% of the precontraction (Fig. 1, right panel and Fig. 2, middle panel). This relaxation represents the maximum response following magnesium withdrawal, since a change in the extracellular magnesium concentration to a value lower than 0.8 mM caused a less pronounced relaxation, whereas at total magnesium withdrawal the tone – after a transient relaxation – stabilized at the initial level (not shown). The effect of variations in the magnesium concentration on the tone of the artery were reversible (Fig. 1, right panel).

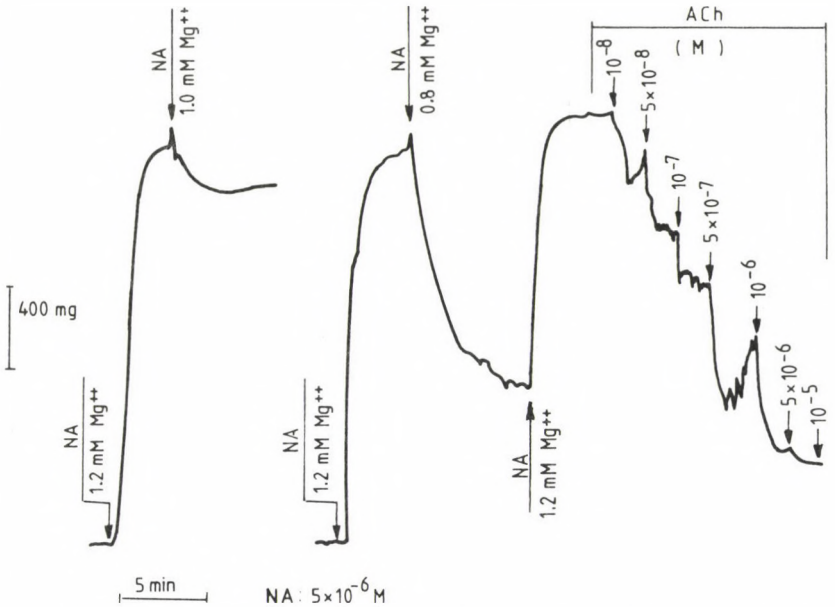

Fig. 1. Typical tracings showing the effect of fast changes in the extracellular magnesium concentration from 1.2 mM to 1.0 and 0.8 mM (left and right panels, respectively) in the feline mesenteric artery precontracted with noradrenaline (NA, $5 \cdot 10^{-6}$ M). In the right panel the effect of restoration of the magnesium concentration to 1.2 mM and then a response to acetylcholine (ACh, $10^{-8} - 10^{-5}$M) is also shown

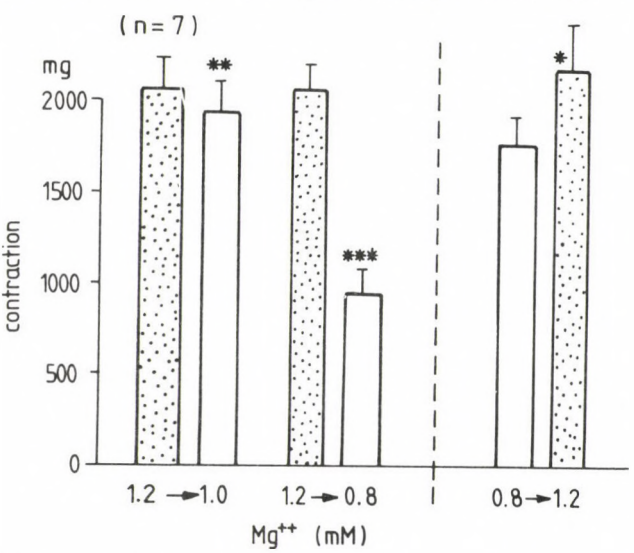

Fig. 2. Effect of fast changes in the extracellular magnesium concentration on the vascular tone of feline mesenteric artery. Absolute values of contractions are expressed in milligrams. Extracellular magnesium concentration was changed from 1.2 mM to 1.0 and 0.8 mM as well as from 0.2 mM to 1.2 mM after precontraction with noradrenaline ($5 \cdot 10^{-6}$ M). Asteriks (*,**,***) indicate significant differences compared to the response observed at 1.2 mM magnesium ($p < 0.05$, 0.01 and 0.001, respectively)

If the vessels were incubated first in a solution containing 0.8 mM magnesium and then magnesium was restored to 1.2 mM, we observed an elevation in the vascular tone (Fig. 2, right panel).

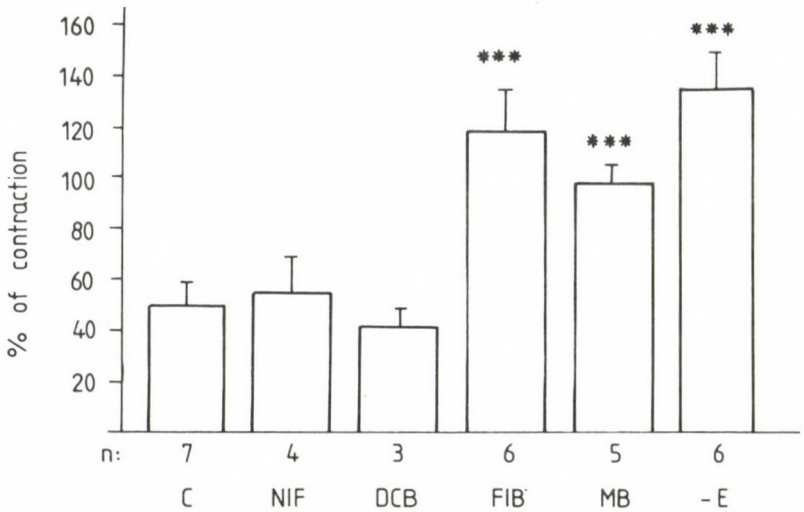

Fig. 3. Effect of nifedipine (NIF, $5 \cdot 10^{-7}$ M), dichlorobenzamil (DCB, $3 \cdot 10^{-5}$ M), hemoglobin (HB, $5 \cdot 10^{-6}$ M), methylene blue (MB, 10^{-5} M) and endothelium removal (-E) on the response to magnesium withdrawal from 1.2 mM to 0.8 mM in feline mesenteric artery precontracted with noradrenaline ($5 \cdot 10^{-6}$ M). The initial contraction was considered as 100%. Asteriks (***) indicate significant differences compared to the control response of the intact vessels (C) ($p < 0.001$)

Figure 3 shows that neither the calcium antagonist nifedipine ($5 \cdot 10^{-7}$ M), nor the sodium/calcium antiport blocker dichlorobenzamil ($3 \cdot 10^{-5}$ M) inhibited the relaxations induced by withdrawal of extracellular magnesium from 1.2 mM to 0.8 mM. On the other hand, oxyhemoglobin ($5 \cdot 10^{-6}$ M), methylene blue (10^{-5} M) or endothelium removal inhibited this relaxation.

Discussion

The present results showed that small changes in extracellular magnesium – similarly to the response of the feline middle cerebral artery [26] – modulate the basal synthesis and/or release of EDRF in the mesenteric artery. The relaxation was not be present in the endothelium-denuded vessels and was blocked by methylene blue and hemoglobin, suggesting the involvement of endothelium and EDRF [17]. Interestingly, decreasing the level of magnesium below 0.8 mM caused much smaller relaxation than a lowering to 0.8 mM. Since magnesium withdrawal induces a dose-dependent elevation in the vascular tone in vessels without endothelium, which is

only about 20% at 0.8 mM (see Fig. 3), but becomes very pronounced at total magnesium deficiency (unpublished observations, see also [1, 2]), probably the direct smooth muscle contractile action of magnesium deficiency counteracting with the relaxant effect of EDRF abolishes the relaxation at total magnesium withdrawal. In a recent work Gold et al. studied the reactions of pulmonary vessels to changes in extracellular calcium and magnesium [8] and found that the algebraic sum of the smooth muscle and endothelial actions of magnesium gives the actual vascular change. These investigators – in accordance with the results of the present study – reported transient endothelium-dependent relaxations followed by sustained endothelium-independent contractions of bovine intrapulmonary vessels in response to total magnesium withdrawal [8].

Similarly to the response of the feline cerebral vessel [8], nifedipine or dichlorobenzamil did not modify the magnesium withdrawal-related dilations, thus, the magnesium deficiency induced calcium influx, which triggers the release of EDRF [8, 13] occurs via a pathway independent from nifedipine-sensitive calcium channels or the sodium/calcium exchange system.

The results of the present and previous [6, 24, 25, 26] studies indicate that marked regional differences exist in the modulatory action of slight changes in extracellular magnesium on the vascular responses: the cerebral vessels are more sensitive than the mesenteric vessels to the modulation by magnesium in respect of agonist-induced contractions [8, 25] and basal EDRF release [26, 28, present study], but there is no regional difference in the modulatory effect of magnesium on agonist-induced, EDRF-mediated dilations [8, 25].

Two opposite effects of magnesium withdrawal have been reported previously: elevation of the vascular tone [1, 2] and endothelium-dependent relaxation [8, 13, 26, 27, 28, 31]. The results of the present work indicate, that – similarly to the findings in feline [26] and human [28] cerebral vessels and feline femoral veins [27] – small changes in the magnesium concentration activate rather the endothelium than the smooth muscle directly. The alterations in magnesium levels studied are in the physiological range since plasma magnesium levels are normally between 1.2 – 0.8 mM under physiological conditions, but magnesium can be as low as 0.4 mM in pathophysiological states [4, 22, 23].

The present findings may also suggest that physiological changes in circulating magnesium levels may play a role in the regulation of EDRF release *in vivo*. This possibility may have important physiological and pathophysiological consequences considering the fact that EDRF (nitric oxide; NO) is an important regulator of blood pressure, tissue perfusion and platelet aggregation [3, 11, 12, 18, 29]. Perivascular magnesium elicits more pronounced actions on the tone of blood vessels than luminal changes in the concentration of this ion [19, 27]. *In vitro* studies showed that this asymmetrical response can be abolished by endothelium removal [27]. Thus,

endothelium exerts a protective action against the vasoconstrictory effect of magnesium deficiency, in vessels with endothelial dysfunction vasospasm may develop [see also 13, 26].

A massive magnesium deficiency has been shown to increase blood pressure in experimental animals [4]. In contrast, slight experimental magnesium deficiency does not elevate or even decrease blood pressure [16]. Similarly, the clinical symptoms of magnesium deficiency usually do not include hypertension – in some cases hypotension occurs – [4, 23]; clinical trials and epidemiological examinations on the relationship between blood pressure and magnesium are controversial as well [30]. A possible cause of this controversy may be the modulatory action of magnesium on EDRF release. In this context, it is noteworthy that essential hypertension is associated with endothelial dysfunction [20] which may shift the balance between the endothelial and direct smooth muscle actions of magnesium towards the smooth muscle.

Sustained release of EDRF is extremely dependent on extracellular calcium influx [9, 14]. Recent work from Moncada's laboratory clearly shows the capability of the endothelial NO-synthesizing pathway to respond to changes in the concentration of calcium around the physiological range, thus, modulating the vascular tone [15]. These authors also conclude, that calcium exerts its physiological actions rather through EDRF than modulating smooth muscle directly, an experimental finding, which may have important pathophysiological consequences e.g. by explaining some controversies which exist on blood pressure regulation in pregnancy [15].

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CALCIUM AS A COUNTERACTIVE AGENT TO STREPTOMYCIN INDUCED RESPIRATORY DEPRESSION: AN *IN VIVO* ELECTROPHYSIOLOGICAL OBSERVATION

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Effect of streptomycin on respiratory function in cats was studied. It was observed that streptomycin at a dose of 40 mg/kg body weight intravenously (i.v.) caused respiratory failure or streptomycin induced respiratory depression (SIRD). This respiratory failure is not linked with Herring-Breuer stretch receptors beacuse the effect remained unaltered in artificially ventilated cats. The involvement of central structures in SIRD can be discarded since intracarotid and intraventricular administration of streptomycin failed to produce any change in respiration. Studies on monosynaptic reflex, dorsal and ventral root activities of spinal phrenic and intercostal nerves, and on fusimotor and α-motor neuron activities of spinal intercostal and phrenic nerves in decerebrated cats indicated clearly that respiratory depression is not only due to blockade at neuromuscular junction but due to functional depression at the level of muscle receptors and spinal cord motor neurons. The respiratory depression induced by streptomycin was more or less completely reversed when calcium was administered intravenously from external source. It is speculated that streptomycin induced respiratory depression may be mediated through calcium inhibition which can be treated with external calcium in conjunction with artificial respiration.

Keywords: streptomycin induced respiratory depression (SIRD), dorsal and ventral root activities, fusimotor neuron; α-motor neuron, calcium ion

Antibiotics were introduced and soon they were recognized as the 'miracle drugs' to the human race in the treatment of infectious diseases like Cholera, Plague, Typhoid and Typhus fever, Dysentery, etc. with their tremendous beneficial effects. Diseases the were believed to be beyond control three decades ago are now being successfully treated, and this has been due to antibiotics, the so called 'life saving drugs'. During the present century the medical practitioners were ready to replace their concept of 'chemotherapy' with that of 'antibiotic therapy' although some went

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to the other extreme. According to them antibiotics are named 'toxins' and 'poisons' [10], due to some undesirable reactions produced occassionally and due to the development of bacterial resistance to some antibiotics.

Acute intoxication caused by aminoglycoside antibiotics (AGS) was first demonstrated by Molitor and Graessle between 1946 and 1950 [16, 15]. Since 1956, clinically important toxic reactions of AGS have been reported. Acute respiratory depression by streptomycin, one of the major AGS antibiotics, was described by Vital Brazil and Corrado [24] and Vital Brazil and co-workers [25] in 1957.

The prior concept that this group of antibiotics suppress serum ionized calcium through chelation is no longer tenable [18]. So, non-availability of calcium ions at the neuromuscular junction or at the sarcoplasmic reticular system (SR System) might be the cause of respiratory paralysis produced by the antibiotics [22]. Endo [7] has given emphasis over calcium inuced calcium release from the sarcoplasmic reticulum. In view of the aforementioned observations, the present investigation was focused to examine whether supplementary calcium administered from external source could counteract the respiratory depression caused by streptomycin.

Methods*

Animals

The investigation was carried out on adult cats of either sex weighing between 1.5 to 2 kg. Cats were kept under control diet for at least 6 days before the experiments and were anaesthetized after overnight fasting. Cats were anaesthetized by intraperitoneal administration of sodium pento-barbitone (Nembutal) in the dose of 35-40 mg/kg body weight, with a maintenance dose of 10 mg/kg body weight intravenously when required.

General preparation of the animal

After anaesthesia cats were placed in the supine position on the operation table. The four limbs were tied with cords and were fixed with the table. The trachea was exposed to a 'T' shaped polythelene cannula was inserted into the trachea after a low tracheostomy for free breathing and also for artificial ventilation with a starling Ideal Respiratory Pump, when required. A thermistor was connected with the tracheal 'T' tube for monitoring respiration through a Beckman RM Dynograph. Right femoral vein was cannulated by inserting a polythelene catheter which was connected to a three-way Stop cock (Pharmaseal, U.S.A.) for administration of pentobarbital and drugs. 5% glucose Saline was administered by drip feed through right-femoral vein to maintain normal body fluid and electrolyte balance. The rectal temperature was noted by using a thermometer (Zeal, U. K.) and the temperature was maintained at $37° \pm 0.5°C$ throughout the experiment using heating pad placed below the operating table. Femoral arterial blood pH was checked and maintained at normal range either by alteration of ventilation or by

* Abbreviations used in the paper: AgS, aminoglycoside antibiotics, SIRD, streptomycin induced respiratory depression; E-C, excitation contraction; SR system, Sarcoplasmic reticular system; Ca^{++}, calcium ion; i.v. intravenous; Ach, acetylcholine; SIU, stimulus isolation unit.

infusion of $NaHCO_3$ (8.4%). Right femoral artery was also cannulated and connected to a Bel-and-Howell (Type 4-327-0129) pressure transducer coupled with the Beckman RM Dynograph. Both respiration and blood pressure (if required) were recorded on Beckman RM Dynograph from time-time to check that they are within normal levels. The animal was kept undisturbed for at least 30 min after completion of the aforementioned surgical procedure.

Recording of fusimotor (γ-motor) neurone activity

The fusimotor neuron activity was recorded both by direct and indirect methods as used by Hunt [14] and Granit and Kadda [12] respectively. In the direct method the spontaneous discharge of Fusimotor neuron was picked up from central cut end of the nerve supplying a particular muscle. In the indirect method the muscle spindle activity of a particular muscle was picked up from dorsal root afferents.

Muscle spindle activity

Spindle activity was recorded in intact anaesthetized cat. A dorsal laminectomy was performed between either C_2-C_5 segment (for diaphragm muscle) or T_4-T_8 segment (for intercostal muscle) of the spinal cord. The animal was fixed on a cat's frame. The skin flaps surrounding the laminectomized area were arranged to form a pool which was filled with paraffin maintained at 37°C by a thermistor regulated heat lamp. The dura was split and the left dorsal root of C_3 or T_4 was cut close to the spinal cord. The peripheral end of the nerve was split into fine strands under a stereoscopic dissecting microscope (Vickers, U.K.) and laid across a bipolar silver-silver chloride electrode with the indifferent electrode balanced with some other tissue strands. The single unit activity of the diaphragm on the intercostal muscle spindle was picked up from the split dorsal root of the corresponding spinal cord segment. As the diaphragm and intercostal muscles were related with respiration, the respiratory movement was also synchronized with the single unit discharge for confirmation with the help of thermistor attached to the tracheal 'T'-tube, coupled with Beckman RM Dynograph and then displayed on an oscilloscope through the preamplifier (Tektronix AM 502) which showed the onset of firing of discharge along with the upward (inspiratory) movement of respiration. Single unit activity was confirmed by heights and shape of the spike in the oscilloscope at a fast sweep speed. The action potentials, that were picked up with bipolar silver-silver chloride electrode was initially amplified with a differential preamplifier (Tektronix AM 502) and displayed on one channel of the Tektronix 5112 oscilloscope. A parallel connection was made to an audioamplifier for monitoring sound. The single unit activity was stored in a Racal 4 FM tape recorder for further analysis of data. Respiration was recorded through the other channel of the oscilloscope. The unit activity and the respiration were recorded on a moving film using Philips Oscilloscope Camera. Identification of spindle afferent discharge was possible by their response to succinylcholine [1].

Ventral root activity

The activity of the fusimotor neurons supplying the diaphragm or intercostal muscle was recorded from the central cut end of the nerve. The rest of the recording arrangements were similar to that described above. For the confirmation of fusimotor activity the method described by Critchlow and Euler [4] was followed with lignocaine (0.25%). The arrangement of the recording system of neural activity from different spinal nerves (both cervical and thoracic level) along with respiration is shown diagrammatically in Fig. 1.

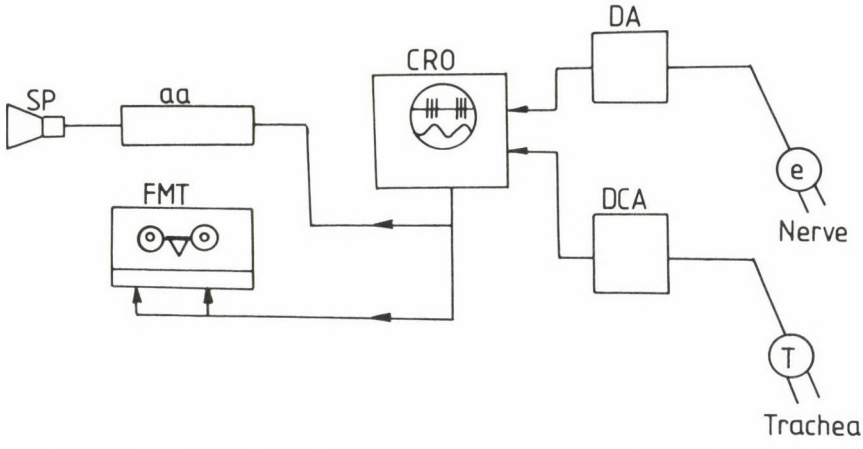

Fig. 1. Schematic diagram of the experimental set up. Abbreviations are: aa, audio-amplifier; DA, differential amplifier; DCA, DC amplifier; e, silver/silver chloride electrode; CRO, cathode-ray oscilloscope; SP, speaker, T, thermistor; FMT, FM tape recorder

Recording of monosynaptic reflex

The surgical procedure for recording monosynaptic reflex activity was similar to that done in case of dorsal and ventral root activity recording. After that the C_3 and/or T_4 dorsal and ventral roots were individually looped over bipolar silver-silver chloride stimulating and recording electrodes. Monosynaptic reflex was elicited by stimulating the dorsal root with monophasic square wave pulses of 0.8-1 ms duration and 3-5 volts from a grass S-48 stimulator, delivered through a stimulus isolation unit (SIU). The action potentials were picked up from the corresponding ventral root and were initially amplified with a differential low noise pre-amplifier (Tektronix AM 502). In order to reduce interference filters were properly adjusted. The output from the preamplifier was fed a Tektronix 5113 storage oscilloscope and photographed with Pentax MX Camera. The height of the monosynaptic reflexes was measured and expressed as a percentage of the average control reflex.

Midcollicular decerebrate preparation

Midcollicular decerebration was done under anaesthesia in cats. After preliminary cannulation of trachea, right femoral vein and artery, the common carotid arteries, were exposed bilaterally. The cerebrum was removed through an opening in the skull, the spatula being slid downwards on the tentorium cerebelli to cut the brainstem at the intercollicular level. Before the decerebration was done, common carotid arteries were occluded and the occlusion was released a few minutes after decerebration. 5% (w/v) glucose saline was infused intravenously to maintain the blood pressure, and the cat was left undisturbed for another half an hour as described by Evans and Mc. Pherson [8]. The decerebration was done after completing all other major dissections.

Drug used

Streptomycin sulphate (Sarabhai Chemical, India), calcium chloride (Sigma Chemical, U.S.A.), succinyl choline (Sigma Chemical, U.S.A.), pentobarbitone sodium (Nembutal, Abott Lab., India). Streptomycin and other drugs were dissolved or diluted in isotonic sodium chloride solution of pH 7-7.2 and introduced intravenously in the least volume possible to produce minimal osmotic shock to the tissues.

Experimental protocol

In order to test the influence of streptomycin on respiration, first respiration was examined under control conditions. Control responses were repeated 20 min following their administration. To test the effect of streptomycin on respiration and the reversal of streptomycin induced respiratory depression by calcium (Ca^{++}), the sequence of experiments was as follows: (a) investigation of the dose dependent control response to different doses of streptomycin (10, 20 and 40 mg/kg); (b) repetition of the responses with 40 mg/kg i.v., streptomycin while studying dorsal root and ventral root activity of spinal intercostal and phrenic nerves: in normally ventilated cats, on decerebate preparations and on monosynaptic reflex activity.

Calcium (Ca^{++}) was injected in a single dose (1.35 mEq/kg i.v.) as calcium chloride solution just after administration of streptomycin. The details of the method have been described in the method section. The respiratory depression caused by streptomycin was completely reversible.

Data analysis

Results were computed as respiratory rate, afferent/efferent single fibre discharge rate before and in the 4th minute (maximum effect) of streptomycin administration under different experimental conditions. Respiratory rate or discharge rate was finally computed and plotted as mean ± SEM. The same procedure was followed in case of monosynaptic reflex too with the only exception that the percentage in the amplitude under different experimental conditions was computed and presented as mean ± SEM.

The statistical analysis was performed with 'Student *t*-test' for paired or unpaired samples.

Results

Effect of streptomycin in normal anaesthetized cats

10 cats were used for this study. Effects of intravenous streptomycin at doses of 10 mg/kg, 20 mg/kg and 40 mg/kg of body weight were studied. It was observed that streptomycin at a dose of 10 mg/kg did not produce any appreciable change in respiration (Figs 2 and 3). However, at dose of 20 mg/kg ($p < .001$) and 40 mg/kg streptomycin caused significant changes in respiratory rate and depth (Figs 2 and 3).

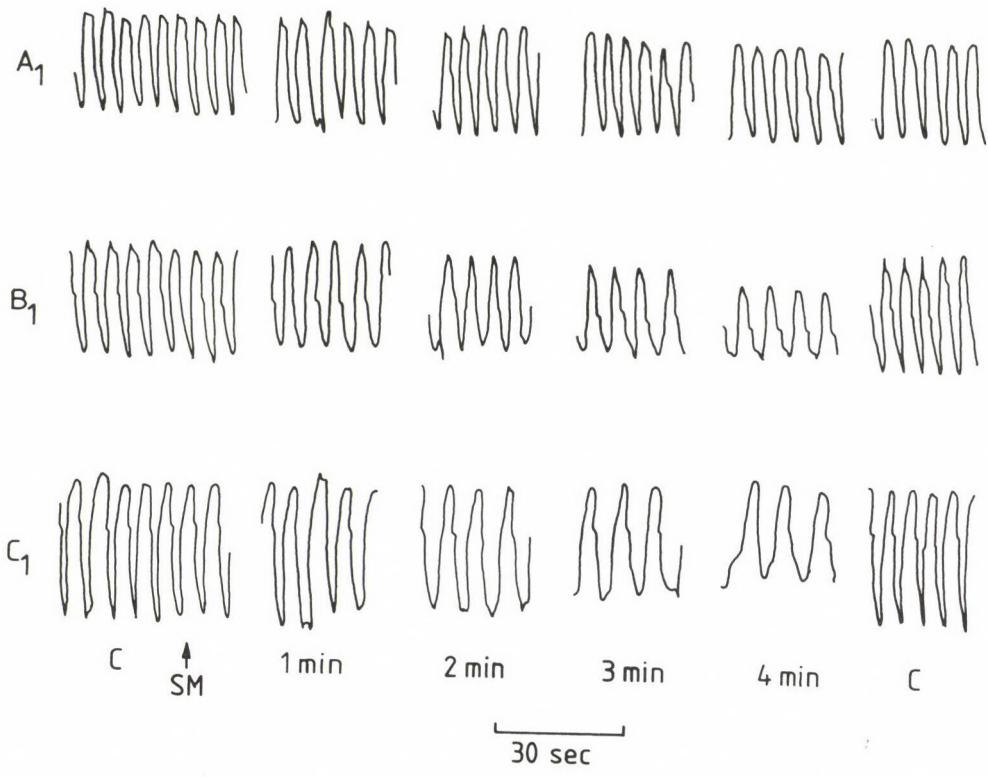

Fig. 2. Typical record showing the effect of streptomycin (intravenous) on respiration at doses of 10 mg/kg (A_1), 20 mg/kg (B_1) and 40 mg/kg (C_1) of body weight in normal anaesthetized cats. C, normal; Sm, point of application of streptomycin

Effect of calcium on streptomycin induced respiratory changes

In normally ventilated cats: Streptomycin at a dose of 40 mg/kg significantly depressed respiration when administered alone (Fig. 4). However, when administered along with calcium chloride (1.35 mEq/kg) streptomycin could not produce such depression (Fig. 4) i.e., calcium counteracted the streptomycin induced respiratory depression (Fig. 5).

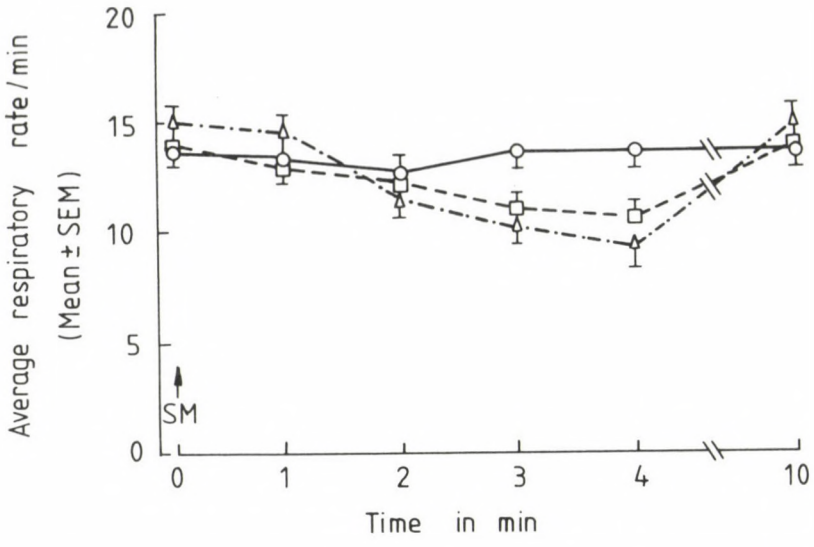

Fig. 3. Dose-response curves showing the effect of 10 mg/kg (O—O), 20 mg/kg (□---□) and 40 mg/kg (Δ-.-.-Δ) streptomycin (Sm) on respiratory rate in normal anaesthetised cats. Points indicate mean ± SEM of 10 experiments

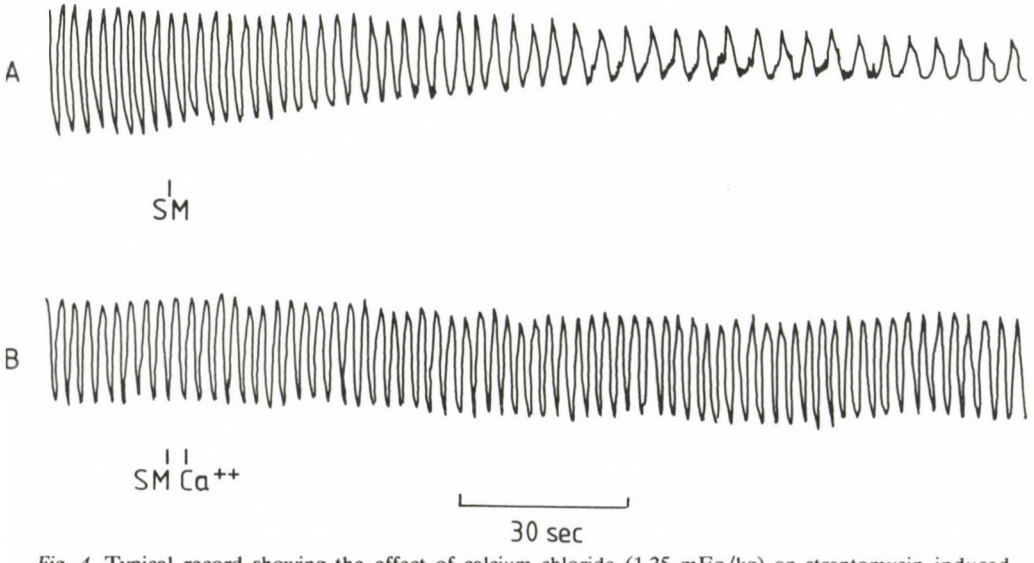

Fig. 4. Typical record showing the effect of calcium chloride (1.35 mEq/kg) on streptomycin induced depression in respiratory rate in normal ventilated cats. A, streptomycin (40 mg/kg, i.v.); B, calcium (1. 35 mEq/kg, i.v.) after streptomycin; SM, point of application of streptomycin; Ca^{++}, point of application of calcium chloride

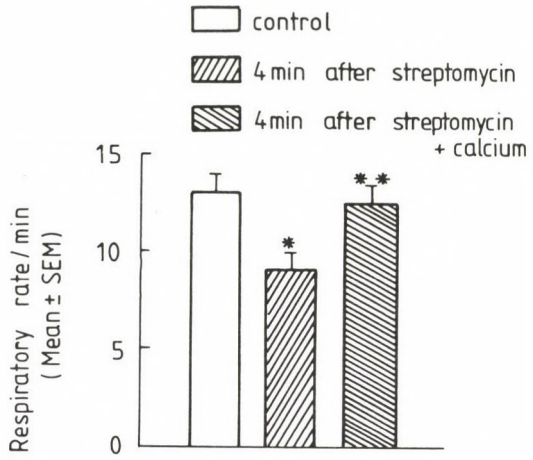

Fig. 5. Histogram showing the effect of calcium chloride (1.35 mEq/kg) on streptomycin (40 mg/kg) induced depression in respiratory rate in normally ventilated cats (n = 10). Values are expressed in mean ± SEM. * Significant reduction from control, ** significant increase from streptomycin effect

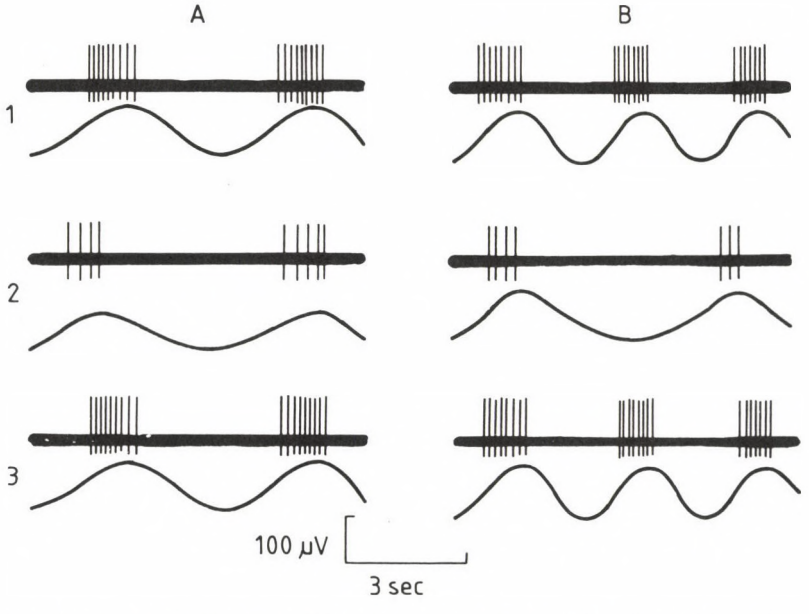

Fig. 6. Typical ventral root (efferent) activities (A) and dorsal root (afferent) activities (B) recorded with respiration (in all cases lower channel) from spinal intercostal nerves (at T_4) of cat; in both A and B:1, Control activities; 2, activities 4 min after i.v. streptomycin (40 mg/kg); 3, activities 4 min after application of streptomycin (40 mg/kg) and calcium chloride (1.35 mEq/kg)

On dorsal and ventral root activities of spinal intercostal nerves: Calcium chloride solution when administered intravenously at a dose of 1.35 mEq/kg. counteracted the streptomycin induced respiratory depression in cats as well as its effect on unit activities of ventral (Fig. 6A) and dorsal (Fig. 6B) roots of the spinal intercostal nerves (Fig. 7).

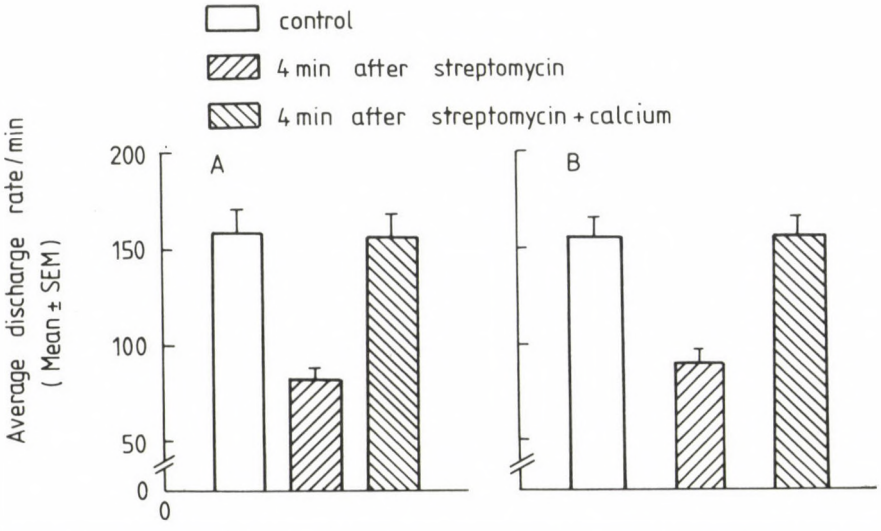

Fig. 7. Histogram showing the effect of calcium chloride on streptomycin induced respiratory depression recorded from the ventral root (A) and dorsal root (B) of spinal intercostal nerves of cat. Values are expressed in mean ± SEM of 10 experiments

On dorsal and ventral root activities of spinal phrenic nerves: When calcium chloride was administered intravenously at a dose of 1.35 mEq/kg, the streptomycin induced respiratory depression along with depression of unit activities recorded from dorsal roots (Fig. 8A) and ventral roots (Fig. 8B) of the spinal phrenic nerves were significantly (Fig. 9) counteracted.

On fusimotor and α-motor neuron activities of spinal intercostal nerves in decerebrate cats: In decerebrate cats the streptomycin induced respiratory depression along with depression of unit activities recorded from the dorsal (Fig. 10A); and ventral (Fig. 10B) roots of spinal intercostal nerves were counteracted significantly (Fig. 11) when streptomycin was administered together with calcium chloride (1.35 mEq/kg, i.v.).

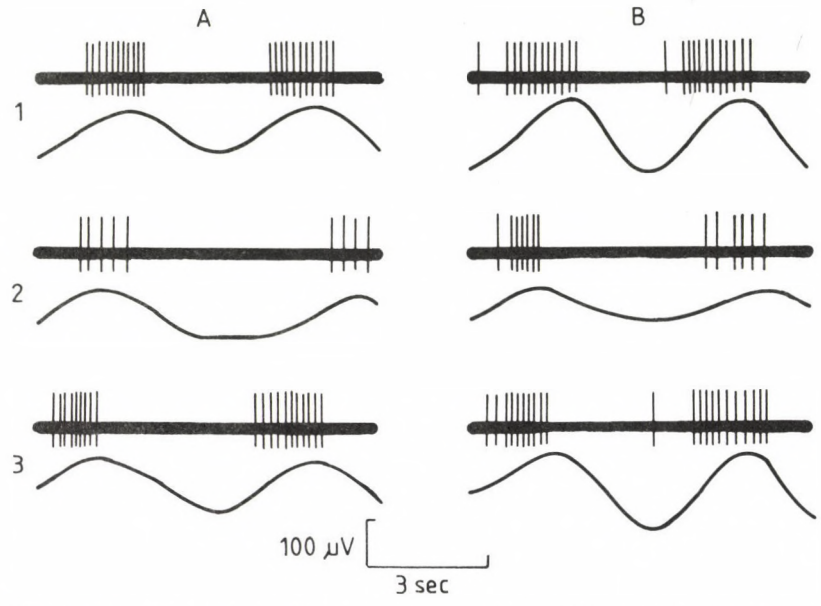

Fig. 8. Typical dorsal root activities (A) and ventral root activities (B) recorded with respiration (in all cases lower channel) from spinal phrenic nerves (at C_2-C_5) of cat; in both A and B: 1, control activities; 2, activities 4 min after streptomycin (40 mg/kg) i.v., 3, activities 4 min after streptomycin and calcium chloride

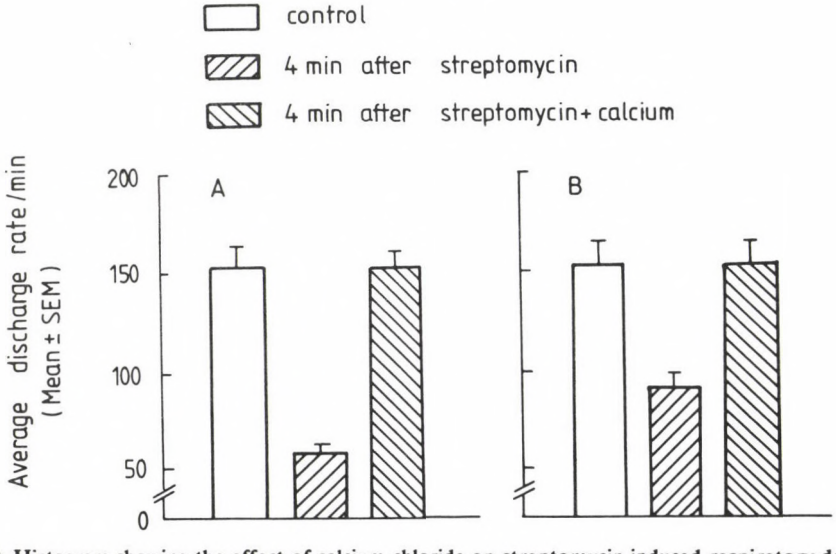

Fig. 9. Histogram showing the effect of calcium chloride on streptomycin induced respiratory depression recorded from the dorsal root (A) and ventral root (B) of spinal phrenic nerves of cat. Values are mean ± SEM of 10 experiments

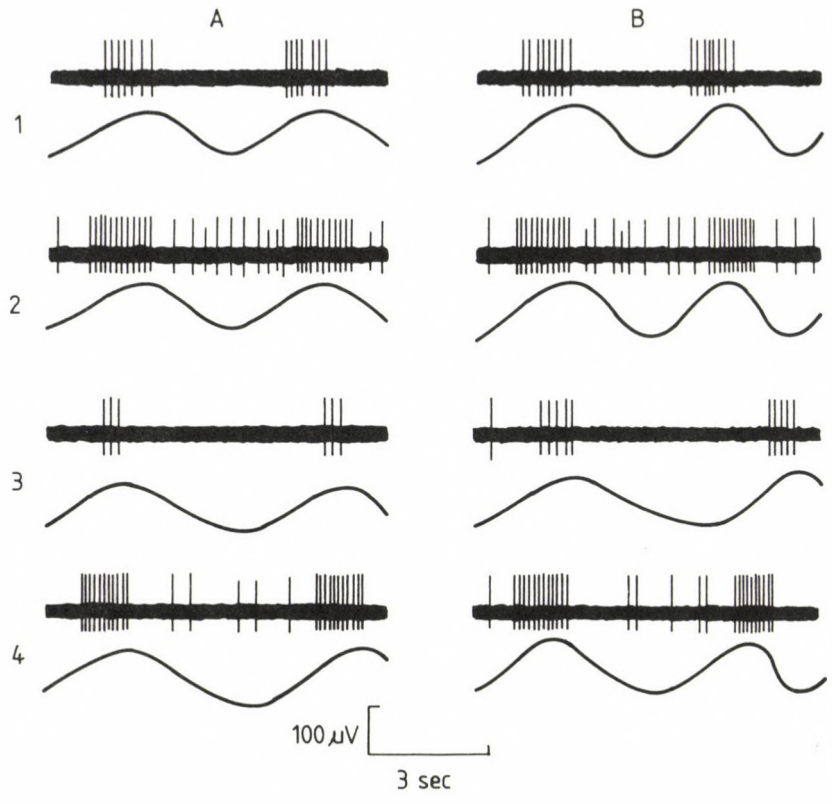

Fig. 10. Typical dorsal root activities (A) and ventral root activities (B) recorded with respiration (lower channels) from spinal intercostal nerves (at T_4) in decerebrated cat. In both A and B: 1, Control activities; 2, activities 15 min after decerebration; 3, activities 4 min after application of streptomycin (40 mg/kg); 4, activities 4 min after application of streptomycin and calcium chloride (1.35 mEq/kg)

On fusimotor and α-motor neuron activities of spinal phrenic nerves in decerebrate cats: In decerebrate cats streptomycin induced depression was also counteracted when streptomycin was administered together with calcium (Fig. 12A) and (Fig. 12B), as in the previous experiment, when fusimotor and α-motor neuron activities were recorded from spinal phrenic nerves (Fig. 13).

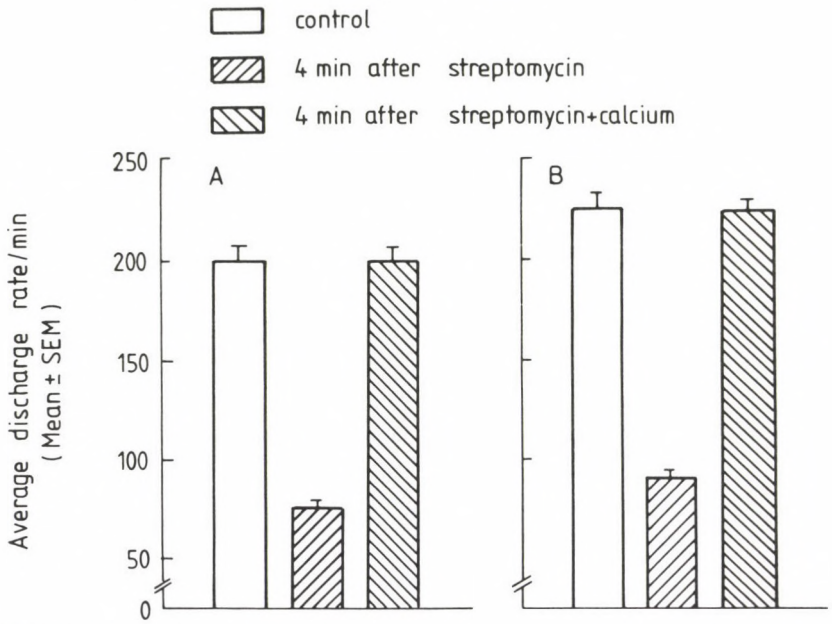

Fig. 11. Histogram showing the effect of calcium chloride on streptomycin induced respiratory depression recorded from the dorsal root (A) and ventral root (B) of spinal intercostal nerves in decerabrated cats. Values are expressed in mean ± SEM of 10 experiments

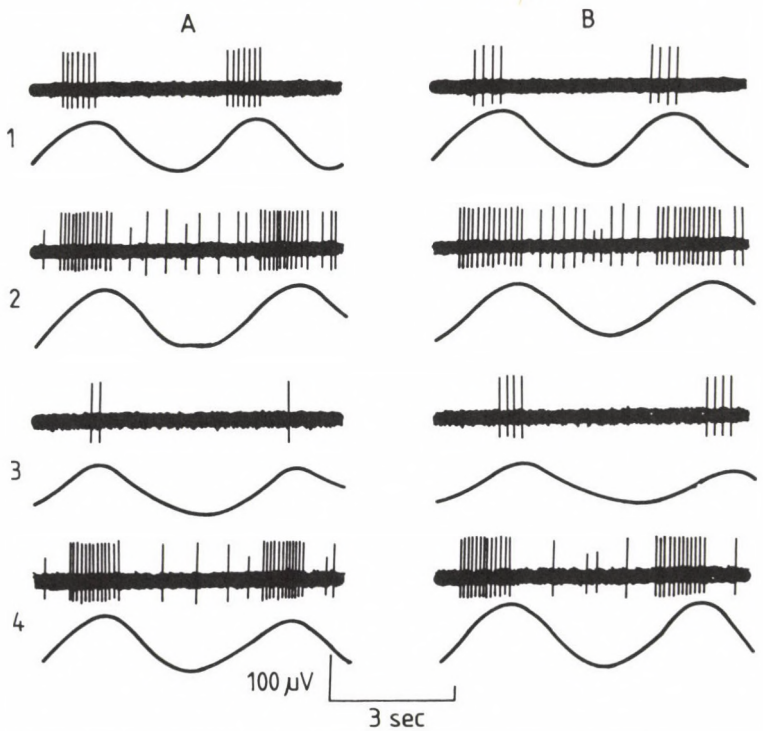

Fig. 12. Typical dorsal root activities (A) and ventral root activities (B) recorded with respiration (lower channels) from spinal phrenic nerves (at C_2-C_5) in decerebrated cats. In both A and B; 1, control activities; 2, activities 15 min after decerebration; 3, activities 4 min after i.v. streptomycin (40 mg/kg); 4, activities 4 min after application of streptomycin and calcium chloride (40 mg/kg)

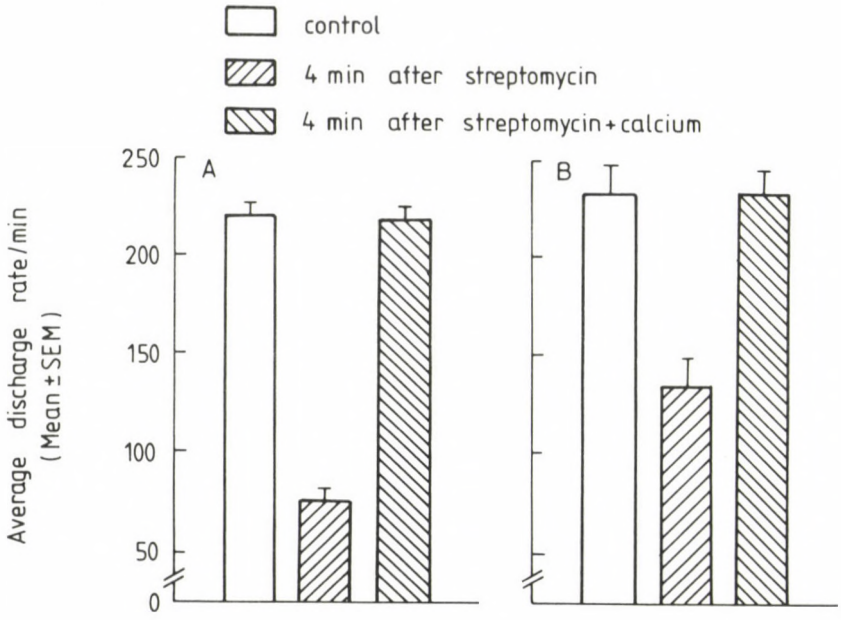

Fig. 13. Histogram showing the effect of calcium chloride on streptomycin induced respiratory depression recorded from the dorsal root (A) and ventral root (B) of spinal phrenic nerves in decerebrated cats. Values are expressed in mean ± SEM of 10 experiments

On monosynaptic reflex activity: The reduced monosynaptic reflex activity, recorded at the thoracic and/or cervical level (Fig. 14), observed after 40 mg/kg streptomycin intravenously was counteracted significantly after administration of intravenous calcium chloride solution at a dose of 1.35 mEq/kg.

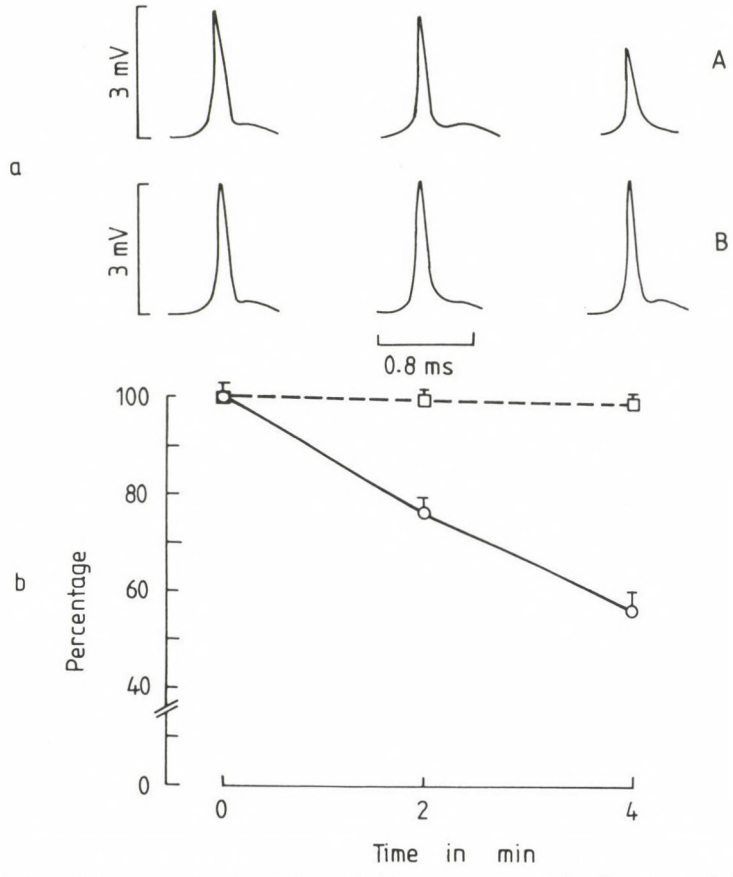

Fig. 14. a) Effect of streptomycin and calcium chloride on monosynaptic reflexes recorded at the level of T_4 ventral root of cat. Samples of the action potentials at 0, 2, 4 min after the application of 40 mg/kg streptomycin (A) and streptomycin with 1.35 mEq/kg calcium chloride (B) are illustrated. b) Percentage recovery of streptomycin induced depression (O—O) of monosynaptic reflex with i.v. calcium chloride (□---□)

Discussion

Many workers have suggested that streptomycin like Mg^{++}, combines with the same receptors as Ca^{++} and make them incapable of releasing acetylcholine (Ach). Thus the antibiotics are considered to be competitive inhibitors of Ca^{++} at receptor sites on the nerve terminals. Hence in the present work efforts were made to study whether calcium from the external source can counteract the depressive effect of streptomycin on respiratory muscles, on muscle receptors and on spinal cord motor neurons.

The study supports earlier reports that aminoglycoside antibiotics (Ags) induced respiratory depression on prolonged administration or when acute high dose were administered [2, 17, 11].

Since a dose of 40 mg/kg i.v., streptomycin was found to produce consistently reproducible respiratory depression, this dose was used in subsequent studies. Though the doses of streptomycin used in the present study seems to be quite high it may be pointed out that similar and even higher doses of Ags have been used in previous studies by other investigators. For example, Corrado [2] has used upto 110 mg/kg of streptomycin and 100 mg/kg of neomycin and kanamycin. Giala and Paradelis [11] have used gentamicin at dose of 80 mg three times daily.

The involvement of Herring-Breuer stretch receptors and central structures in the respiratory depression produced by streptomycin can be ruled out since studies in artificially ventilated cats and intracarotid administration of streptomycin towards the brain and also its intraventricular administration failed to produce any change in respiration [21] respectively.

On the basis of the results of the present investigations it can be stated that the streptomycin induced respiratory depression is mainly due to the muscular paralysis of the respiratory muscles like intercostal and diaphragm muscles. This muscular paralysis could be the resultant cause of non-availability of calcium ions at the neuromuscular junction [19] or at sarcoplasmic reticular (SR) system. It is well known that in skeletal muscle the depolarization of the muscle fibre membrane permits or promotes the entrance of extracellular Ca^{++} ions into the sarcoplasm and that these Ca^{++} ions in some manner activate the mechanical response leading to E-C coupling in skeletal muscle [9, 20].

It is well established that the contractile activation of skeletal muscle is the consequence of a rise in free cytoplasmic calcium concentration. Calcium binds to the divalent ion binding sites on troponin and removes the inhibition for the interaction of the myosin heads with actin. The role of the central root of the SR in regulating the level of free Ca^{++} in E-C coupling and in relaxation is undisputed [13].

The mechanism of the neuromuscular blocking property of the AgS [2, 3 18,] is not yet clear. It was initially demonstrated that myographic characteristics of the neuromuscular blockade produced by streptomycin in dogs were quite similar to those induced by Mg^{++} [24]. It should be recalled that Mg^{++} besides inhibiting the action of Ca^{++} in transmitter release also has a depressing effect on the excitability of muscle fibers [6]. These two properties of Mg^{++} were also demonstrated to be induced by streptomycin [5, 24, 23] and other related AgS [18]. These reports clearly suggest that the streptomycin neomycin group of antibiotics primarily inhibits the prejunctional release of Ach and also depresses post junctional sensitivity to the humoral agent by competing with Ca^{++}.

The present investigation indicates that the streptomycin induced respiratory depression observed in normally ventilated cats and its effects on dorsal and ventral root activities of spinal phrenic and intercostal nerves, of fusimotor and α-motor neuron activities of spinal intercostal and phrenic nerves in decerebrated cats, and on monosynaptic reflex activities are all counteracted by supplementary calcium administered from external source.

So, from the present data we suggest that the respiratory paralysis induced by streptomycin is mediated through inhibition of calcium and that in case of respiratory depression or paralysis caused by streptomycin, the treatment of choice is artificial respiration coupled with intravenous administration of calcium.

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PATHOLOGY OF WAVE III OF BRAINSTEM AUDITORY EVOKED POTENTIALS (BAEPs)

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In a group of 195 consecutively examined patients with brainstem neurological symptomatology parameters of the wave III BAEPs were pathological in 20 patients. In 70% of them the clinical symptomatology and/or the cranial computed tomography examination results pointed to a lesion at the level of pontomedullary junction and lower pons Varoli where according to the recent opinion the possible generators of this BAEP wave are situated (superior olivary complex, nuclei cochleares, corpus trapezoideum). These results support the supposed localization of possible generators of the wave III BAEPs.

Keywords: BAEPs, generators, wave III, clinical correlation

The brainstem auditory evoked potentials (BAEPs) have attained wide clinical usage in audiology, neurology and intraoperative monitoring. Yet considerable controversy exists about the precise localization of the generators of their various component waves. Identification of these generators would maximize the power of the BAEPs in the topographical diagnosis of the brainstem lesions. New knowledge about the localization of the generators of BAEPs component waves can be gained by several ways:

1) intracranial recordings within or near the subcortical auditory pathway (in human or animals) [7, 9, 10]

2) analysis of the effect of experimental lesions in animals [15, 16, 17]

3) investigation of far-field topographies [6, 12]

4) correlation of clinical, pathological and by cranial computed tomography or magnetic resonance imaging localized brainstem lesions with pathologic patterns of the BAEPs [3, 4, 5, 13, 14, 18].

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Each of these methods has its advantages and disadvantages. The optimal understanding of the generators comes from integration of all the available data.

Direct application of the data gained from experimental studies in animals to the interpretation of human BAEPs is not possible because of the cross-species differences in the brainstem and the central auditory pathways' anatomy [11]. Studies of the brainstem auditory evoked potentials in patients with discrete lesions of the brainstem can be helpful in identification of the BAEPs generators.

Subjects and method

In 195 patients with symptoms of a brainstem lesion during neurological examination BAEPs were evoked using a rarefaction click, 90 dB HL, 20/s, monoaurally. Recording electrodes were on the vertex and processus mastoideus ipsilaterally to the stimulated ear. Three averages of 2000 responses were made and superimposed to test the reproducibility of the response, all 6000 being used for definitive processing (Fig. 1). For exact measurement and analysis a special computer assisted method was used [1]. For each of the seven component waves the following parameters were automatically computed and printed: peak latency, peak amplitude, interpeak time interval and central conduction time. Values outside +- 2SD interval were considered pathologic. From the group of all 195 consecutively examined patients were chosen those with pathologic parameters of the wave III uni- or bilaterally and with normal values of the wave I and II parameters in order to exclude the influence of a more peripheral pathology on the wave III BAEPs parameters. Characteristics of this subgroup are in Table I.

Table I

Characteristics of the patients with wave III BAEPs pathology

	Number of patients
Male	13
Female	7
Together	20
Age range	17–64 years

Results

Standardized diagnoses of the patients with wave III BAEPs pathology are summarized in Table II.

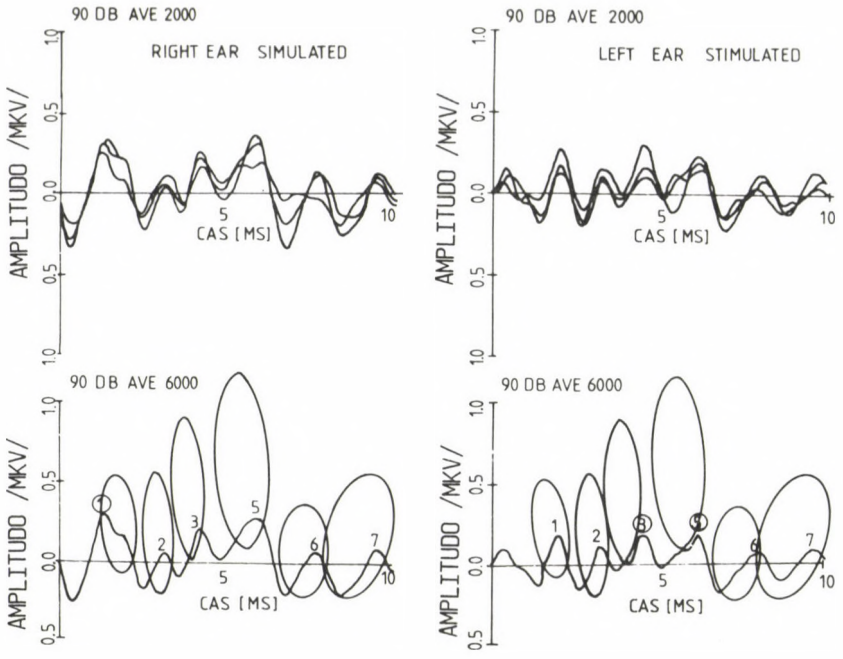

Fig. 1. Brainstem auditory evoked potentials in a patient with multiple sclerosis. Increased peak latency of wave III and interpeak II-III interval on the left side

Table II

Standardized diagnoses of the patients with wave III BAEPs pathology

	Number of patients
Tumor	2
Encephalomalacia	8
Multiple sclerosis	5
Inflammation	2
Trauma	2
Other	1
Together	20

Where it was possible, auditory function of each patient using routine audiometry was examined before the BAEP recording. The results are demonstrated in Table III.

Table III

Auditory function of the patients with wave III BAEPs pathology – results of routine audiometry

	Number of patients
Normal	11
Hypacusis perceptive	6
Not performed	3
Together	20

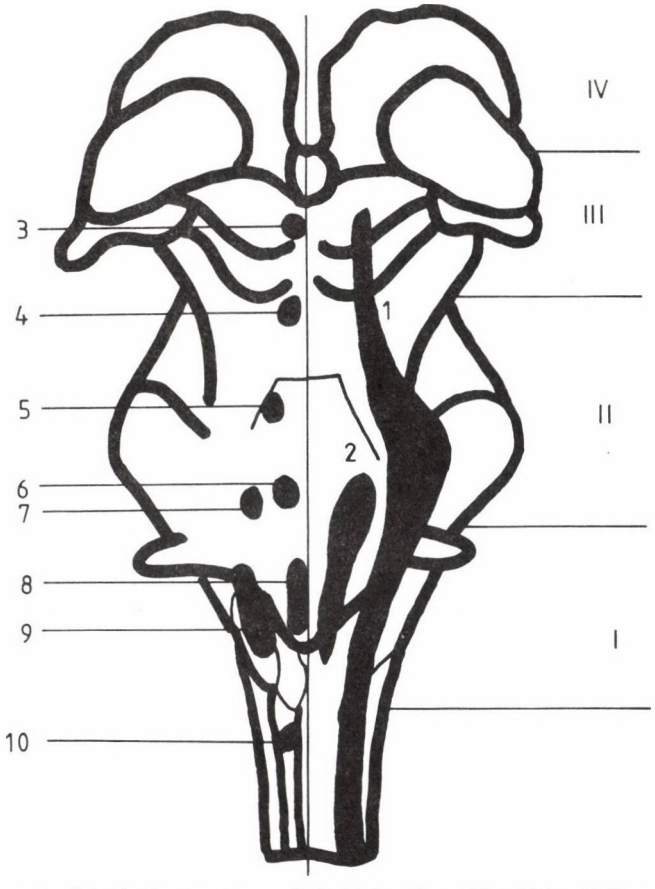

Fig. 2. Functional anatomy of the brainstem-motor (left side) and sensitive (right side) nuclei of cranial nerves: 1-n.V, 2-n.IX, VII and X, 3-n.III, 4-n.IV, 5-n.V, 6-n.VI, 7-n.VII, 8-n.VIII, 9-n.IX and X, 10-n.XI. I - oblongata, II - pons, III - midbrain, IV - thalamus. (Printed with permission from L. Cigánek, I. Koprdová [2])

The clinical symptomatology of this group was rather extensive and multiform, what becomes understandable knowing the functional anatomy of the brainstem (Fig. 2). As it can be seen in Table IV, it pointed in 70% to a lesion at the level of medulo-pontine junction (35%) or lower pons Varoli (35%). By cranial computed tomography (CCT) 13 of these patients were examined. The results of CCT are in Table V. In 4 cases it confirmed a hypodense lesion at the pontine level, in 7 patients with wave III BAEP pathology the CCT was normal.

Table IV

Clinical symptomatology of the patients with wave III BAEPs pathology

Symptom	Number of patients	Lesion level
hemiparesis	1	
quadroparesis	6	
hemisensitive symptom	2	
central vestibular symptom	1	
vertigo	6	
nystagmus	12	pons Varolin
strabismus	2	
diplopia	1	7 /35%/
laesio n.V sensit.	3	
laesio n.VI	1	
laesio n.VII centr.	5	
		pontomedulary junction 7 /35%/
laesio n.IX	2	
laesio n.X	3	
laesio n.XI	3	oblongata
laesio n.XII	4	1
bulbar symptomatology	7	
neocerebellum	5	
paleocerebellum	2	
ataxia	1	
		mesencephalon
laesio n.III	2	2
peripherial vestibular symptom	2	n.VIII 2
diffuse lesion	1	elsewhere 1

Table V

Cranial computed tomography imaging in patients with wave III BAEPs pathology

	Number of patients
Normal	7
Hypodension (pontis, oblongatea vermis cerebelli)	4
Atrophia cerebelli	2
Not performed	7
Together	20

Discussion

According to the experimental studies with brainstem lesions in guinea pigs and cats the wave III of the BAEPs originates from the caudal pons in the contralateral superior olivary complex [15, 16, 17]. Similar results have been obtained also in studies correlating BAEPs findings with clinical, pathological and radiological findings of localized lesions of the brainstem in human [13, 14], as well as the results of intracranial recordings directly from the structures of the brainstem during neurosurgical operations [7]. According to the results of BAEPs investigation in patients with unilateral brainstem lesions the origin of the wave III BAEPs is supposed (opposite to experimental studies) to be mainly in the ipsilateral structures of the superior olivary complex to the stimulated ear [3, 4, 5, 18]. These results are supported also by the dipole model of BAEPs generators in human [12] and intracranial recordings of the BAEPs with deep electrodes [4, 9, 10]. Moreover, the recent works of Moller and Janneta with intracranial BAEPs recordings suggest the generator of this wave to be in the cochlear nuclei at the level of pontomedullary junction [9, 10]. According to the well known length of the auditory nerve in man and the impulse conducting velocity in it as well as the values of interpeak interval I-III Scherg and von Crammon, in accordance with the results of the above-mentioned authors, it is suggested that the wave III BAEPs represents the first activity of the second-order neurons in the cochlear nuclei or in their vicinity [12].

The results of our observations are in accordance with these suggestions, too. Pathology of the wave III BAEPs correlates with a rather extensive neurological symptomatology, which in 70% of cases points to a lesion at the level of pontomedullary junction or lower pons Varoli. Pathology of this BAEPs wave can be associated also with normal hearing function as revealed the routine audiometry (55% of our patients).

Similar results of normal hearing in patients with BAEPs pathology were obtained also in our previous work [2]. The possible explanation of this fact is that the various component waves of the BAEPs could be generated not only by structures involved in behavioral mechanisms of hearing.

Conclusion

The wave III BAEPs pathology correlates with a rather multiform extensive neurological symptomatology, pointing to a lesion at the level of pontomedullary junction and lower pons Varoli, where the supposed generators of this wave are localized.

The wave III BAEPs pathology can occur with normal function of hearing.

The problem of wave III BAEPs generators has not been closed so far. According to the recent works this wave can originate from the activity of various brainstem structures. Besides the cochlear nuclei and superior olivary complex neurons certain role can be played also by the activity of an impulse spreading through the corpus trapezoideum and lemniscus lateralis fibres as well [8].

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IMPACT OF A SINGLE INSULIN TREATMENT (IMPRINTING) APPLIED DURING LIVER REGENERATION ON HEPATIC INSULIN RECEPTOR DEVELOPMENT, BLOOD GLUCOSE LEVEL AND LIVER FUNCTION PARAMETERS IN ADULT RATS

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Rats subjected to partial hepatectomy (surgical removal of two thirds of the liver) showed no appreciable change in serum cholesterol, bilirubin, albumin, total protein and A/G values at 2, 5, 12 and 21 days after the intervention. The enzyme activities characteristic of liver damage (GOT, GPT, LDH, AP) were high in the control group and low in the insulin-imprinted group at 2 days, tended to normalize in both groups at 5 days and changed slightly at 12 days. The blood glucose level was markedly decreased in the control group and to a lesser degree also in the experimental group at 2 and 5 days of sampling. Insulin treatment (loading) performed at 2 and 5 days accounted for a drop of blood glucose which was followed by normalization within 2 h. Starving value and response to insulin loading uniformly fell into the physiological range at 21 days, whereas at 12 days no normalization occurred in either group within 2 h of insulin loading, although the starving value was physiological. The binding capacity of the insulin receptor was markedly low in the control group as long as 12 days, and tended to normalize by 21 days. In the insulin-imprinted group the binding capacity increased over the control at 2 and 5 days and normalized by 12 days.

Keywords: liver, insulin imprinting, receptor development, regeneration

Although receptor development is – like immune response and behaviour – genetically encoded in higher organisms, environmental influence plays a decisive role in receptor maturation (Csaba 1981). For example, hormone receptors mature

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only after primary interaction with the adequate hormone (hormonal imprinting) that involves a qualitative and quantitative adaptation of the binding structure to the hormones produced by the given organisms [6, 7]. Hormonal imprinting takes usually place in the perinatal period and plays a decisive role in the further development of the receptor structure [8]. Appropriate quantity of the adequate hormone accounts for normal receptor maturation in the critical period, whereas presence of a foreign molecule that is able to bind to the developing hormone receptor biases receptor maturation and causes mainly quantitative changes in receptor availability. The changes induced by faulty imprinting persists for a lifetime, regardless whether it was physiological or pathological [9].

The maturity of an organism does not mean that all its cell components are mature. Certain cell types, for example the haemopoietic cells, are being formed continuosly and appear therefore in an undifferentiated state that requires imprinting for maturation in the adult organism, too [11]. It follows that imprinting is related to the developmental stage of the cells rather than to the chronological age of the macroorganism. If cells requiring imprinting in their early stage of development are present, imprinting does take place, and it also takes place in connection with dedifferentiation and subsequent redifferentiation processes in a given mature organ, for example in the course of liver regeneration, when both the microsomal enzyme system [13] and the developing insulin receptors [12] undergo imprinting.

In the present study we investigated receptor development during liver regeneration, with special regard to the extraordinarily complex change consequent upon the surgical removal of the greater part of the liver [1]. In view of this we followed up not only the fluctuations in hepatic insulin binding capacity and the associated changes in the blood sugar level, but also certain biological parameters that are characteristic of the functional state of the liver.

Materials and methods

Male Wistar rats of our own closed breed, weighing 250 g on the average were used. The rats were subjected to partial hepatectomy by the method of Higgins and Anderson [14] and were assigned to two groups. The rats (group 1) simultaneously with partial hepatectomy received a single subcutaneous dose of 1 IU/kg body mass insulin (Insulin Semilente, Mc, Novo, Copenhagen, Denmark), whereas the control rats (group 2) received saline. Liver function tests, such as determination of serum cholesterol (CHOD POD method) bilirubin (modified Jendrassik method), albumin (bromocresol green method), total protein levels (biuret reaction) and of the albumin/globulin (A/G) ratio, as well as of GOT (Seropack, Reanal), GPT (Seropack, Reanal), LDH (Clinisotest) and AP (p-nitrophenylphosphate optimated test) activities were determined with a Hitachi 704 C automat at 37°C in both groups 2, 5, 12 and 21 days after treatment (the blood was collected from the tail vein).

The blood glucose level was determined (by GOD-Period method) after starvation for 24 h immediately before as well as 30, 60, and 120 min after intravenous administration of 0.66 IU crystalline insulin (Actrapid MC Novo, Copenhagen, Denmark), to check up sensitivity to insulin.

Hepatocellular membrane preparations secured at the above sampling intervals were examined for competitive insulin binding capacity. 0.1-1.0 ng/ml I monoiodine porcine insulin (Isotope Institute Hung. Acad. Sci., Budapest) and a rat liver plasma membrane preparation (Neville, step 11, 0.1-0.5 mg protein/ml) were incubated together in Ca-free Krebs-Ringer phosphate buffer, pH 7.5, for 16 at 4 °C, in the presence and absence of unlabelled insulin. 1.5% (bovine serum albumin (BSA) was also added to the insulin containing systems. Each sample was used in 500 µl final volume, and was tested in 2-4 replicas. At the end of the incubation period, the bound hormone was separated by centrifugation (at 10 000 *g* for 2 min at 4 °C) and the pellet was assayed for radioactivity. The radioactivity associated with the pellet in the sample containing 4x10 µg/ml unlabelled insulin was attributed to non-specific binding and was subtracted from the total activity measured in each sample.

The significance of inter-group variations was evaluated by Student's *t*-test.

Results

The serum cholesterol, bilirubin, albumin and total protein levels and the A/G ratio did not(appreciably) differ between the experimental and control groups at any sampling time (Table I). The enzyme activities differed appreciably, not yet significantly, 5 and 12 days after partial hepatectomy. The experimental rats showed a relative decrease (to control) in GOT and GPT activity 2 days after the surgical intervention, and a relative increase in AP and LDH activity 12 days after it (Table II). The precise evaluation of the results was greatly hampered by extreme individual variations. The fact nevertheless remains that the extreme activity increase that indicated liver damage 2 days after the operation tended to subside by 5 days, although it still remained over the control.

Table I

Liver-dependent serum indexes
(mean ± S.D.)

	Pretreatment	Number of animals	Cholesterol mmol/l	Bilirubin µmol/l	Albumin g/l	Total g/l	Albumin/ globulin
2 days	saline	26	1.29 ±0.25	6.6 ±1.78	24.6 ±1.44	47.7 ±3.49	1.07 ±0.12
	insulin	25	1.44 ±0.28	7.12 ±1.82	25.6 ±1.72	49.2 ±3.43	1.09 ±0.10
5 days	saline	18	1.60 +0.28	3.50 +0.70	24.6 +2.02	51.5 +3.10	0.92 +0.10
	insulin	26	1.69 ±0.31	4.3 ±0.78	25.2 ±1.76	51.5 ±3.56	0.96 ±0.09
12 days	saline	19	1.88 ±0.31	3.54 ±1.14	27.0 ±1.66	57.3 ±2.77	0.91 ±0.12
	insulin	12	1.90 ±0.41	3.85 ±0.66	29.08 ±2.14	57.5 ±3.16	1.04 ±0.10
21 days	saline	14	2.22 ±0.30	5.46 ±0.65	30.3 ±1.85	60.1 ±2.80	1.02 ±0.10
	insulin	18	2.04 ±0.27	5.25 ±1.17	29.6 ±1.92	62.3 ±4.1	0.93 ±0.15

Table II

*Serum levels of enzymes**
(mean ± S.D.)

	Pretreatment	GOT U/l	GPT U/l	Aph U/l	LDH U/l
2 days	saline	826.8 ±260.0	787.6 ±247.9	725.7 ±160.6	1561.5 ±366.3
	insulin	433.8 ±254.8	363.0 ±350.8	761.6 ±136.4	1308.3 ±272.2
5 days	saline	220.1 ±47.6	66.7 ±20.2	750.9 ±164.4	1502.6 ±391.1
	insulin	211.8 ±50.20	67.9 ±15.5	760.6 ±176.3	1550.4 ±457.1
12 days	saline	192.6 ±37.23	72.5 ±26.00	719.0 ±236.2	1484.2 ±384.1
	insulin	192.2 ±33.04	72.0 ±20.62	661.3 ±149.9	1831.3 ±378.7
21 days	saline	214.3 ±27.45	94.0 ±19.55	609.4 ±154.3	1695.8 ±227.4
	insulin	203.1 ±22.8	83.7 ±13.1	467.0 ±132.7	1738.6 ±259.8

*Number of animals as in Table I

The blood glucose level dropped below the lower physiological limit in both groups 2 and 5 days after the operation (Fig. 1), and tended to normalize after 12 days. The (experimental) rats had higher blood glucose levels than the controls at all sampling intervals, except at 12 days.

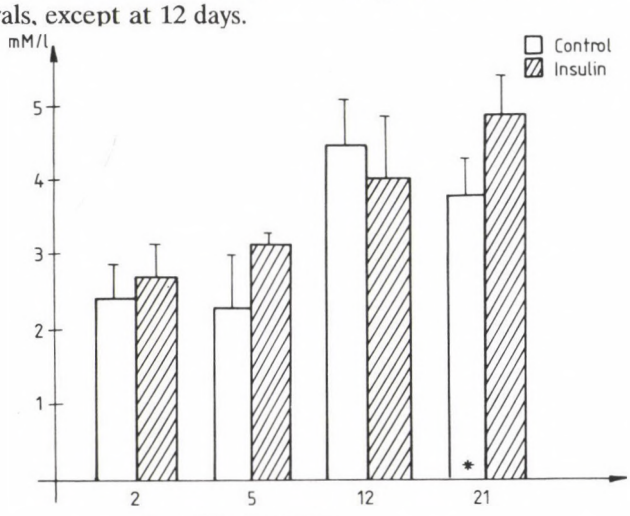

Fig. 1. Basal blood sugar levels (mean ± S.D.) at different days after hepatectomy. * = significant difference ($p < 0.01$) to the paired column

The experimental and control rats responded to insulin treatment (loading) at all post-operative sampling intervals (Fig. 2). At 5 days, insulin loading accounted for normalization of the reduced blood glucose level in the pretreated group within 2 h, at 12 days a drop was observed in 2 h of treatment and at 21 days rise of blood glucose indicated a physiological response to loading.

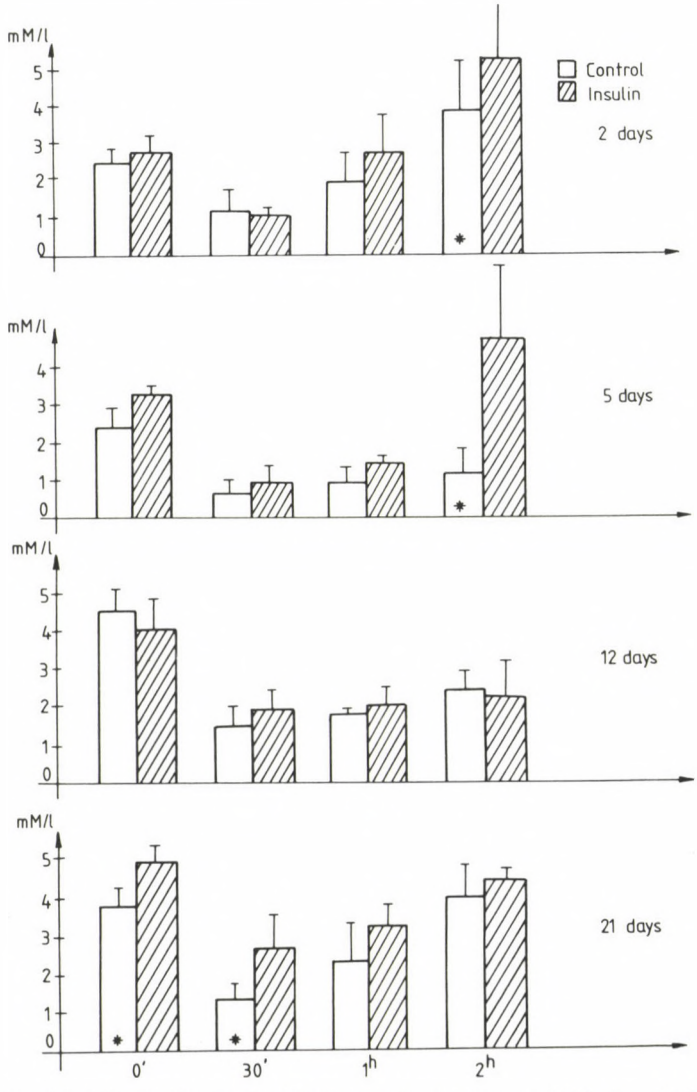

Fig. 2. Influence of insulin loading on the blood sugar level (mean ± S.D.). * = significant difference ($p < 0.01$) to the paired column

Insulin binding tended to decrease in the experimental group until 12 days (Fig. 3), then tended to normalize by day 21. Competitive insulin binding increased over the control in the initial stage, then normalized completely by 21 days.

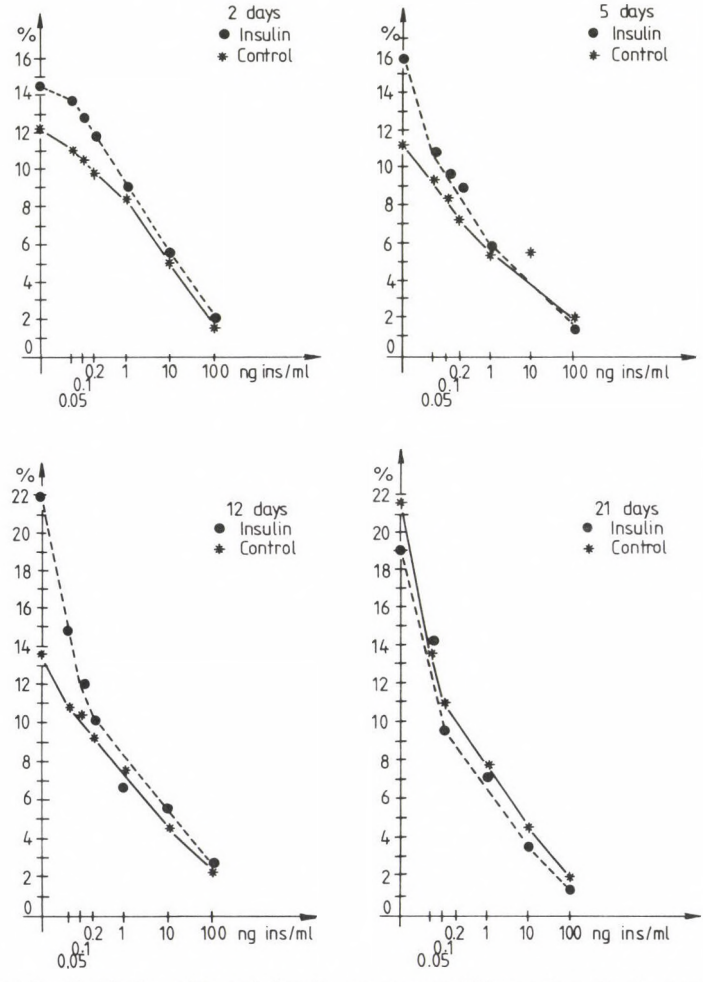

Fig. 3. Insulin binding of hepatic insulin receptors at different days after hepatectomy

Discussion

According to the literary data, partial hepatectomy (removal of two thirds of the liver in the present study) is followed by an enormous cell proliferation that attains its peak by 24 h after the operation, and stabilizes at a relatively stationary level later [1, 20]. The biochemical parameters of liver function are less effected by

the intervention; the enzyme activites characteristic of liver damage tend to increase and the blood glucose level tends to decrease after partial hepatectomy [1, 5, 20]. Liver regeneration takes about three weeks, as was also obvious in the present study.

Regenerative cell proliferation requires the collaboration of certain hormones that enhance hepatocellular division. Insulin and glucagon are most active in this respect [5, 16, 18]. This can explain why the rats given after hepatectomy a single dose of insulin showed a lesser increase than the controls in the enzyme activities characteristic of hepatocellular damage. The measured parameters of liver function changed hardly, if at all, after hepatectomy, indicating the enormous functional potential of the remaining one third of the liver.

Thus, a functioning, but at the same time vigorously dedifferentiating liver tissue was subjected to imprinting by an excessive dose of exogenous (pig) insulin that differed both qualitatively and quantitatively from the endogenous insulin available in the organism of the hepatectomized rats. That foreign insulin was used to assess the receptor-level effect of imprinting in terms of insulin binding and impact on receptor kinetics. It should be noted that, since hepatectomy is followed by blood glucose drop, the blood glucose changes presumably reflected also other than receptor-level events.

The present experiments demonstrated that, as shown already earlier [12], a single insulin treatment (imprinting) applied in the initial stage of liver regeneration had the same effect on receptor development as neonatal imprinting. The binding values of the experimental rats increased over the control already two days after hepatectomy. However, unlike neonatal insulin imprinting, post-operative imprinting accounted for a relative increase rather than a decrease [10, 15] of the blood glucose level in response to insulin loading. It follows that although insulin imprinting did take place during regeneration, certain regeneration-associated events also played a role by modifying the physiological response at the level of the organism. Thus, the experimental rats, which had more insulin receptors than the controls as a result of imprinting, responded to insulin loading by blood glucose elevation instead of a drop in consequence of greater insulin binding. Probably an increased down-regulation that could not be measured under the conditions given in this experiment was responsible for the phenomenon, because there is evidence [5] that liver regeneration is associated with an increased hepatocellular insulin intake. Reversion of the general tendencies of response at 12 days after hepatectomy deserves special consideration. Abrupt increase in the formerly even level of AP and LDH activity, drop of blood glucose below the control level, and the failure of blood glucose to normalize within 2 h of insulin loading indicated that association of increased insulin binding with a greater glycogen storage gave rise to depression of the blood glucose level. Normalization of the hepatocellular insulin binding capacity took in fact about 12 days in the insulin imprinted rats.

Since insulin plays an important role in the regeneration response of the liver [2-5, 17, 19], it stands to reason that insulin imprinting accelerated regeneration by enhancing the binding and thereby the action of insulin.

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PHYSIOLOGY – PATHOPHYSIOLOGY

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CHANGES IN THE NUCLEIC ACIDS AND PROTEIN CONTENT OF THE OVARIES IN RELATION TO A SHORT AND LONG PHOTOPERIOD IN A FRESHWATER CATFISH *HETEROPNEUSTES FOSSILIS* (BL)

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This study indicates the role and possible correlative involvement of ovarian total protein and nuleic acids in the developmental changes and cellular multiplication during the reproductive cycle of the female *Heteropneustes fossilis*.

Keywords: vitellogenesis, oogenesis, photoperiod

Attempts have been made to understand the role of different environmental factors in controlling the reproductive cycle of freshwater fish. Among various environmental factors, the decisive role of photoperiod in modulating the reproductive physiology has attracted considerable attention [16, 23, 31, 32]. Following the line of the above studies it seems to be important to estimate the regulatory impact of photoperiod on certain biochemical components in female fish.

The aim of the work was to study the functional correlation between quantitative variation in protein and nucleic acid content of the ovary and their involvement in its growth, maturation and oogenesis under a short and a long photoperiod during the ovarian cycle of an Indian freshwater catfish *Heteropneustes fossilis*.

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Materials and methods

Fish were procured from local Ramgarh lake and acclimatized in the laboratory condition for 15 days.

The fish *Heteropneustes fossilis* shows sexual dimorphism [21], therefore only female fish were used. The mature, female fish were divided into three groups, each group consisted of 30 individuals. Aquaria of the same size were filled with tap water up to the same level. The water temperature did not vary more than ±1°C from the control group. Aquaria covered with a wide wire mesh were kept in wooden photoperiodic chamber lighted by a 4 ft tubelight of 40 watts, connected to an automatic timer. The control group was provided the natural photophase.

The following three groups were maintained:

Group I (Control): The fish received natural photophase

Group II (1L:23D): This group of fish was kept in total darkness for 23 h per day and was illuminated with fluorescent tube only for 1 h per day from 12 noon to 1 p.m.

Group III (18L:6D): Fish in this group received artificial illumination for 18 h per day from 4 p.m. to 10 a.m.

The water of the aquaria was changed and fish were fed with prawn powder and liver pieces every day during the light period.

Experiments were performed for a duration of 45 days during the preparatory phase, active phase and post-spawning phases of the reproductive cycle. At the end of the experiment, fish were blotted and then dissected to take out the ovaries.

The determination of total protein and nucleic acids was made using the method of Lowry et al. [20] and Schneider [25], respectively. Mean and standard error (mean ± S.E.M.) were given. Significant differences were calculated by Student's '*t*' test [3] at $p<0.05$ or less.

Results

The quantitative variation in protein, RNA and DNA content of the ovary depends upon the state of its maturity, the synthesis of RNA and also the influx of protein from exogenous sources such as liver. Additionally the stages and tempo of nuclear division also plays perceptible role.

Protein

Compared to that of control, the protein contents of the ovary (Table I) under short photoperiod (1L:23D) shows an uniform increase. This increase, however, is more significant during the preparatory and post-spawning phases while in the active phase the enhancement is much less. The preparatory phase is characterized by the process of active multiplication and development when enhanced protein synthesis is highly required. This, however, stabilizes during the active phase. But the proportionate increase exclusively during post-spawning phase indicates that a short photoperiod has an appreciable impact on early oogenesis as compared to the long photoperiod.

Under 18L:6D, though the protein content of the ovary in the preparatory phase is fairly high in comparison to the control, however, it appreciably falls during the active and post-spawning phases. It is interesting to note that the quantitative decrease in protein content during active and post-spawning phases is much below to that of the control. It may, however, be correlated with the high rate of conversion of oocytes to their more advanced stages during active phase and a total absence of mature oocytes in the post-spawning phase.

RNA

The data on the RNA content under the aforesaid photoregimes do not corroborate the amount of protein found during preparatory, active and post-spawning phases (Table I).

Table I

Contents of total protein (mg/g of wet weight) and nucleic acids (DNA and RNA) (μg/mg of wet weight) in the ovary of female Heteropneustes fossilis (Bloch) under short and long photoperiods during the entire reproductive cycle

	Control	1L:23D	Ratio Exp/cont	18L:6D	Ratio Exp/cont
Preparatory phase:					
Protein	428.666 ± 0.120	818.293*** ± 0.335	1.908	561.066 ± 0.260	1.308
RNA	130.958 ± 0.084	103.275 ± 0.070	0.788	116.174*** ± 0.046	0.887
DNA	31.520 ± 0.112	27.100*** ± 0.064	0.860	28.825*** ± 0.130	0.914
Active phase:					
Protein	463.626 ± 0.302	499.733*** ± 0.209	1.077	312*** ± 0.123	0.672
RNA	167.236 ± 0.085	157.578*** ± 0.038	0.942	246.348*** ± 0.048	1.473
DNA	24.890 ± 0.041	15.426*** ± 0.0061	0.619	27.213*** 0.054	1.093
Post-spawning phase:					
Protein	276.026 ± 0.261	480.640*** ± 0.775	1.741	247.600*** ± 0.260	0.897
RNA	125.752 ± 0.084	92.979*** ± 0.082	0.740	101.794*** ±0.146	0.809
DNA	14.776 ± 0.056	10.894*** ± 0.062	0.737	18.668*** ± 0.035	1.263

Each value represents mean ± S.E.M. *** = $p<0.001$

However, it has been recorded that under 1L:23D in preparatory active and post-spawning phases, the amount of RNA is the lowest in comparison to the long photoperiod, of 18L:6D including control. Under long photoperiod, the highest level of RNA has been measured in the active phase. Although in preparatory and post-spawning phases the RNA level is higher than that recorded during the short photoperiod but it is lower than that of their respective controls.

DNA

The amount of DNA is associated with the rate of nuclear division and it shows a gradual fall from preparatory to post-spawning phase under short and long photoperiod including the control. (Table I) Its highest level is registered during preparatory and its lowest during post-spawning phase. However, under long photoperiod (18L:6D), the amount of DNA is higher in all the phases in comparison to short photoperiod including control. This appears to be a significant observation which points towards stimulated oogenesis under the long photoperiod.

Present biochemical parameters, however, do not provide a multidimensional support to the histo-cytological observations. Nevertheless, 18L:6D can be regarded as an overall stimulatory photoperiod while 1L:23D has a selective impact on ovarian functions.

Discussion

Earlier authors [4, 5, 19, 27, 28] have tried to describe the role of carbohydrate protein and lipid contents in the development and maturity of the fish gonad. They have, however, pointed out changes in the carbohydrate, protein and lipid content in different tissues during the reproductive cycle and also tried to correlate the gonadal maturation and associated processes particularly with the spawning activity in freshwater fish.

Growth, maturity and spawning requires a higher energy level. Such a demand is obviously met with the large amount of nutrient stored in the different parts of the body such as liver and muscle. The carbohydrates, proteins and lipids are transferred and made avaible to gonads to commence, continue and conclude the reproductive processes [8, 14, 15]. It is evident in *Esox lucius* (L) [7, 21] that a high energy requirement is involved in the gonadal growth and maturation which is covered by endogenous sources. Similarly the kinetics of spermatogenesis during its peak, also demands a high level of protein and lipid along with the carbohydrate for its completion [29, 30]. Such endogenous nutrient is accumulated during the excess food supply during the time of low energy demand [9, 18, 19, 28].

In the present study in *Heteropneustes fossilis* it can be seen that under a short photoperiod of 1L:23D the protein content in the ovary steadily decreases from the preparatory to the post-spawning phase. Such a change leading to active oogenesis, may be attributed to the enhanced protein synthesis/supply to meet the level of requirement during the preparatory phase which later becomes stable during the active phase. But the proportionate increase in the later part of the post-spawning phase indicates its impact on the initiation of early oogenesis. Cytological features during active and post-spawning phases under long photoperiod (18L:6D), point that utilization of exogenous source is almost inhibited. Thus this may be considered that a long photoperiod is only stimulatory for enhanced vitello-genesis followed by quick spawning.

In catfish *Mystus vittatus* [1] the amount of protein, RNA and DNA increases from April to June and decreases from June to September. The transportation of exogenous protein is necessary for the development of gonad and maturation of gametes because the greater demand for protein during active phase cannot be fulfilled through the dietary protein alone [19]. This has been further supported by recording the cyclical changes in total protein, protein nitrogen, nitrogen, water, fat and ash content in the muscle of *Clarias batracus* [2].

Protein supplementation for ovarian development from different exogenous sources is well documented. In *Blennius pholis* and female *Gobius niger* [26], the protein yolk is synthesized in ovary from the blood serum. An increase in the protein content of the circulating serum with the onset of maturity and its subsequent decrease during spawning period has been recorded [17]. However, in Zebra fish *Brachydanio rerio* [12], the source of protein yolk platelets is reported to be the liver.

The involvement of cytoplasmic RNA is found instrumental in the formation of protein yolk. Thus the presence of protein and RNA in the oocytes has been reported [10, 11, 33]. In Zebra fish [13] and in *Gambusia affinis* [33], the cytoplasmic RNA is recorded to be involved in the formation of protein yolk. In the present study, the RNA content in the ovary of *Heteropneustes fossilis* increases from preparatory to active phase and declines during the post-spawning phase. However, except during the active phase where the RNA content points towards a stimulatory effect of long photoperiod, an overall decrease in the value of RNA content is observed during the preparatory and post-spawning phases.

From these data though on one hand a correlation between RNA and protein synthesis is difficult to establish but on the other hand, additional exogenous sources to supplement protein requirement during ovarian maturation may well be anticipated. The amount of DNA may vary during various biochemical activities of the cell in several animals [34]. In the nucleolus of oogonia, a DNA positive reaction has been described in *Amphipnous cuchia* [24]. Maximum intensity of Feulgen reaction [6] during maturation phase of male *Channa punctatus* is additionally

reported [29]. It may therefore, be conjectured that the cellular differentiation and biochemical maturation vigorously run parallel to each other. Thus during the active phase, the cell division and differentiation can be associated with the biochemical events taking place in testicular lobules. However, in *Gambusia affinis*, a Feulgen negative test [33] is also reported though without any valid explanation.

In the present study, a gradual fall is observed in the DNA content from preparatory to post-spawning period under all the photoregimes. However, under the long photoperiod (18L:6D) the amount of DNA is higher in each phase pointing towards stimulated gametogenesis under this photoperiod.

From a comparative evaluation of the impact of a long and a short photoperiod on the ovarian activity in *Heteropneustes fossilis* it may be inferred that a short photoperiod includes a proportionate increase in the protein content during the post-spawning phase exclusively. Whereas the long photoperiod has been recorded to be stimulatory for the vitellogenesis. Seemingly both photoperiods are found to be selectively beneficial for the entire process of oogenesis.

These photoperiods may consequently be utilized to shorten the tenure of different phases of the sexual cycle inducing the fish for spawning earlier than the usual.

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EFFECT OF MK-801 ON DOPAMINE RELEASE EVOKED BY HYPOXIA COMBINED WITH HYPOGLYCEMIA

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[^{3}H]dopamine ([^{3}H]DA) release was measured from rat striatal slices under normoxic and hypoxic conditions. In some experiments hypoxia was combined with glucose withdrawal. Hypoxia increased the evoked release of dopamine without affecting resting release. Hypoglycemia itself increased only the resting release of [^{3}H]DA. In the absence of glucose hypoxia provoked a dramatic rise in both resting and stimulation-evoked release of dopamine. This effect was partly reduced by Ca^{2+} withdrawal, and was abolished in the presence of tetrodoxin (1 μM). The NMDA-receptor antagonist MK-801 (3 μM) attenuated the effect of hypoxia and hypoglycemia on [^{3}H]DA release. It was suggested that activation of NMDA receptors is involved in dopamine release during hypoxia and energy deprivation.

Keywords: hypoxia, hypoglycemia, dopamine release, striatal slices

The brain is very sensitive to ischemia because of its high metabolic rate and small reserves of energy-rich phosphates and carbohydrates [22]. Several investigators have shown that ischemia/anoxia in the brain alters neuronal activity and the release of neurotransmitters, and this correlates with the functional changes which precedes irreversible neuronal damage [11, 17, 18, 19, 21, 23]. In addition, evidence has been obtained that hypoglycemia results in membrane depolarization and energy failure, accompanied by neuronal necrosis [1, 15, 34].

Many *in vivo* and *in vitro* studies have shown that ischemia/anoxia releases glutamate and dopamine (DA) [2, 3, 7, 8, 9, 10, 12, 16, 19, 35]. Similarly, hypoglycemic conditions provoke a substantial increase in the extracellular concentrations of dopamine [13] and glutamate [4], as assayed by microdialysis, as

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well as *in vitro* studies [24]. Only a few authors, however, have analyzed the effect of combined oxygen and glucose withdrawal on transmitter release, a situation very frequently occurring during ischemia [14, 20]. It is not clear whether hypoxia and energy deprivation directly affected the release of DA or it is mediated through the effect of glutamate released in excess during this condition. This question seems to be very critical, because Lindvall et al. [13] have shown that selective lesion of mesostriatal dopaminergic neurons prevents the neuronal damage produced by insulin-induced hypoglycemia in the caudate-striatum.

We used an *in vitro* model of hypoxia combined with hypoglycemia in which both resting and evoked release of [^{3}H]DA from rat striatal slices can be measured in microvolume perfusion conditions. The aim of our study was to explore whether or not the effect of hypoxia and energy deprivation on DA release is mediated by the action of excitatory amino acids through their specific receptors.

Method

Male Wistar rats (140 g) were killed by decapitation and the brain was rapidly removed. The striatum was dissected and sliced into 400 μM thick coronal sections with a McIlwain chopper. Slices were preincubated at 37 °C in the presence of [^{3}H]-DA (10 μCi/ml, specific activity 42 Ci/mmol) in Krebs solution containing 0.3 mM ascorbic acid and 0.03 mM Na_2EDTA for 60 min and gassed with a mixture of 95% O_2 + 5% CO_2. After 90 min of preperfusion (flow rate 1 ml/min), 3-min fractions were collected and analyzed for tritium by liquid scintillation spectroscopy. Slices were placed in a microvolume perfusion chamber [30] and stimulated by a stimulator "Eltron" twice for 3 min using biphasic pulses starting at the 3rd (S_1) and the 13th (S_2) fractions. The release of tritium elicited by electrical field stimulation (2 Hz, 1 ms, 360 shocks, 10 V/cm) was expressed as a fractional rate, i.e., as a percentage of the amount of radioactivity in the tissue at the time when the release was determined. The effect of drugs on the release was expressed as a ratio of S_2/S_1. Drugs were given 20 min before S_2.

When the effect of hypoxia was studied the preparations were subjected to hypoxic condition (95% N_2 + 5% CO_2) starting at the 6th sample. In the case of hypoxia combined with hypoglycemia glucose was withdrawn from the Krebs solution. Changes in the resting release were expressed by the ratio of R_2/R_1 (R_2 and R_1 – mean values of two fractions before S_2 and S_1, respectively).

Statistics

Statistical analysis of data was carried out using the paired Student's *t*-test and ANOVA followed by Dunnett test. The means ± S.E.M. are presented. Differences with *p* value of less than 0.05 were considered significant.

Drugs

All of the chemicals used to prepare Krebs solution (NaCl 113, KCl 4.7, $MgSO_4$ 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25, KH_2PO_4 1.2, and glucose 11.5 mM) were of analytical grade. The sources of the drugs used were: MK-801 [(5R, 10S)-(+)-5-methyl-10, 11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10 imine hydrogen maleate] from RBI; tetradotoxin (TTX), and EGTA [(ethylene glycol-bis(β-aminoethylether) N, N, N, N,,-Tetraacetic acid)] from Sigma; ascorbic acid and Na_2-EDTA (ethylenediamine tetraacetate dissodium) from REANAL, Budapest, Hungary, [^{3}H]dopamine (spec. activity 42 Ci/mmole) from Amersham.

Results

At the beginning of the collection period the tissue contained 125 000 ± 16 000 Bq/g radioactivity (n = 40). During the experiments 40.1 ± 2.1% (n = 40) of the total tritium content was released. The resting release, during a 3-min collection period, was 1.1 ± 0.4% (n = 40) of the total content of radioactivity. In response to electrical field stimulation (2 Hz, 1 ms, 360 shocks) the evoked release increased to 3.92 ± 0.08% (n = 40) and was fairly constant after repeated stimulation. The ratio S_2/S_1 was 0.85 ± 0.08 (n = 4) (Fig. 1).

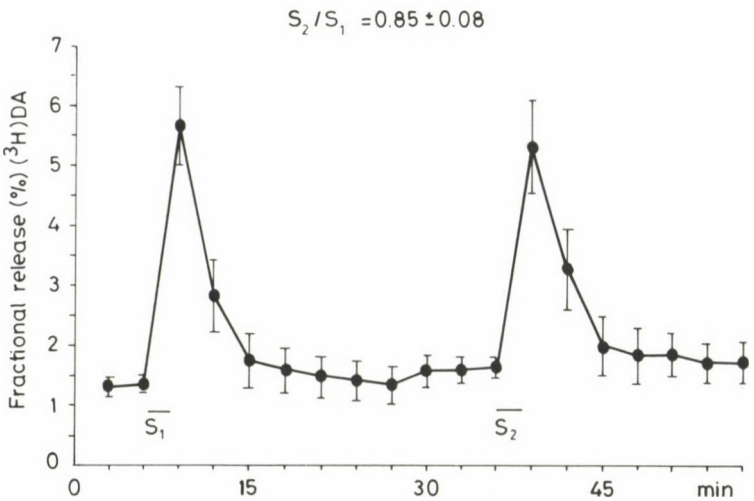

Fig. 1. Release of [^{3}H]dopamine ([^{3}H]DA) from rat striatal slices. The slices were stimulated twice ($\overline{S}_1$ and $\overline{S}_2$) as indicated. Field stimulation was used (2 Hz, 1 ms, 360 shocks). Krebs solution was perfused at a rate of 1 ml/min. Number of experiments (n = 4)

Exposure of the slices to a hypoxic condition, beginning at the 18th min after starting the collection increased the evoked release. The S_2/S_1 ratio was enhanced to 1.72 ± 0.04 (n = 6) without any significant change in resting release (Fig. 2). In order to examine whether activation of NMDA-receptors is involved in the effect of hypoxia on [^{3}H]DA release, we studied the effect of MK-801, a selective non-competitive NMDA-receptor antagonist. MK-801 (3 μM) itself had no effect on DA release, but it antagonized hypoxia-induced increase of evoked [^{3}H]DA release (Fig. 2).

When the effect of energy deprivation on [^{3}H]DA release was examined, slices were perfused with a glucose-free Krebs solution. Unlike hypoxia, the absence of glucose gradually enhanced the resting release (data not shown). The evoked release was not affected in hypoglycemic condition.

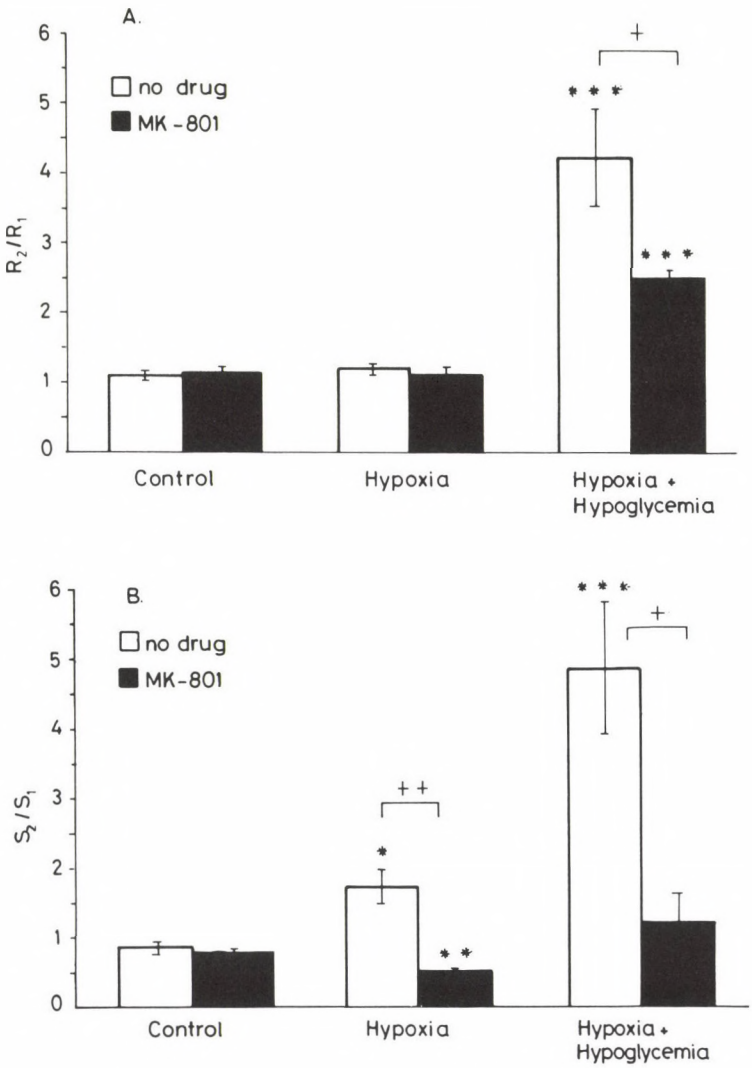

Fig. 2. Effect of MK-801 on: (A) resting (R_2/R_1) release of [^{3}H]DA; (B) stimulation-evoked (S_2/S_1) release of [^{3}H]DA. Blank columns – values under normoxic condition (control), in hypoxia, and under condition when hypoxia was combined with glucose withdrawal (hypoxia + hypoglycemia); Filled columns – values obtained in the presence of MK-801 (3 μM) under the same conditions. MK-801 was added to the perfusion fluid 20 min before S_2. The tissue was exposed to hypoxia and glucose-free condition for 30 min from the 18th min of perfusion. (*) significance as compared to the control (*$p<0.05$; **$p<0.01$; ***$p<0.001$) (ANOVA followed by Dunnett test). (+) level of significance between the releases in the presence and absence of MK-801 ($^+p<0.05$; $^{++}p<0.01$) (Student's *t*-test)

However, when the tissue was exposed to hypoxia in combination with hypoglycemia a massive release of [^{3}H]DA was observed (Fig. 2). When Ca^{2+} was withdrawn and 1 mM EGTA was added to the Krebs solution the effect of hypoxic-hypoglycemic condition on [^{3}H]DA release was partly reduced (data not shown). Tetrodotoxin (TTX) at a concentration of 1 μM completely blocked the release of [^{3}H]DA. Under conditions when hypoxia was combined with hypoglycemia the stimulation-evoked (S_2/S_1) and resting release (R_2/R_1) were enhanced to values of 4.85 ± 0.95 and 4.22 ± 0.69, respectively (Fig. 2). MK-801 (3 μM) significantly decreased the resting release, and also prevented the increase of stimulation-evoked tritium outflow under hypoxic-hypoglycemic condition (Fig. 2).

Discussion

Rat striatal slices perfused with Krebs solution deprived of either oxygen or oxygen and glucose as a model of cerebral ischemia were used to study the effect of these conditions on the release of [^{3}H]DA. Hypoxia increased the stimulation-evoked release of [^{3}H]DA, however, the resting release remained unchanged. Since MK-801, an NMDA-receptor antagonist and $[Ca^{2+}]_o$ withdrawal reduced the release of [^{3}H]-DA evoked by electrical field stimulation in hypoxic condition, it is very likely that glutamate release and Ca^{2+} entry underline this effect. Probably both NMDA-gated Ca channel and N-type voltage sensitive calcium channel (VSCC) are involved. Therefore, it is conceivable to suggest that in response to hypoxia glutamic acid is released from the cortico-striatal axon terminals and it activates the NMDA-linked channel with a consequent opening of N-type VSCC.

When hypoxia was combined with hypoglycemia an even more massive release of DA was obtained. The increase in resting release was partly Ca^{2+}-independent and TTX-sensitive. Since the application of 1μM TTX inhibited the release of DA in response to hypoxic-hypoglycemic condition, it is very likely that the release was associated with axonal firing. However, Cheramy et al. [5] have shown that glutamate stimulates the release of DA in a TTX-resistant manner. Moreover, our results have shown that MK-801, a NMDA-receptor antagonist attenuated the effect of hypoxia in combination with hypoglycemia on DA release, indicating that this effect is mediated by glutamate released in excess during hypoxic-hypoglycemic condition (Fig. 3b). The finding of Wang [33] that dopaminergic axon terminals are equipped with glutamate (NMDA) receptors and stimulation of these receptors resulted in a direct release of DA (Fig. 3a) from striatal synaptosomes corroborates this conclusion. Probably hypoxia in combination with hypoglycemia induces glutamic acid release as a result of the increased firing rate of cortico-striatal glutamatergic neurons, and this release is TTX-sensitive (Fig. 3c). Glutamic acid released under this conditions, in turn, releases DA from dopaminergic axon terminals by increasing $[Ca^{2+}]_i$. The

interaction of cortico-striatal glutaminergic and nigro-striatal dopaminergic axon terminals is either synaptic or non-synaptic [29, 31], but in both cases glutamate released stimulates DA release (Fig. 3a).

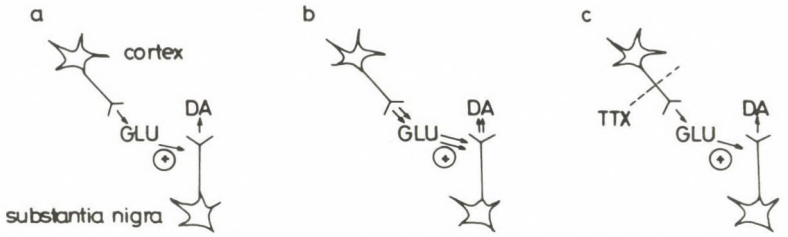

Fig. 3. Stimulation of striatal dopamine (DA) release by glutamate: a. Glutamate released from cortico-striatal pathway stimulated either synaptically or non-synaptically the release of DA from dopaminergic axon terminals; b. During hypoxic-hypoglycemic conditions the release of glutamate increased due to the increased firing rate of glutaminergic neurons which in turn increased the release of DA via activation of presynaptic NMDA-receptors; c. In hypoxia combined with hypoglycemia TTX attenuated the release of DA because of the blockade of cortico-striatal glutamatergic axon terminals

It was suggested by Vizi et al. [26, 28, 32] that while influx of $[Ca^{2+}]_o$ into the nerve terminals is mainly responsible for axonal stimulation-evoked release of transmitters, the increase of $[Ca^{2+}]_i$ mainly from intracellular stores, may result in an increase of resting release of the transmitters. Removal of glucose may produce a rapid reduction of ATP whose lack results in an inhibition of Na^+/K^+-ATPase. Since the inhibition of this enzyme results in an increase of transmitter release [25, 26, 27], the effect of glucose withdrawal may be mediated via this mechanism. This means that the loss of ATP results in an inhibition of Na^+/K^+-ATPase activity when glucose was omitted. Thereby the $[Na^+]_i$ increases, which in fact reverses the Na^+/K^+ exchanger. In addition, energy-dependent Ca^{2+} transporters being able to pump cytosolic Ca^{2+} out across the cell membrane, or into intracellular compartments (endoplasmic reticulum, mitochondria) are impared under low-energy conditions. In both cases the resting release is expected to be increased.

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CRF-DEPENDENT AND CRF-INDEPENDENT MECHANISMS INVOLVED IN HYPOPHYSIAL-ADRENAL SYSTEM ACTIVATION BY BACTERIAL ENDOTOXIN

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The immune system and the hypothalamic-pituitary-adrenal (HPA) axis play important role in the overall inflammatory response. The mechanism through which lipopolysaccharide (LPS, endotoxin) stimulates the HPA axis is not well understood. In order to clarify the role of hypophysiotropic peptides of paraventricular origin in the effect of LPS on ACTH and corticosterone secretion, the effect of LPS was studied on rats with lesions of hypothalamic paraventricular nucleus (PVN). It was shown that 90 min after 2 mg/kg LPS i.p. the ACTH, but not the corticosterone response was effectively blunted in PVN-lesioned rats, as compared to sham operated animals. However, in PVN-lesioned rats 240 min after treatment with LPS a significantly higher plasma ACTH and corticosterone level was monitored.

It is, therefore, suggested that in response to LPS activation of HPA both CRF(s)-dependent and CRF(s)-independent mechanisms are involved, even a direct effect of the adrenal cortex should be taken into account.

Keywords: hypothalamic-pituitary-adrenal axis, endotoxin, cytokines, median eminence, neuro-immune communication

Evidence has been accumulated that there is an interaction between the neuroendocrine and the immune system, and they are able to regulate each other's function. Recently, application of bacterial lipopolysaccharide, a subcellular component of Gram-negative bacteria [4], has been often used to study how the stimulation of the immune system activates the hypothalamo-pituitary-adrenal axis

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(HPA) [8, 14, 16, 31]. Interleukin-1 (IL-1) [5, 21] and tumor necrosis factor (TNF) [2, 22] cytokines produced mainly by LPS-activated macrophages and monocytes have been shown to be implicated in the activation of HPA. It was shown [19] that stimulation of ACTH secretion in mice treated with LPS, is at least partially mediated by or dependent upon the action of IL-1.

However, the mechanism through which LPS and related cytokines stimulate the HPA and the exact site(s) of their action still remains an enigma. While some authors using hypothalamic lesions [31] or pharmacological blockade of CRF release [16] have suggested that hypothalamus mediates endotoxin stimulation of HPA, others [14, 24] have observed that LPS could stimulate corticosterone secretion even after removal of the medial hypothalamus. There is now increasing consensus that the main site of action of IL-1 [1, 21] and TNF [2] is located in the hypothalamus, through the production of corticotropin releasing factor (CRF). However, it is currently under debate whether IL-1 or other cytokines may act directly on the pituitary level [3] or not [1, 21]. To clarify whether *in vivo* the endogenous CRF is crucial in the action of LPS on ACTH and corticosterone secretion, the effect of LPS was studied in rats with hypothalamic paraventricular nucleus (PVN) lesion. This nucleus is the major source of neuropeptides (CRF-41, vasopressin, etc.) involved in regulation of ACTH synthesis/release from the pituitary gland.

Material and methods

Male Wistar rats (weighting 200–250 g) were housed under controlled conditions (lights on: 07.00-19.00; temperature: 24 ± 1 °C; humidity 65%) and fed laboratory rat chow and water *ad libitum*.

Paraventricular nucleus lesion (PVL): Five days before the experiment the rats were anaesthetized by i.p. injection of 4 mg/100 g body weight pentobarbital. The hypothalamic paraventricular nucleus was lesioned surgically as described [13, 14]. Briefly, animals were placed in a stereotaxic frame. A specially designed microknife was lowered to the base of the skull through a burrhole behind the Bregma suture, rotated 360 ° and withdrawn. Rostrally this lesion usually damaged the anterior comissural nucleus; posteriorly it extended into the dorsomedial nucleus. Laterally it did not reach beyond the fornix. For sham-operation the skull was opened and the knife was lowered to the base of the skull as in the lesioned animals, but not rotated. The placement of the lesions was checked after the decapitation by registering the trace of the knife on the basal hypothalamus.

One day before the experiment the rats were weighed and placed into individual cages. On the next morning the animals were injected i.p. either with *E. coli* 0101/RG/W LPS (produced in "F. Joliot-Curie" National Research Institute for Radiobiology and Radiohygiene by the method of Westphal, using phenol extraction) 2 mg/kg or with saline. 90 and 240 min later they were decapitated under minimal stress. The trunk blood was collected into chilled tubes containing Na2EDTA and centrifuged. The plasma was stored at −20 °C until assayed.

Radioimmunoassays: Plasma adrenocorticotropin (ACTH) and corticosterone were determined by RIA, as previously described [12].

Statistical analysis: Data were transformed to logarithms before analysis of variance, which was followed by Dunn's test for multiple comparisons.

Results

Changes in plasma adrenocorticotropin concentration (Fig. 1)

In control animals injected with saline in both sham-operated and paraventricular lesioned groups the plasma ACTH concentrations were in the range of 8–22 fmol/ml. This value of ACTH corresponds well to values routinely obtained by our direct radioimmunoassay. Plasma ACTH levels 90 and 240 min after *E. coli* LPS administration were significantly higher in sham-operated animals ($p<0.01$) than in control animals (Fig. 1a).

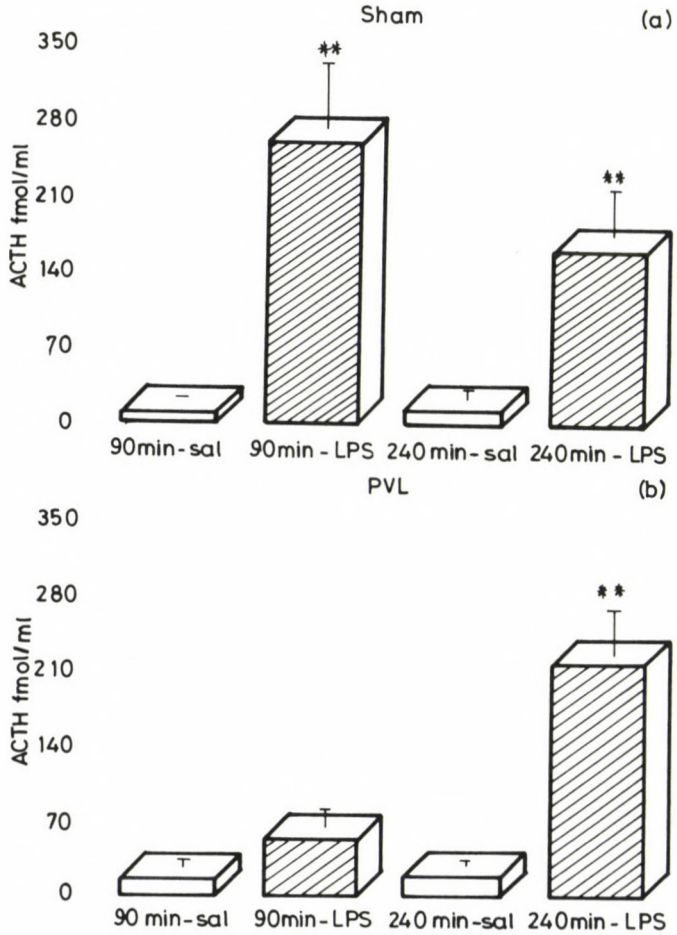

Fig. 1. Effect of 2 mg/kg i.p. bacterial lipopolysaccharide on plasma ACTH level in sham operated rats (Fig. 1a) and in rats with lesions of paraventricular nucleus (PVL) (Fig. 1b). Animals injected with saline (sal) were used as controls. Means ± S.E.M. from six animals in each group. **$p<0.01$ compared with the corresponding saline-treated group

In paraventricular lesioned rats the elevation of plasma ACTH, 90 min after LPS administration, was not significant compared to the saline-treated controls. Four hours later, however, plasma ACTH raised to the level which was not different from the hormone levels measured in sham-operated group at the same time (Fig. 1b).

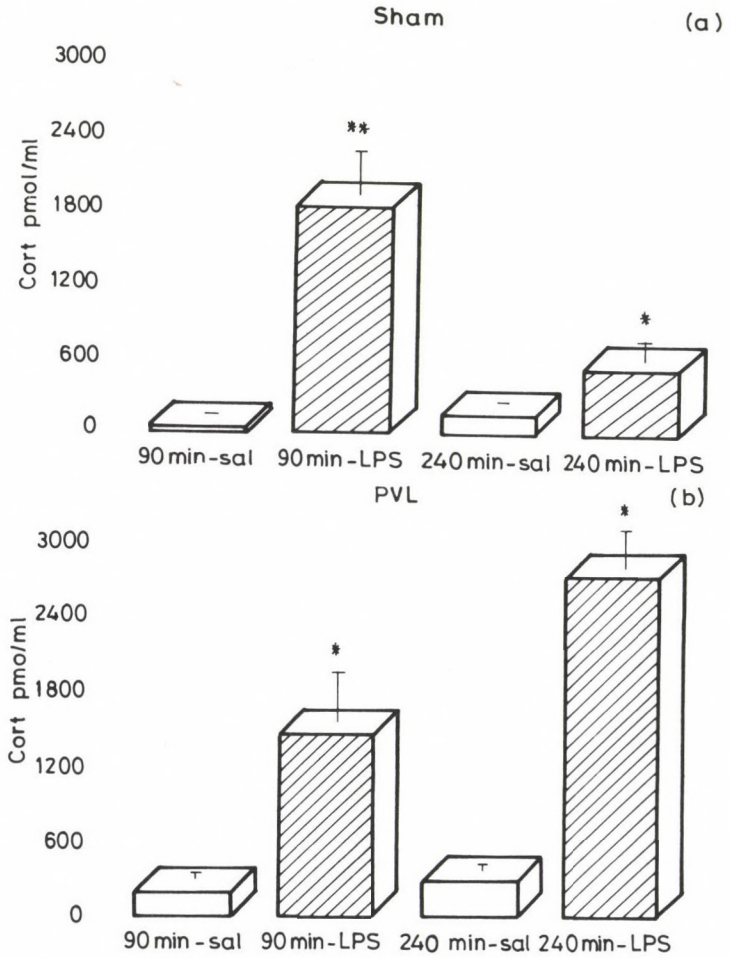

Fig. 2. Effect of 2 mg/kg i.p. bacterial lipopolysaccharide on plasma corticosterone level in sham-operated rats (Fig. 2a) and in rats with lesions of paraventricular nucleus (PVL) (Fig. 2b). Animals injected with saline (sal) were used as controls. Means ± S.E.M. from six animals in each group. *$p<0.05$ and ** $p<0.01$ compared with the corresponding saline-treated group

Changes in plasma corticosterone concentration (Fig. 2)

The basal corticosterone concentration in the plasma of saline-treated animals was in the range of 32–200 pmol/ml. In sham-operated rats LPS induced 7–56-fold elevation of corticosterone levels above the baseline. The maximal elevation was at 90 min (1810 pmol/ml) (Fig. 2a).

In paraventricular lesioned rats, 90 and 240 min after i.p. injection of LPS, significantly higher corticosterone levels were monitored, as compared to the controls. In contrast to the not significant elevation of plasma ACTH 90 min after the treatment, plasma corticosterone was induced to reach the a level comparable to that of the sham-operated control (Fig. 2b).

Discussion

In the present study evidence was obtained that *E. coli* LPS directly or indirectly is able to activate hypophysial-adrenal system even in the absence of hypophysiotropic neuropeptides of paraventricular origin. In PVN-lesioned rats, 90 min after LPS treatment, the elevation of plasma ACTH was blocked. This finding suggest that the action of LPS at this time requires CRF-41, AVP or other releasing factors of the paraventricular nucleus. However, after four hours a significant increase of ACTH level was observed indicating that the effect of LPS is not mediated through hypophysiotropic factors of paraventricular origin. Therefore, two different mechanisms might be involved in the effect of LPS: an early, CRF(s)-dependent, and a late, CRF(s)-independent mechanism. Our data are supported by the observation of Moberg [16]. He has shown that lesion of median eminence (ME), a hypothalamic structure where neurosecretory projections from the PVN terminate, totally abolished the effect of LPS. Therefore, it is conceivable to suggest, that *in vivo* the effect of LPS and/or related cytokines is located at the level of ME.

LPS and/or related cytokines released in the periphery may be able to reach ME, since this structure is devoid of blood-brain barrier. It has recently been shown that IL-1 [1, 21] and TNF [2] cytokines, which are released during LPS stimulation of the immune system, activate HPA through a CRF-dependent mechanism. In support if this hypothesis Sharp et al. [22] showed that intraparenchymal injections of IL-1β, adjacent to the hypothalamic ME, resulted in an increase of ACTH level. This finding also indicates that CRF-containing nerve terminals in the ME are the targets. Another possibility is that CRF/ACTH release [reviewed in 18] is modulated by catecholamines. Recently substantial stimulation-evoked and frequency-dependent release of NA from isolated ME was demonstrated [25]. The release was not subject to so-called alpha-2 adrenoreceptor mediated negative feedback modulation and it has been suggested that NA in this region exerts sustained tonic modulation through

alpha-2 adrenoreceptors exclusively located on the axon terminals of the hormone-containing neurons [25]. Using isolated ME, we have shown, that TNF was able to inhibit the stimulation-evoked release of NA from isolated ME [Elenkov, Kovách, Duda, Stark and Vizi, unpublished results]. These data suggest that TNF might influence the noradrenergic inputs in the ME and consequently modulate the release of CRF. Since central noradrenergic inhibitory influences on CRF release were proposed by Ganong [9], desinhibition of this inhibitory pathway by TNF might result in an increase of CRF release and subsequently ACTH production. This suggestion is supported by the findings of Matta et al. [15] who demonstrated that chronic neurotoxic ablation of central catecholaminergic neurones and acute depletion of central catecholamine pools were both associated with a substantial reduction in the ACTH secretory response to IL-1β instilled into ME. This means that this response depends on the availability of catecholamines to be released. It is also interesting that the ACTH response to intraventricular injection of IL-1 was effectively blocked by prazosin [30], an alpha-1 and alpha-2B adrenoreceptor antagonist.

It was demonstrated that ACTH response induced by IL-1 is mediated by hypothalamic PgE through CRF secretion [29]. In fact it was shown [17] that PgE levels in the ME were remarkably higher than in the medial basal hypothalamus and the anterior pituitary. Since PgE reduces the release of NA [10], and LPS and TNF enhances PgE_2 production in the hypothalamus of rats [6] it is conceivable to suggest the following (see Fig. 3): LPS or other immunological stimulus provokes the release of TNF and other interleukins mainly from monocytes and macrophages. TNF and probably also other cytokines being increased in the serum, act at the level of ME and release PgE. The increased production of PgE in turn inhibits the release of NA in the ME, thereby resulting in an increase of CRF release and subsequently ACTH and corticosterone production. This fine tuning of CRF release, based on non-synaptic communication [26, 27] and presynaptic transmitter modulation [27, 28], might result in a considerable increase in ACTH and corticosterone levels. The elevated serum level of hormones could prevent the excessive production of cytokines. This feedback modulation between IL-1 and glucocorticoids was proposed by Besedovsky et al. [5].

Concerning the CRF-independent mechanism in the late phase of the response, indicated by excessive ACTH response after four hours in PVL animals, apparently can be suggested that there is an other pathway involved in this mechanism. Our data suggest, however, that LPS or related cytokines, *in vivo*, during this time, might have a direct effect on the pituitary gland to release ACTH. However, LPS alone [31] was unable to induce ACTH secretion from cultured pituitary cells and other data available concerning direct effect at the pituitary level of different cytokines related to LPS are still contradictory [1, 3, 21]. Another possibility is, that LPS acts through an extrapituitary, non-paraventricular pathway. To explain

the phenomenon of "non-CRF" stimulation of ACTH production by LPS needs further evidence. It was shown recently that opioids are released in two secretory pools during endotoxin treatment [7]. In addition, intrinsic pituitary IL-1 and IL-6 was found to be induced by bacterial LPS [11, 23]. Therefore endogenous opioids and intrinsic interleukins might be involved in this process, however, this and other possibilities for the CRF-independent mechanism of activation of hypophysial-adrenal system needs further investigation.

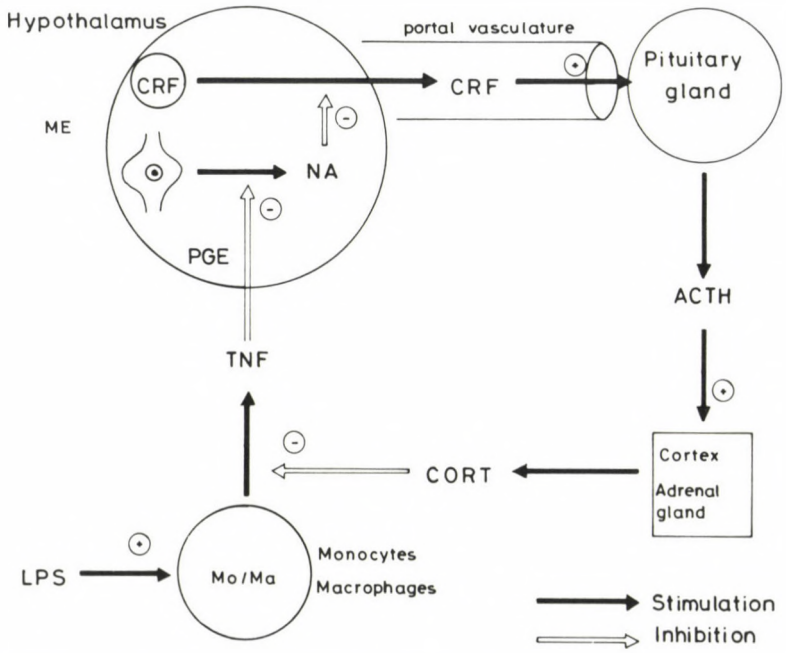

Fig. 3. Proposed model for CRF-dependent mechanism of activation of hypophysial-adrenal system by bacterial lipopolysaccharide and related cytokines. For more details, see the text

An increased plasma corticosterone level was observed after endotoxin treatment [8, 16]. The fact, that in our experiments there was an apparent corticosterone response in PVN-lesioned rats after 90 min, and at the same time the ACTH response was substantially blunted, suggests, but does not prove, that LPS and/or related cytokines have a direct effect on the adrenal steroid secretion. This possibility has been proposed for the effect of TNF [2].

In summary our results suggest that both CRF-dependent and CRF-independent mechanisms are involved in pituitary-adrenal axis activation by bacterial endotoxin, mechanisms in which ME might play an important interface role in the bidirectional communication between the neuroendocrine and immune systems.

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GROWTH OF THE ALIMENTARY TRACT WITH AGING IN CHICKENS

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There is a mathematical model supporting that when growth rate of the chickens is maximized and not constrained by the food-availability, the optimal relationship between body mass and alimentary tract mass should conform to a two-segmented straightened line with different slopes. In the present work we have studied the model using the mass of the intestines as an indicator of growth of the alimentary tract of the *Gallus gallus domesticus* L. We have observed the slope change of the two segments around a body weight of 90 g that corresponded to two-week-old animals and, at this age it was supposed that the differentiation of the intestine reach the maximum.

Keywords: growth, intestine, chicken, age

Growth is a process in which the organism undergoes change of form due to differentiation of the organs and tissues. There are different explanations for variations in growth rate. That one includes factors of the environment, such as availability of food, quality of diet [1]. The limitation of growth rate would be related to the maturation of the tissues or the rate at which animals can process food and covert it to biomass [4, 7, 8].

Lilja and Sperber [8] stated that the rate of postnatal growth is at least partly determined by the distribution of energy devoted to growth between the alimentary tract and the rest of the body. The rate of energy assimilation is limited by the size of the alimentary tract, so that growth rate is limited by the rate of assimilation. Konarzewski et al. [6] developed a mathematical model of growth, describing optimal allocation of energy between development of the alimentary tract and the rest of the body in birds. They compared the predictions of their model with the data of growth

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of 12 bird species and its assumption in a posterior study [6]. Their study did not include *Gallus gallus domesticus* L and purpose of the present paper was to check this model in male Leghorn chicken.

Materials and methods

250 male Leghorn chickens (Deklab XL) from 1 to 130 days old were used. The animals were sacrificed with chlorofrom. The alimentary canal was removed, carefully emptied and cleaned of adherent fat. The weight of intestines was determined to the nearest 0.01 g. The body weight was also determined.

For the broken-stick model proposed by Konarzewski et al. [5] to changes in body mass and emptied intestine mass we applied a computer program. The program was based of the least-square method and it loose for the best fit of the two-segmented linear models.

The best-fit models were chosen after comparison of their residual sum of squares with the other models by the means of the F-statistics. For comparison of the slopes and evaluations of the segments, *t*-test and covariance analysis were applied.

Results and discussion

Figure 1 shows the relationship between the coefficient intestinal-weight/body-weight and the animal age. This coefficient increased strongly in the first week and reached the maximum around the 15th day. This was followed by a progressive decrease to the age of 9 – 10 weeks.

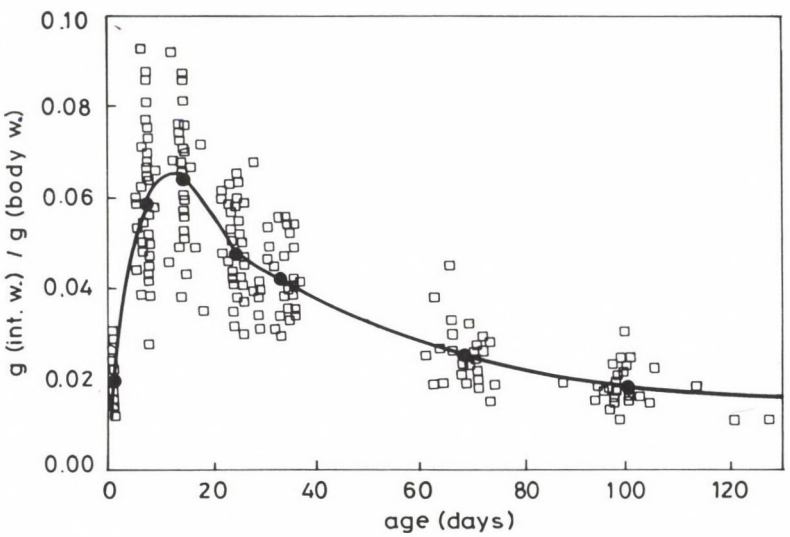

Fig. 1. Intestinal weight/body-weight at different ages in chickens. Individual values (□) and mean values (●)

The evolution of this parameter and the maximum observed in two-week-old chicken is in accordance with other authors who supposed that the differentiation of the intestinal tract begins in the embryonic period, continues after the hatching and reaches the maximum at the second week of life [1].

Figure 2 shows the relationship between body weight and intestinal weight in animals from 25 g to 400 g. The calculus was made with animals from 25 g to 2000 g but in the figure there are no represented all the animals because of the scale differences. We could observe two lines with statistically different slopes ($p < 0.001$). The first line was from 25 g to 90 g and the second from 90 g to 2000 g. The animals around 90 g were the 2-week-old ones and they corresponded to the animal age at we observed the maximum coefficient intestinal weight/body weight, described in Fig. 2.

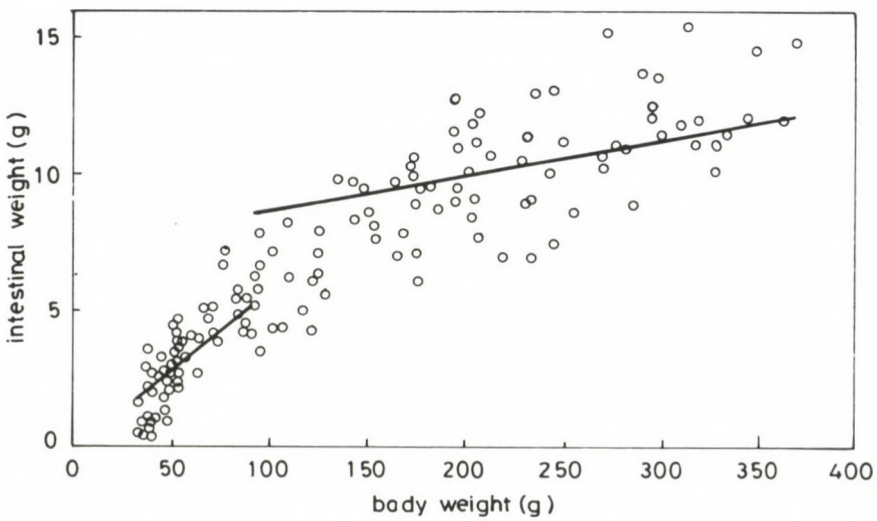

Fig. 2. The relationship between body mass and intestine mass in *Gallus gallus domesticus* nestling described by two-segmented straight lines. Individual values of animals from 25 g to 400 g

The two-segmented model was sufficient to fit the data. Its lines were described by the following equations:

first line: $y = 0.0567x$

second line: $y = 0.0129x + 7.3700$

According to the model proposed by Konarzewski the relationship between weight of body and digestive tract is linear. The data points on the body weight/digestive tract weight should therefore be best fitted by a two-segmented straight line with the slope of the first and second segment different from the first.

With the present study we confirm the model proposed by Konarzewski et al. [5] and the maximum differentiation of the intestinal tract of chicken around the second week of life.

The decrease observed thereafter would be indicative of the decline observed in some activities of the intestine such as nutrient transport [3, 9, 10].

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ANIMAL MODELS OF ANXIETY: A CRITICAL REVIEW

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Anxiety has been one of the most challenging targets for drug research. Despite the successful carrier of the benzodiazepines which revolutionized the pharmacotherapy of certain types of anxiety disorders in the past decades, there have still remained a lot of problems. One of the most important is that benzodiazepines have serious adverse side effects which limit their use in the practice: sedation, muscle relaxation, amnestic action, and addiction inducing properties [36]. Another serious problem is that several anxiety disorders do not have adequate pharmacotherapy at present [26, 31]. These two factors stimulate research for discovering new, non-benzodiazepine-like anxiolytic compounds.

Since animal models of anxiety are the main experimental tools in searching for compounds with potential anxiolytic activity, validation of these models is obviously a crucial point. One can evaluate them in the aspects of predictive and face validity. In the ideal case an animal model is considered to possess predictive validity if 1. the model does not yield false positives (i.e. drugs which show activity in the model but prove to be inactive in the human disorder), 2. neither does it yield false negatives (i.e. drugs which are thrown away on the basis of their ineffectiveness in the animal model, yet they would be potent in the human disorder), and 3. there is a strong positive correlation between the clinical efficacy of the tested drugs and their activity in the model [53].

Criteria of face validity involve 1. a clear resemblance to the human clinical phenomena 2. that the underlying neural and biochemical mechanisms of the animal model should correspond to those of the human disorder and 3. that pharmacological manipulations should influence the animal model in the same manner like they affect

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the clinical illness. A possible classification of some commonly used behavioural models of anxiety is shown in Table I (only rodent models):

Table I

Classification of rodent behavioural models of anxiety

Test	Reference	Test	Reference
1. Approach-avoidance paradigms:			
Geller–Seifter test	20, 40	light-dark box	10, 11, 13
lick conflict	38, 40, 52	social interaction	15, 22, 26
shock-probe conflict	34, 50	neophobia	5, 36, 41, 55
four-plate test	1, 46	passive avoidance	27
elevated plus maze	26, 37, 45, 54	shuttle-box self-stimulation	21
hole-board	2, 17, 23, 35		
2. Instinctive behaviours:			
aggressive behaviour		defensive burying	6, 50
– shock induced fighting	47, 51	ultrasonic	14, 31
– isolation induced fighting	28	distress calls	
3. Interaction with anxiogenic drugs:			
pentetrazole drug discrimination	29, 32		
4. Other models:			
handling order	3, 30		
PAG stimulation	19, 24		

Approach-avoidance paradigms: this group contains the vast majority of the behavioural models. The common paradigm of these methods is shown in Fig. 1. It exploits natural drives of the animal resulting in approach behaviour which is in turn confronted with aversive environmental stimuli inducing avoidance behaviour. This situation results in the suppressing of the approach behaviour which is considered as the model of the anxiety state. Anxiolytic drugs release the suppressed behaviour, in other words they exert a disinhibitory effect.

Probably, the most widely used paradigm is the so-called "lick conflict" or "punished drinking" test [52], which uses thirst as the approach drive and shock as the aversive (punishing) stimulus. The method of Geller and Seifter [20] is a similar paradigm – in fact this was the "ancestor" of the former one. It is also based on the hunger/thirst – shock coupling, but it involves a conditioned behaviour component, as well. These two methods have many variations in the literature [38, 40].

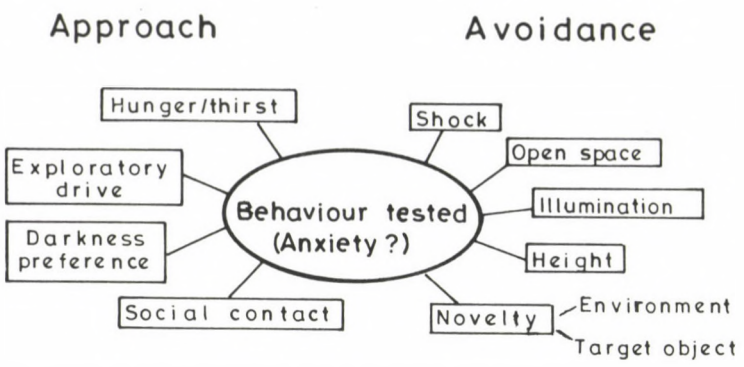

Fig. 1. A general scheme of the approach-avoidance models of anxiety. See explanation in text

A big disadvantage of these methods is the use of food- or water-deprivation, since drugs increasing water or food intake on their own may emerge as false positives. Two other models eliminate the deprivation factor: the four-plate test [1, 46] and the shock-probe conflict test [34, 50] in which the exploratory drive is used instead of hunger or thirst as the source of the approach behaviour and that is punished by shock.

However, applying shock as aversive stimulus also bears certain problems. First, it is far from being a physiological, naturally occurring stimulus. Second, it gives the possibility of detecting false positives: compounds which increase the number of tolerated shocks by raising the pain threshold of the animal.

For these reasons, models of the approach-avoidance type involving neither deprivation nor shock came into use. Exploratory behaviour is exploited against height + open space in the elevated plus maze [26, 37, 45, 54], against novelty in the hole-board [2, 17, 23, 35, 37] and against bright illumination in the light-dark discrimination test [10, 11, 13]. Novelty + bright light are also used to suppress social contact between pairs of rats in the social interaction model [15, 22, 26].

Other approach-avoidance pairs worth mentioning are hunger vs. novelty of food [36], or of environment [5, 42, 55] or both [41] in variations of the neophobia model, darkness preference vs. shock in the passive avoidance test [27], and rewarding vs. aversive self-stimulation of the brain in the shuttle-box self-stimulation method [21].

2. Instinctive behaviours: in these models a genetically innate behaviour, such as intraspecies aggression, distress vocalization or defensive burying is evoked experimentally. Aggression can be induced by isolation [28] or by electric shock [47, 51]. Ultrasonic distress calls can be evoked in rat pups by taken them away from their mother [31] or in adult rats by electrically shocking them [14]. Defensive burying can

be stimulated by a shock-probe [50], or by glass marbles [6]. Anxiolytics inhibit these behaviours, in contrast to their disinhibitory (releasing) effect in the approach-avoidance models.

3. Interaction with anxiogenic drugs: Interesting, while among the models of other mental disorders like depression or schizophrenia interactional methods are widely used and accepted as valid models (e.g. antagonism of various effects of apomorphine in the case of antipsychotics or antagonism of tetrabenazine-actions in the case of antidepressants), the overwhelming majority of behavioural anxiety models is 'situational', that is the "state of anxiety" is evoked by placing the experimental animal in a certain situation and not by treating it with a specific compound. Among behavioural anxiety models the antagonism of the effect of the anxiogenic compound pentetrazol (PTZ) is the only interactional one. The method is actually a drug discrimination paradigm for PTZ [29, 32]. A disadvantage of this model is that only drugs acting via the same machinery on which PTZ acts (the BZD-GABA-Cl^- ionophore complex) can be detected. On the other hand, a very nice correlation has been found ($r=0.89$) between the activities of anxiolytics in this method and their average clinical daily doses [29].

4. Other (non-grouped) models: Handling order: a new and simple model of anticipatory anxiety: animals taken out last from the home cage show hyperthermia compared to those which are handled first. Anxiolytic drugs can reduce this hyperthermia [3, 30].

Stimulation of the dorsal part of the periaqueductal gray matter (PAG): electric stimulation of this part of the brain in man evokes a state of anxiety which resembles a panic attack. In rats stimulation of this area results in freezing behaviour alternating with vigorous flights (running and escape attempts) and apparently aimless vertical jumps, accompanied by vegetative signs such as piloerection, midriasis, increase of respiratory rate. Elevation of the current threshold inducing these behaviours is considered to be an anxiolytic effect [19, 24].

Models of anxiety disorders have some characteristic features which one must take into consideration when screening anxiolytic drugs: 1. large variance of behavioural variables due to individual differences, 2. large variance due to sensitivity to environmental factors: the tested behaviour is extremly sensitive to even minor changes in environmental factors: shock parameters [40, 47, 49], level of illumination [15, 31, 37], height of the plus maze [37, 45], size of the experimental chamber especially when exploration or behavioural interaction are measured [10, 13, 31, 51], familiarity with the experimental chamber [15, 37, 38], handling procedure prior to the sessions [3], degree of deprivation [49], conditioning schedule parameters [40, 48], duration of measurement [37]. These factors all influence the aversiveness of the aversive stimuli and the drive of the approach behaviour, consequently the overall behaviour of the animal in the experimental situation. There are many conflicting

results in the literature about the actions of new non-benzodiazepine-like anxiolytics. Discrepancies can be in a large degree attributed to the differences in these "details" of the experiments, 3. inverted U-shaped dose-response curves: they are very typical in the approach-avoidance types of models, contrasting to the PTZ antagonism where classical dose-response curves can be plotted.

The inverted U-shaped dose-response curve bears the risk that with too high or too low screening doses one can miss an active compound (Fig. 2). The situation is more complicated due to the experience that the height of the curve (i.e. the percentual effect) the width of the curve (i.e. the anxiolytic dose range) can change from experiment to experiment and even the curve itself can shift, and all these can happen simultaneously. For this reason finding anxiolytic activity is much more difficult than finding e.g. antidepressant or antipsychotic activity.

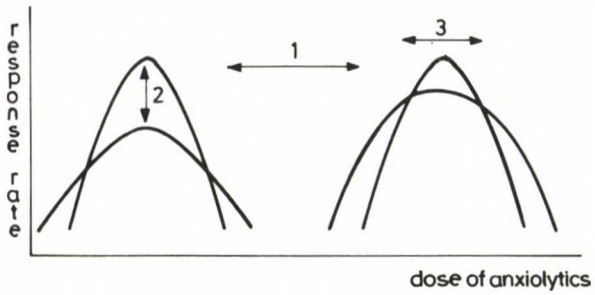

Fig. 2. Characterization of the inverted U-shaped dose-response curves of anxiolytics. 1 – shift of the curve, 2 – change in the maximal effect, 3 – change in the anxiolytic dose-range

Consequently, some practical guidelines for screening anxiolytic drugs can be established. 1. Dose-range studies are necessary instead of the use of a single screening dose. 2. Rigorous control of environmental factors (including handling!) is unavoidable. 3. Experiments must be conducted with relatively large samples. 4. Application of at least three different types of models is recommended, since the different models do not detect the same set of compounds. The false positive – false negative pattern varies from test to test. 5. Experiments must be repeated whenever a tendency of anxiolytic effect has been observed in the marginal doses since one may "catch the tail" of the inverted U curve in a single experiment.

In Table II activities of certain anxiolytic drugs in various models of anxiety are compared.

Table II

Activity of some anxiolytic compounds in various models of anxiety (Numbers of appropriate references)

Model	BZDs	Buspirone	Ritanserine	Ondansetron	TCAs
lick conflict	+ (14, 40, 52)	+ (14, 40, 44)	+ (9, 40)	– (7, 9, 25, 39)	–(C+) (18, 40)
Geller–Seifter test	+ (14, 40)	+ (4, 9, 14, 40, 49)	+ (4, 40)	– (7, 39)	–(C+) (40)
elevated plus maze	+ (12, 31, 37, 54)	+ (7, 45)	+ (7, 9, 12, 31)	– (16, 39)	– (31, 37)
social interaction	+ (14, 22, 31)	+ (9, 14, 22)	– (4)	+ (7, 9, 25, 39)	– (7, 31)
light-dark discrimination	+ (10, 11)	+ (4, 9, 11)	+ (4, 9, 10)	+ (7, 9, 11, 25)	– (11)
four-plate test	+ (1)				– (1)
shock-probe conflict	+ (34, 50)	+ (50)	+ (9, 34)		+ (34)
neophobia	+ (5, 8, 36, 41, 42, 55)		+ (42)	+ (42)	–(C+) (18, 41, 55)
passive avoidance	+ (27)	+ (27)			– (27)
antiaggressive activity	+ (14, 28, 49)	+ (14, 49)			+ (28, 33, 43)
defensive burying	+ (6, 50)	– (7)		– (7)	– (7)
ultrasonic vocalization	+ (14, 31)	+ (14)			
handling order	+ (3, 30)	+ (30)			– (30)
PAG stimulation	+ (19, 24)		– (19)	– (7, 24)	– (19)

BZDs: benzodiazepines
TCAs: tricyclic antidepressants
C: chronically given
+: positive findings have been found in the literature, together with or without negative results
–: only negative findings have been found in the literature
blank cells: no relevant data have been found in the literature

Benzodiazepines represent the classical anxiolytics acting through the GABA-BZD receptor complex. Buspirone, ritanserine and ondansetron (GR 38032F) are drugs which exert their effect through the serotonergic system. Buspirone is a 5-HT_{1a} partial agonist [7, 13] and has been on the market for a few years. Ritanserine, a 5-HT_2 antagonist [7, 13] and ondansetron, a 5-HT_3 antagonist [24] are in clinical trial phase. The tricyclic antidepressants also are used in the treatment of certain anxiety disorders [7, 26, 31].

As it can be seen from Table II benzodiazepines show activity in all the models. However, this is the direct consequence of the fact that these compounds are the first to validate a new model with. Despite the well-known contradiction in the literature about the effect of buspirone and ritanserine in behavioural models of anxiety, yet, there are quite a lot of studies which could detect the activities of these molecules. The picture is more confused in the case of ondansetron: this drug did not prove to be active in about half of the models in which it was tested. Nevertheless, its clinical efficacy is still to be proven, too.

The inactivity of tricyclic antidepressants in the majority of the models seems to be unsatisfactory, nevertheless, there are some more promising aspects. First, the effect of imipramine and desipramine could be detected in the traditional lick conflict paradigm (desipramine also in the neophobia model) after chronic treatment – a factor which is also necessary to get a clinical effect of these drugs. Second, imipramine was found to be active after single doses in two other tests. However, there is not a single behavioural model of anxiety at present which would be able to detect all the clinically used or investigated anxiolytic drugs. Although this fact is depressive from one hand, it is at least very stimulating for inventing new models of anxiety from the other hand.

The human anxiety disorders are classified into several subtypes. The relevance of the methods mentioned so far to model the different subtypes of human anxiety disorders is an important question. Following the classification of the DSM-III-R [26, 31] some models are listed in Table III. These models have been suggested to be up to a certain degree model of a given subtype of anxiety disorders.

Conflict procedures as models of generalized anxiety disorder (GAD) are verified by their predictive validity: the classical and reliably detectable target molecules of these paradigms are the benzoidiazepines – the compounds, the main indication of which is GAD in the human practice [31, 40]. However, with respect to face validity conflict procedures cannot be considered a fair model of GAD for two reasons. 1. The animal models bring about a kind of 'state-anxiety', related to a concrete situation, while GAD is typically a form of trait-anxiety [26, 31]. 2. In the models the 'anxiety state', i.e. the suppression of approach behaviour, is a rational, reasonable reaction of the experimental subject to the environmental aversive stimuli, whereas perhaps the most characteristic feature of GAD is the irrational nature of anxiety, the "causeless fear".

Table III

Anxiety models which supposedly correspond to DSM-III categories of anxiety disorders

Disorder	Model	Predictive validity	Face
generalized anxiety disorder	conflict procedures	+	-
panic disorder	PAG stimulation	-	+
agoraphobia	elevated plus maze	-	?
social phobia	social interaction	-	+
obsessive-compulsive disorders	defensive burying	+?	+?
posttraumatic stress disorders	ultrasonic vocaliz.	?	+

? means questionable or no experimental evidence

PAG stimulation, social interaction, and ultrasonic distress vocalization are suggested as models of panic disorder, social phobia, and posttraumatic stress disorder (PTSD), respectively, obviously on the basis of their phenomenological similarity to the human disorder [7, 19, 24, 31]. Unfortunately, their predictive validity has not been verified yet: on one part, tricyclic antidepressants, which are the usual drugs of choice in panic disorder and PTSD [31] have been found to be ineffective in PAG stimulation [19]. (Their effects have not been investigated yet in ultrasonic vocalization.) On the other part, compounds, active in these methods (benzodiazepines first of all) are not suggested to be chosen in the human illnesses. In the case of social phobia no adequate pharmacotherapy exist at present [26, 31]. The latter statement also applies to agoraphobia thereby questioning the predictive validity of the elevated plus maze as a model of this anxiety subtype. The latter idea was tentatively hypothesized by Pellow et al. [37], on the basis of the observation that during repeated trials habituation did not occur – as it is the case in agoraphobia, as well (a faint kind of face validity).

Defensive burying as proper model of obsessive-compulsive disorder (OCD) was suggested by Broekkamp et al. [7] on the basis that serotonin uptake inhibitors show potent activity both in the animal model and in the human disease. It can be further argued that there is some similarity between the method and OCD if one considers obsessive-compulsive activities as such notorious actions which have lost their once existing significance and relevance in the actual environment (e.g. hand washing in order to avoid infection). Defensive burying has the same character: an

originally purposeful behaviour which became irrelevant, notwithstanding being notoriously performed, in the experimental environment. Further experimentation is needed of course to prove or to disprove the predictive and face validity of this model.

Reviewing the relations among the members of the triad: disease – animal model – drug effect, it is clear that the present situation is far from being satisfactory. The overall answer to the question: can the animal models reflect the subtypes of anxiety disorders?, is no, or at least not yet. One of the main target of anxiolytic research is to invent models which are more refined and specialized to the different forms of human anxiety, thus giving the chance to discover drugs for subtypes of anxiety disorders which do not have adequate pharmacotherapy at present.

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PHOSPHOLIPIDS AND FATTY ACIDS IN HUMAN BRAIN TUMORS

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Phospholipid and fatty acid composition of human brain tumors is presented. The white matter contains a greater amount of phosphatidylinositol and a very low level of lysophosphoglycerides, as compared to the grey matter. Glioma and meningioma tumors contain a greater amount of phosphatidylinositol, sphingomyelin, and lysophosphoglycerides, as compared to normal cortex tissue. A significant rise in oleic, linoleic and arachidonic acid content in tumor tissue was observed. It is suggested, that changes in lipid composition, may play a role in structural and functional membrane perturbations in neoplastic cells.

Keywords: glioma, meningioma, phospholipids, fatty acids

The lipid bilayer of cellular membranes tends to exist at the transition point between gel and liquid crystalline state with a change in environmental temperature, the lipid composition and structure are varied to maintain the existence of both phases [21, 37]. A consequence of this partition of phases is in addition to the sidedness of lipid families, both fluid and solid-like domains exist with different fatty acid composition [27]. Between these domains there exist, boundary defects and these interfacial areas may correspond to binding sites for enzymes or transport proteins or may even accomodate pockets of water. The lipid bilayers are thus not homogeneous mixtures containing a cross-section of the total fatty acids and the influence of different types of fatty acids is, in consequence, much greater than if their properties were simply averaged. The size of the fluid domains is in large extent dependent on the proportion and nature of the unsaturated fatty acid components of the membrane lipids.

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The principle forces binding integral proteins to the lipid bilayer are hydrophobic [30], although ionic forces may have been involved in the initial steps of membrane assembly and are a major factor in the binding of peripheral proteins to the head-group region of the bilayer.

In a fluid bilayer, the proteins undergo conformational changes and are in a "fluid-like" state in which accessibility to other molecules is increased [13]. Increased viscosity decreases the conformational freedom of the proteins and their ability to respond to external effectors [26]. Heron et al. [18] have considered that a decreased membrane fluidity brought by increased cholesterol to phospholipid ratio or sphingomyelin/phosphatidylcholine ratio or increased saturation degree of the acyl chains, causes increased lipid–lipid interaction and decreased lipid–protein interaction. Increased fluidity, on the other hand, may result in vertical displacement of the protein receptors into the membrane [10].

Activity of most membrane-bound enzymes, increases with increased degree of membrane lipid unsaturation [4]. On the other hand (Na^+, K^+, Mg^{2+}) ATPase had the reverse behaviour, indicating a possible inactivation of the enzyme by polyunsaturated fatty acids [8]. The polar head groups of the membrane lipids have been shown to have a definite influence on the enzyme activity, as well [34].

The central nervous system is characterized by a high lipid content, occupying more than 50% in dry weight [22, 32]. Although the brain tumors are also rich in lipids, there are only few reports concerning brain tumors based on lipid biochemistry. However, up to now, the correlation between membrane phospholipids and membrane functions in brain tumors has not been clarified.

In this study, we analyzed phospholipids obtained from human brain tumors and compared these results with control samples of grey and white matter of the brain.

Materials and methods

Eighteen brain tumors, including 8 gliomas (5 glioblastomas and 3 astrocytomas) and 10 meningiomas (3 meningiotheliomatous, 5 transitional and 2 fibroblastic) were obtained during surgery. The diagnosis in each case was confirmed histologically. Portions of adjacent normal-appearing cortex and white matter were obtained for control study.

Frozen tissue samples were homogenized in 100 mM Tris-HCl buffer, pH = 7.2, containing 0.25 M sucrose. Lipid extraction was performed according to Folch et al. [14]. Phospholipids were analyzed by thin-layer chromatographic method on precoated silica gel G plates (E. Merck, Darmstadt, Germany) as previously described [38]. Fatty acid composition of particular phospholipid classes was analyzed by gas chromatography (Perkin Elmer F 30 apparatus, Perkin Elmer Ltd., Beaconsfield, Bucks., England) as described in another paper [29]. Phosphorus content was estimated according to Bartlett [6].

Statistical analyses were performed by Student's t test for unpaired data.

Results

Table I shows the per cent composition of phospholipids in brain tumors and in control grey and white matter. Phosphatidylethanolamine and phosphatidylcholine in the cortex are in equal amounts, however, in the white matter a greater amount of phosphatidylethanolamine was found. In gliomas the levels of phosphatidylethanolamine and phosphatidylserine were significantly lower, in meningiomas high level of sphingomyelin was observed.

Table I

Brain tumor phospholipids (% of total)

	Grey matter	White matter	Glioma	Meningioma
	A	B	C	D
PE	32.1 ± 1.5^{a}	36.2 ± 1.6	28.3 ± 1.5^{bc}	31.5 ± 1.6^{e}
PC	34.2 ± 1.6^{a}	28.1 ± 1.4	30.7 ± 1.6^{b}	29.2 ± 1.5^{e}
PS	16.3 ± 1.1	17.3 ± 1.0	10.5 ± 0.9^{bc}	6.2 ± 0.5^{de}
PI	1.4 ± 0.1^{a}	4.1 ± 0.3	4.0 ± 0.3^{b}	3.1 ± 0.3^{de}
PA	0.3 ± 0.1	0.4 ± 0.1	0.3 ± 0.1	0.3 ± 0.1
Sph	11.3 ± 0.9	13.2 ± 1.0	15.3 ± 1.1^{bc}	22.1 ± 1.5^{de}
LpC+LpE	4.4 ± 0.3^{a}	0.7 ± 0.1	10.9 ± 1.0^{bc}	7.6 ± 0.6^{de}

Mean values ± S.D. Abbrevations used: PE – phosphatidylethanolamine, PC – phosphatidylcholine, PS – phosphatidylserine, PI – phosphatidylinositol, PA – phosphatidic acid, LpC – lysophosphatidylcholine, LpE – lysophosphatidylethanolamine, Sph – sphingomyelin. a $p<001$ A vs B, b – $p<0.001$ C vs A, c – $p<0.001$ C vs B, d – $p<0.001$ D vs A, e – $p<0.001$ D vs B

Table II shows fatty acid composition of phospholipids in tumors. The grey matter is copious in arachidonate and docosahexanoate. Tumor tissues are rich in linoleic; linoleic and arachidonic acids, as compared to non-neoplastic tissue. The basal difference between "indolent" meningioma and "malignant" glioma is the palmitoleic acid content.

Table II

Fatty acids of brain tumor phospholipids (mole %)

	Grey matter	White matter	Glioma	Meningioma
	A	B	C	D
16 : 0 DMA	1.9 ± 0.2^{a}	0.4 ± 0.1	1.8 ± 0.2^{c}	2.1 ± 0.2^{e}
16 : 0	25.2 ± 1.5^{a}	19.3 ± 1.4	24.1 ± 1.6^{c}	23.4 ± 1.6^{e}
16 : 1 (n−7)	0.5 ± 0.1^{a}	3.3 ± 0.3	3.3 ± 0.3^{b}	0.4 ± 0.1^{e}
18 : 0 DMA	1.5 ± 0.2^{a}	1.1 ± 0.2	0.9 ± 0.1^{b}	0.6 ± 0.1^{de}
18 : 0	22.1 ± 1.6^{a}	16.5 ± 1.4	16.7 ± 1.5^{b}	16.7 ± 1.2^{d}
18 : 1 (n−9)	18.4 ± 1.2^{a}	34.2 ± 1.8	24.2 ± 1.4^{bc}	27.5 ± 1.4^{de}
18 : 2 (n−6)	1.6 ± 0.2^{a}	0.4 ± 0.1	3.0 ± 0.3^{bc}	5.2 ± 0.4^{de}
18 : 3 (n−3)	0.8 ± 0.1^{a}	0.6 ± 0.1	1.1 ± 0.2^{c}	1.2 ± 0.2^{de}
20 : 0	2.1 ± 0.2^{a}	2.6 ± 0.2	1.8 ± 0.2^{c}	1.2 ± 0.2^{de}
20 : 4 (n−6)	6.6 ± 0.5^{a}	5.1 ± 0.4	8.2 ± 0.6^{bc}	12.3 ± 1.0^{de}
22 : 0	3.5 ± 0.4	3.4 ± 0.3	2.2 ± 0.2^{bc}	1.1 ± 0.2^{de}
22 : 6 (n−6)	10.9 ± 1.0^{a}	2.5 ± 0.3	5.1 ± 0.5^{bc}	2.2 ± 0.2^{d}
Others	4.9 ± 0.4^{a}	10.6 ± 0.8	7.6 ± 0.6^{bc}	6.1 ± 0.5^{de}

Mean values ± S.D. DMA – dimethylacetal. Significance of differences as in Table I

Discussion

Stein et al. [38] analyzed the fatty acids in various primary brain tumors. Their results were as follows: primary glial tumors were characterized by an increase in percentage of linoleic and palmitoleic acids, and a reduced percentage of stearic and palmitic acids; meningiomas and neurinomas showed a fatty acid pattern resembling a glioma. Similar results were obtained by Hattori et al. [17]. However, these changes did not correlate with morphological subtypes.

Nayyar [31] discovered a reduction of cephalin and sphingomyelin content in brain tumors. Gopal's studies [16] also revealed that the lecitin/cephalin ratio, which was 0.9 in normal brain tissues, was higher than 1.0 in brain tumors and reached 2.2 in meningiomas. Cristensen Lou et al. [12] also reported an increase in the relative content of phosphatidylcholine and sphingomyelin at the expense of phosphatidylethanolamine in brain tumors. They supposed that a high phosphatidylcholine/phosphatidylethanolamine ratio was indicative of a low degree of cellular differentiation and organization, because this ratio decreased during intrauterine life. This was confirmed by us [23, 24] in the case of phospholipid time-dependent changes in the organs of pig during ontogenesis.

Interestingly, we have observed an increase in phosphatidylinositol content in brain tumors, in comparison with the cortex. This phospholipid is a major reservoir of arachidonic acid in the cell [7, 25]. Poduslo et al. [33] also reported a high level of this phospholipid in human glioma cell line. High content of phosphatidylinositol may be the evidence for increase of easily accessible arachidonate pool in neoplastic tissue.

Cooper et al. [11] have shown, that brain tumors produce large amounts of prostaglandins, prostacyclin and thromboxane. Highest production of these compounds was observed in meningiomas.

The role played by prostaglandins in brain tumors is not known. Gliomas have been shown to excrete PGE_2 [15] and only little amounts of neuromodulatory PGD_2 [36]. Approximately 20% of meningiomas invade bone, with bone resorption and new bone formation occurring simultaneously. Since PGE_2 is a potent bone resorbing agent [3], it may be that this prostaglandin released locally from the tumor cells plays a role in this process. Because the accessibility of arachidonate is a limiting factor in prostaglandin production [35], the increase in this fatty acid in brain tumors, observed in our study, may be the expression of increased supply of this prostanoid precursor.

Although some authors have shown an increase in plasmalogen content in brain tumors [1, 2, 40], our data could not verify these findings.

In previous work [28] we have investigated the cytoprotective effect of prostaglandins on red blood cells of atherosclerotic patients, and we have stated that in these cells a compensatory mechanism exists, concerning decrease in palmitic and stearic acid content, with concomitant increase in linoleic and arachidonic acids, to maintain the proper membrane fluidity [29]. Thus, it is possible, that the increase in linoleic acid content in brain tumor phospholipids observed by us in this work, plays a similar role.

Hattori et al. [17] and Barnett et al. [5] have shown, that meningiomas have more fluid membranes, than normal brain cells. This increase in fluidity may be a result of increase in unsaturated fatty acid content. However, there are many other factors modulating membrane fluidity besides the content of phospholipids and fatty acid composition [9, 19, 20]. The increase in mono- and polyunsaturated acid content in brain tumors, observed by us, seems to support this hypothesis.

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THE EFFECT OF THYROIDECTOMY AND THYROXINE ON THE REACTIVITY OF RAT DIAPHRAGM MUSCLE TO ELECTRICAL STIMULATION *IN VITRO*

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The effect of thyroidectomy and thyroxine on the reactivity of diaphragm muscle to electrical stimulation was studied in adult albino Wistar rats. Thyroidectomy significantly affected the contractility of the diaphragm muscle. The result shows that thyroidectomy predisposes the muscle of the diaphragm to fatigue.

Keywords: thyroidectomy, thyroxine, electrical stimulation, diaphragm muscle, contractility, fatigue

It has been demonstrated that contraction and relaxation in skeletal muscle occurs primarily by means of intracellular Ca^{2+}, and that there is only a minimal dependence on extracellular Ca^{2+} [6]. The functional state of the diaphragm – a skeletal muscle – is very important for the normal functioning of the respiratory system [7]. Thyroid gland secretes calcitonin a hormone which is known to have significant influence on intracellular Ca^{2+} store and on the magnitude of extracellular Ca^{2+} in the body [4]. Thyroxine and triiodothyronine are also produced by the thyroid gland [5]. These two hormones are known to be involved in protein metabolism and therefore are of great importance in determining the functional status of most skeletal muscle such as the diaphragm [1, 7]. From the foregoing it is apparent that the thyroid gland could be playing an important role in regulating the function of the diaphragm. The present study therefore examines the role of thyroidectomy and chronic thyroxine administration on the contractility of the diaphragm muscles *in vitro*.

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Materials and methods

Adult male Wister strain rats were used. They were divided into four groups: (i) control (normal rats); (ii) thyroidectomized; (iii) thyroidectomized rats treated with thyroxine; and (iv) thyroxine-treated rats.

At the onset of the experiment, surgical extirpation of the thyroid gland was carried out in the rats in groups (ii) and (iii), care was taken to ensure that the external parathyroids were intact. Rats in groups (iii) and (iv) were fed thyroxine tablets (AH Cox & Co. Barnestaple, UK) ground and administered in the drinking water *ad libitum* at a dose of 6-8μg/100g body weight per day. All rats (groups i – iv) were given rat chow and tap water *ad libitum* for 30 days.

At the end of the experimental period of 30 days, the rats were stunned and killed by exsanguination. The diaphragm muscle was dissected and mounted in a standard organ bath under an initial load of 2 g. The organ bath contained double glucose tyrode solution (mMol/l) Na^+ 149.2; K^+ 2.7; Ca^{2+} 3.6; Mg^{2+} 2.1; Cl^- 145.3 H_2PO_4; HCO_3^- 11.9; and glucose 10. The bath was maintained at body temperature (approximately 37°C) and the pH of the solution was 7.3 – 7.4. Contractions of the hemidiaphragm were elicited by direct stimulation using rectangular pulses of 2 ms at a frequency of 1 Hz and a supramaximal voltage. Isometric contraction was recorded using a force – displacement transducer (Grass FT. 03.) and a Grass polygraph (Model 7D, Grass instruments, Quincy MA).

Dose response curves to procaine HCl (Fig. 1) were obtained by cumulative addition of graded concentrations. The maximum dose so obtained was then used on a fresh hemidiaphragm of the same animal, to obtain the time to maximum inhibition of the muscle (Fig. 2)

A high concentration of KCl (85 mMol) was added into the organ bath after maximum inhibition of the muscle by procaine HCl. The tension developed by the muscle was then recorded.

Statistics

Results were expressed as mean values ± SE of the mean. Significance was determined by the Student's *t*-test for unpaired data.

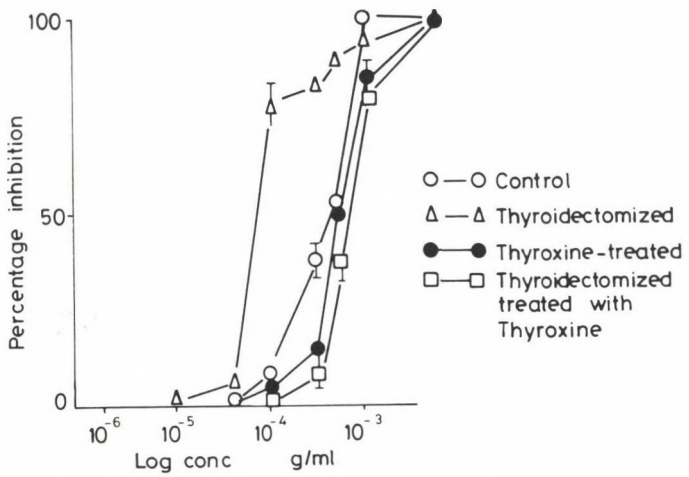

Fig. 1. The dose response curve for procaine HCl

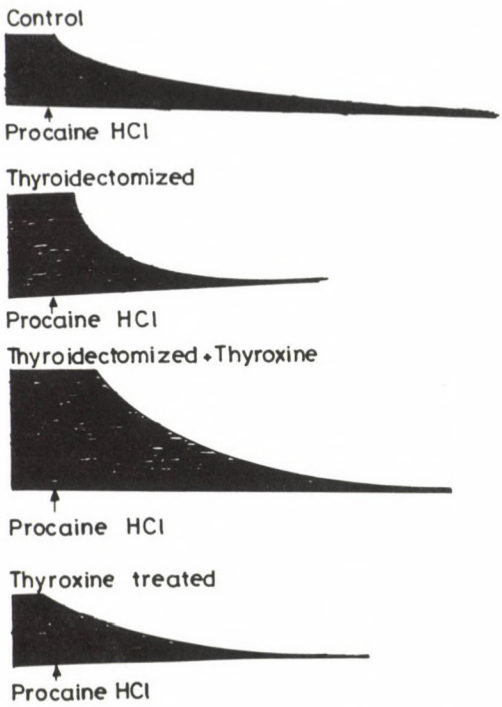

Fig. 2. The activity profiles of diaphragm muscle to procaine HCl ($13x10^{-14}$ g/ml)

Results

The dose response curve for procaine HCl (Fig. 1) shows that there was no significant difference in the contraction of the diaphragm from the control (i), thyroidectomized rats treated with thyroxine (iii) and thyroxine-treated rats (iv). The diaphragm from the thyroidectomized rats showed a significant decrease in IC50 of procaine HCl (Fig. 1).

The activity profiles of the diaphragm muscles of the rats from the various experimental groups to procaine HCl (Figs 2 and 3) showed that the time to maximum inhibition for control rats (33.18 ± 6.66 min), thyroidectomized rats, and thyroxine treated rats (24.11 ± 2 of min) were not significantly different ($P>0.05$). However thyroidectomy significantly decreased the time to maximum inhibition from 33.18 ± 6.66 min to 17.11 ± 3.23 min ($P<0.01$).

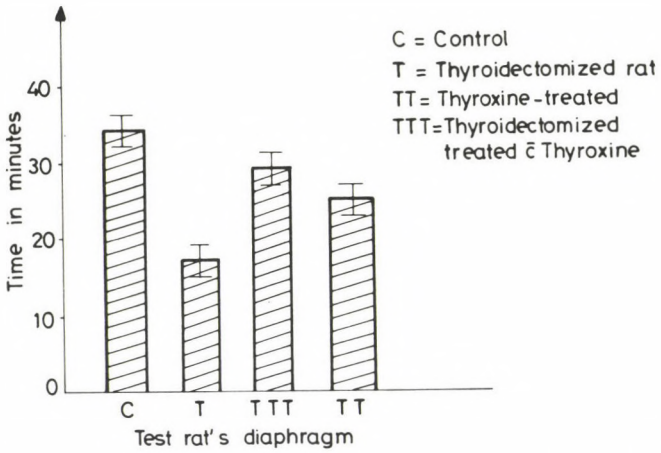

Fig. 3. The histogram showing the time course of diaphragm muscle reactivity to procain HCl

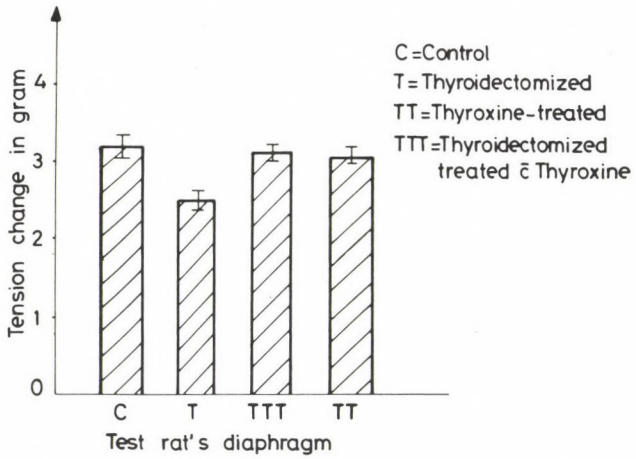

Fig. 4. The histogram showing rat-diaphragm reactivity to KCl (85 mmol)

Figure 4 shows that thyroidectomy significantly decreased ($P<0.05$) tension in diaphragm muscle in response to 85 mMol KCl when compared with the results obtained for control rats, thyroidectomized rats treated with thyroxine and thyroxine-treated rats.

Discussion

The results have clearly shown that thyroidectomy affects the contractility of the rats diaphragm muscle *in vitro*.

The significantly low IC50 observed for the thyroidectomized rats showed that the diaphragm muscle of this category of rats were more susceptible to inhibition by procaine HCl. This is an indication that the diaphragm of the thyroidectomized rats could be more easily fatigued. The results obtained in the activity profile (Figs 2 and 3) and those obtained in the tension change with KCl further confirmed this tendency. The action of thyroidectomy on the contractility of the diaphragm muscle observed presently could be due to the direct effect of thyroid hormones on biochemical processes relating to the promotion of protein synthesis in all the cells of the body [1, 3, 4]. It might also be due to the action of thyroidectomy on calcium metabolism in the body. What this work has clearly showed is that thyroidectomy weakens the diaphragm muscle and predisposes it to fatigue.

The observed effect of thyroidectomy on diaphragm muscle could explain the low oxygen consumption [3] and reduced metabolic rate [4] in clinically hypothyroid patients.

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THE EFFECT OF GRADED SINGLE ORAL DOSES OF PROSTACYCLIN ON THE NUCLEIC ACID CONTENT OF RAT GASTRIC MUCOSA

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Graded single oral doses of prostacyclin significantly enhance in dose-dependent manner the DNA and RNA content of rat gastric *fundic* mucosa, – resulting in a decreased RNA/DNA ratio. This latter phenomenon is convincing sign of new cell formation.

In the *antral* region no significant changes could be encountered but the RNA content showed a tendency-like elevation, evoking an elevated RNA/DNA ratio, which corresponds to *de novo* protein synthesis.

The diversity of the noted changes gives further evidence to the tenet the antral and the fundic gastric mucosa of the rat reacts differently toward external stimuli.

Keywords: prostacyclin, RNA, DNA, gastric mucosa, antrum, fundus, rat

According to the literature the cytoprotective effect of prostacyclin (PGI_2) could partially be explained by an influence on protein synthesis in the gastric mucosa [2].

In a pilot-study 60 min after orally administered 100 μg/kg PGI_2 there was an increase of the DNA content and a decrease of the RNA/DNA ratio in the gastric (fundic) mucosa of the rat [9].

To establish whether the above-mentioned effect of PGI_2 is a real effect of the molecule the following dose-response investigations were carried out.

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Material and methods

Five groups (n = 10/group) of adult female Wistar rats (220 – 230 g of body weight) were used. Prior to experiment the animals were fasted for 24 h but allowed water *ad libitum*.

The animals received the following oral treatment:

Group I. Control animals;

Group II. 10 μg/kg PGI_2;

Group III. 50 μg/kg PGI_2;

Group IV. 100 μg/kg PGI_2;

Group V. 200 μg/kg PGI_2.

All doses were given through a gastric tube.

A stock-solution of PGI_2-methylester (Chinoin, Budapest, Hungary) was made up (1mg/ml) freshly in 0.05 M Tris-buffer, pH 9.6. Dilutions were made immediately prior to use in ice-cold isotonic sodium-bicarbonate solution. The control animals received isotonic sodium-bicarbonate solution in appropriate amounts, also through a gastric tube.

After 120 min of PGI_2 treatment the animals were killed their stomachs were removed and opened.

It is a well-known fact that the basic mechanism of action of PGI_2 is a typical "hit and run" effect [3] therefore after 120 min we have investigated only the so-called "late" effects of PGI_2.

The fundic (oxyntic cell area) and the antral mucosa was separately scraped off and both divided into two parts; which were homogenized either in ice-cold normal saline for protein determination or in 0.8 M ice-cold perchloric acid, for DNA and RNA determination. Emphasis was put throughout on rapid processing and the whole procedure was carried out in an ice-bath.

The protein content of the mucosa has been determined according to Lowry et al. [7].

The RNA content of the homogenizate was determined by the orcinol-reaction [5], while the DNA content was measured using the diphenylamine method [6] and both expressed in μg/mg protein.

Within each group mean ± SEM was calculated and analyzed statistically using Student's *t*-test. Significant differences were assumed (vs the control group) when the probability was less than 5%.

Results

The experimental results are presented in Table I.

According to results obtained it seems that the different, graded, single oral doses of PGI_2 exerted a linear dose-response effect on the gastric (antral and fundic) mucosal DNA and RNA content of rat.

Table I

The effect of single oral graded doses of prostacyclin on the DNA and RNA content of rat gastric mucosa

Group	RNA		DNA		RNA/DNA	
	μg/mg protein		μg/mg protein			
	Antrum	Fundus	Antrum	Fundus	Antrum	Fundus
Control	42.61 ± 3.88	23.88 ± 2.11	24.02 ± 1.82	13.60 ± 2.27	1.77	1.76
10 μg/kg	44.36 ± 3.90	24.24 ± 2.64	24.97 ± 2.74	14.91 ± 2.29	1.78	1.63
50 μg/kg	47.39 ± 4.04	25.42 ± 2.77	23.61 ± 2.79	17.70 ± 2.44	2.01	1.38
100 μg/kg	48.80 ± 4.14	26.75 ± 2.61	23.01 ± 2.95	20.83 ± 2.71+	2.12	1.28
200 μg/kg	49.95 ± 4.32	29.68 ± 2.66+	23.18 ± 2.81	23.76 ± 2.99++	2.16	1.25

Mean ± SEM
n = 10 ^{O}/group
+ = $P<0.05$ vs control ++ = $P<0.01$ vs control

Discussion

Analyzing the experimental results the following conclusions might be drawn:

(i) After 120 min of different (increasing) single oral doses of PGI_2 there is a significant and dose-dependent elevation of nucleic acid levels in the *fundic* mucosa of rat.

(ii) No such relation could be detected in the *antral* mucosa.

(iii) In our opinion more significant conclusions might be drawn taking into consideration the changes of the RNA/DNA ratio [4, 8].

An elevation of this ratio refers to *de novo* protein synthesis, while its decreasing is convincing sign of new cell formation (in the gastric mucosa.) Therefore it seems that:

a. After PGI_2 treatment the *fundic* RNA/DNA ratio showed a dose-dependent *decrease* indicating new cell formation; while

b. the *antral* RNA/DNA ratio *increased*, signalling *de novo* protein synthesis.

This opposite change of the RNA/DNA ratios corresponds to our previous results namely: The two parts of the gastric mucosa of the rat have different properties in due course of gastric ulceration, i.e. the fundic mucosa is more vulnerable toward noxious agents, therefore ulceration takes place almost entirely in the oxyntic cell area. Antral ulceration in the rat is virtually unknown [4].

Therefore the reparative processes – which usually take place concomitantly with the ulcer formation [4] – in the fundic mucosa facilitate new cell formation,

while in the more durable antral region the reaction toward ulcerogenic and any other external noxae point to *de novo* protein synthesis. (Mucus secretion?)

It is not clear yet that the higher antral nucleic acid levels have any connection with ulcer-resistance or not? According to our previous data [1] it seems that a thicker fundic mucosa which contains more cells (hyperplasia) and more nucleic acids is more resistant toward ulceration. The problem needs further investigation.

(iv) Considering finally that PGI_2 is one of the most effective cytoprotective drugs which exerts very significant anti-ulcerogenic action [2, 10] it seems that the molecule itself has a *real, dose-dependent effect* on the gastric mucosa of rat; – stimulating new cell formation in the fundus and evoking *de novo* protein synthesis – most probably mucus secretion – in the antrum.

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INFLUENCE OF ENDOTOXIN ON EXPERIMENTAL POSTALCOHOLIC LIVER INJURY

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The morphological and biochemical changes of the liver after endotoxin intake were analyzed in rats receiving 20% ethanol during 60 days.

Besides morphological changes, concentration of serotonin and histamine in liver homogenates, the activity of asparagine and alanine aminotransferases (AspAT, ALAT), gammaglutamylotranspeptidase (GGTP) and alcohol dehydrogenase (ADH) in blood serum were determined, too.

The most extensive morphologic changes of the liver were seen in group of animals intoxicated with 20% ethanol during 60 days and single dose of endotoxin *E. coli* 0127:B8 intraperitoneally. These changes included necrosis most hepatocytes, focal steatosis of liver parenchyma, considerable hyperemia and parenchymatous degeneration of the liver cells. The cells lining liver sinuses showed considerable swelling as well as necrotic changes. Figures of cell division and haemorrhagic focuses were seen, too. The clusters of mononuclear cells, surrounding necrotically changed hepatocytes were seen in the central part of the liver lobule.

Among the inflammatory mediators estimated in liver homogenate only serotonin reached a high level in the group of experimental animals receiving only endotoxin. Increased activity of aminotransferases AspAt and ALAT were associated with these morphologic and biochemical changes in liver tissue observed in animals receiving ethanol and endotoxin.

Keywords: experimental postalcoholic liver injury, endotoxin

There is relatively little information concerning influence of endotoxin on the liver.

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The clinical observations showed that an increased concentration of endotoxin in the peripheral blood appears in patients with chronic – i.e. postalcoholic liver diseases [1, 5, 8].

This peripheral endotoxemia in these patients is often accompanied with activation of platelet functions, the coagulation-fibrinolysis system and the complement system and other biological effects which give worse prognosis concerning their lives [1, 7, 11].

The aim of this paper is the evaluation of the influence of endotoxin on morphological and biochemical changes of experimental postalcoholic liver disease in rats.

Material and methods

Examinations were carried out on 80 male Wistar rats with average body weight 220 – 280 g. They were allowed free access to a standard pellet diet and water and 20% ethanol. Rats received 20% ethanol instead of drinking water *ad libitum* (11 – 20.5 ml/24h/rat) during 60 days.

Endotoxin as a solution of 3 mg/kg body wt in 1.5 ml of physiological saline solution – 30% of the LD_{50} value was injected intraperitoneally. Endotoxin (lipopolysaccharide from *E. coli* serotype 0127:B8, Sigma, USA) was freshly reconstituted in sterile 0.9% NaCl, at the time of injection. Time exposure was 96 h.

The rats were divided into the following groups:

I group – healthy rats,

II group – 20% ethanol,

III group – endotoxin,

IV group – 20% ethanol + endotoxin.

Blood samples from the abdominal aorta were taken for liver function tests 96 h after the administration of endotoxin under ether anesthesia. The animals were killed, and their livers were extirpated to determine morphologic changes and concentration of serotonin and histamine in liver homogenate.

Liver specimens were fixed in 10% neutral formalin and embedded in paraffin. Sections were stained with haematoxylin and eosin for histopathological examination.

In liver homogenate, the concentration of serotonin and histamine was determined according to Curzon and May, respectively [2, 6].

The activity of asparagine aminotransferase (AspAT) and alanine (ALAT) according to Reitman-Frenkel, the activity of gammaglutamylotranspeptidase (GGTP) according to Szewczuk-Orlowski method based on Technicon RA-1000 analyzer with using standard kits (LACHEMA – CSRS) were determined in blood serum [9, 10]. Moreover, the activity of alcohol dehydrogenase (ADH) in pH 7.4; 9.2; 9.6; 10.4 was determined in blood serum using Technicon RA-1000 analyzer [10].

Liver function tests, concentration of serotonin and histamine were expressed as means ± SD and analyzed statistically by the Student's group *t* test [4].

Results

Histological changes in the liver specimens are shown in Figs 1, 2 and 3.

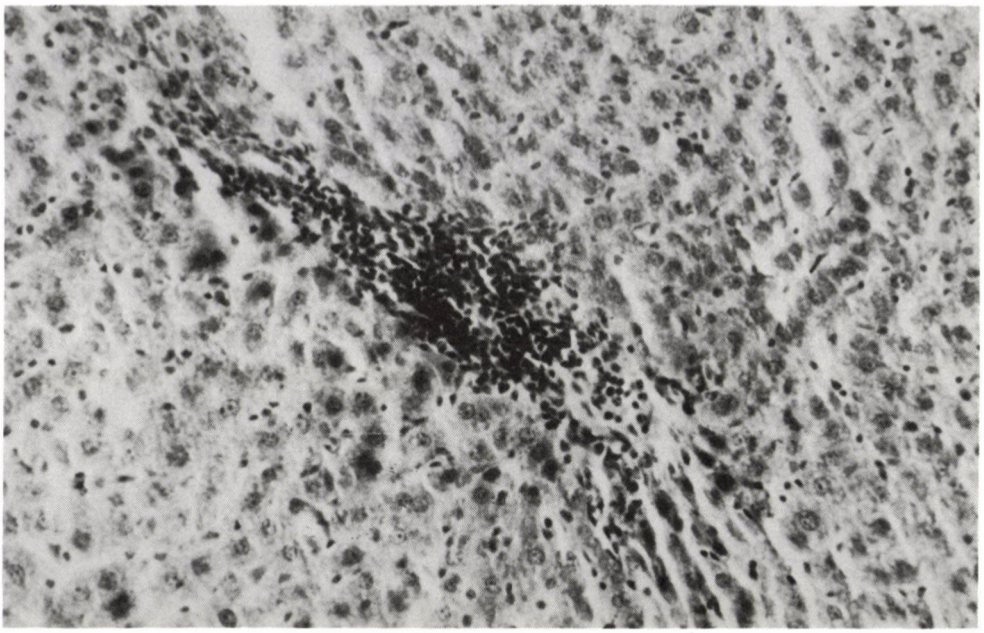

Fig. 1. Morphological changes in liver of rats which after a 60-day intoxication with 20% ethanol, received a single dose of endotoxin *E. coli* 0127:B8 (group IV) intraperitoneally. H + E × 150

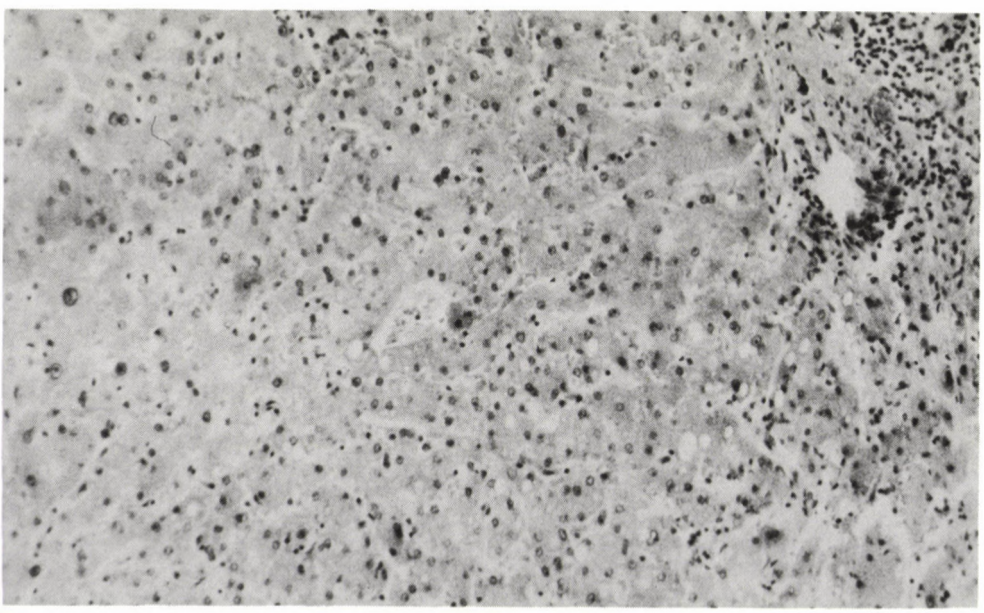

Fig. 2. Histopathological changes observed in rats intoxicated with 20% ethanol during 60 days (group II). H + E × 150

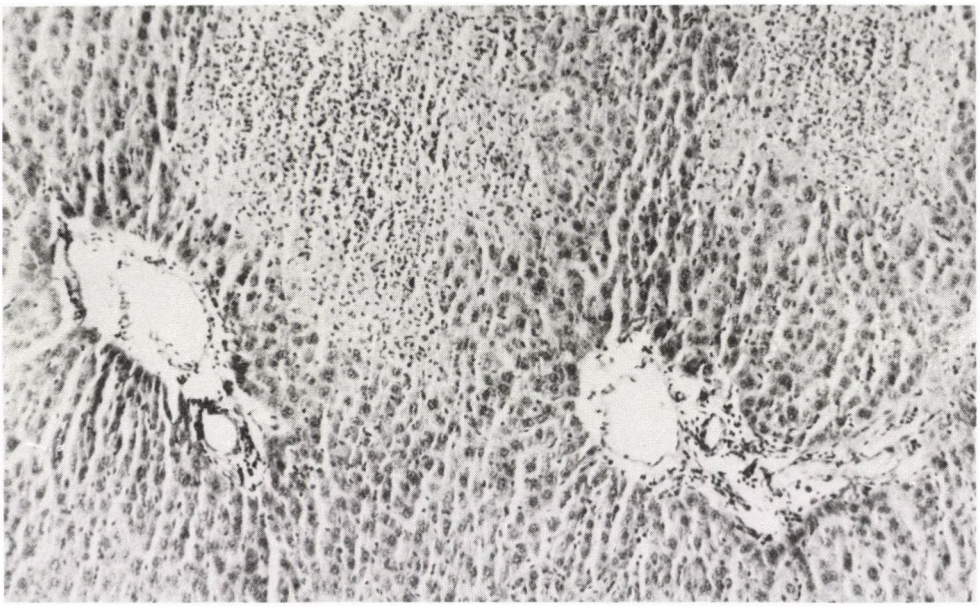

Fig. 3. Morphological changes observed in rats receiving a single dose of endotoxin *E. coli* 0127:B8 (group III) intraperitoneally. H + E × 150

The most extensive morphological changes in the specimens were seen in group IV of the experimental animals i.e. receiving a single dose of endotoxin *E. coli* 0127:B8 after 60 days of the intoxication with 20% ethanol.

These changes included necrosis of hepatocytes, focal steatosis of the liver parenchyma, significant congestion and degeneration of parenchyma liver cells.

The Browicz-Kupffer cells showed significant swelling as well as necrotic changes. Figures of cell division and haemorrhagic focuses were seen in the specimens. Clusters of mononuclear cells surrounding necrotically changed hepatocytes were observed in the central part of lobule (Fig. 1).

Necrosis of single liver cells, focal steatosis of hepatocytes in the central part of lobule, parenchymal degeneration of hepatocytes were observed in the liver specimens of group II of rats receiving 20% ethanol *ad libitum* during 60 days. Congestion of parenchyma, focal infiltrations composed of the mononuclear cells around the central vein of the liver lobule were also observed (Fig. 2).

The microscopic picture of the liver specimens showed the swelling of Browicz-Kupffer cells and the necrosis of single hepatocytes in group III of rats receiving intraperitoneally a single dose of endotoxin *E. coli* 0127:B8 – 30% LD 50.

The figures of cell division were seen in some liver cells. The presence of a large number of neutrophilic granulocytes was observed in the sinusoidal vessels, the liver parenchyma showed a significant congestion (Fig. 3).

Figure 4 and Table I showed the changes of the level of serotonin and histamine in the liver homogenate of the experimental animals.

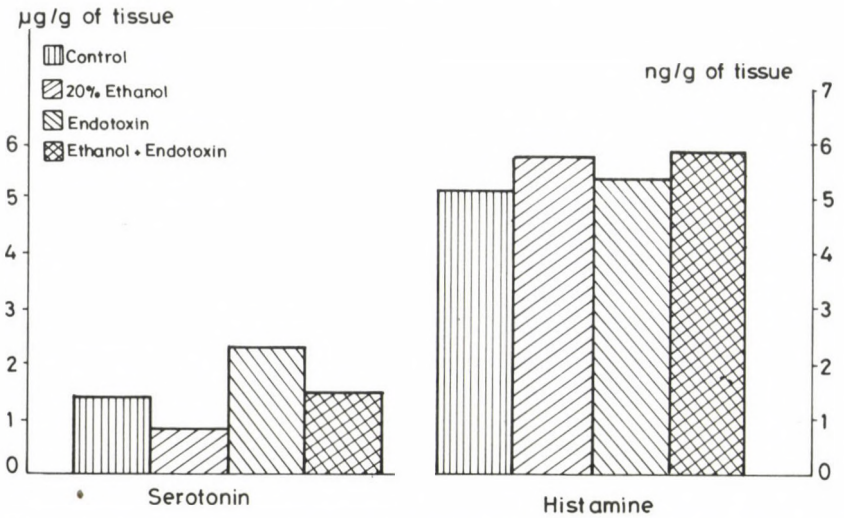

Fig. 4. The level of serotonin and histamine in liver homogenates in experimental groups of rats

Table I

Serotonin and histamine concentration in liver homogenate of experimental animals

Group	n	Serotonin µg/g tissue	p	Histamine µg/g tissue	p
I	20	1.1 ± 0.6		5.9 ± 1.1	NS
II	20	0.5 ± 0.2	I – II <0.05	6.5 ± 1.2	NS
III	20	2.0 ± 0.7	II – III <0.001	6.1 ± 1.4	NS
IV	20	1.2 ± 0.5	II – IV <0.01	6.6 ± 1.7	NS

I group – healthy rats
II group – 20% ethanol
III group – endotoxin
IV group – 20% ethanol + endotoxin

The highest, but in comparison with control group statistically non-significant concentration of serotonin in the liver homogenate was observed in group III ($x = 2.0$ μg/g) of rats receiving a single dose of endotoxin.

It is interesting to observe that intoxication with 20% ethanol during 60 days (group II) has provoked statistically significant decrease of concentration of serotonin in liver homogenate in comparison with control group ($p<0.05$).

The mean concentration of histamine in the liver homogenate of the experimental animals was raised no significantly in comparison with the control group.

Figure 5 and Table II illustrated activity of the analyzed enzymes-AspAT, ALAT, GGTP in blood serum of analyzed rats.

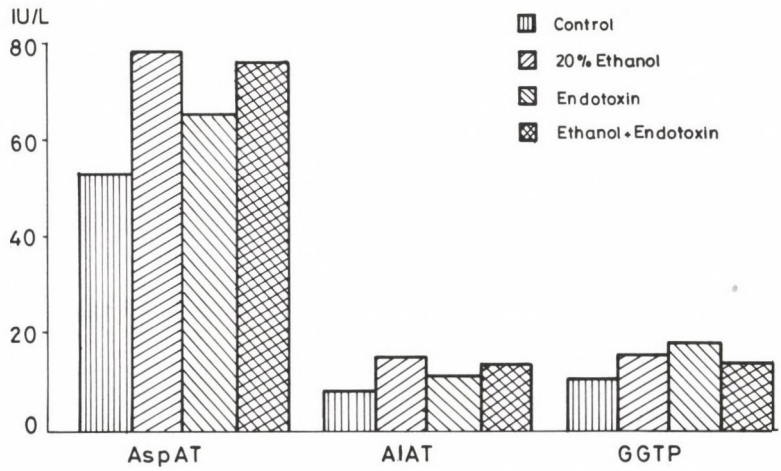

Fig. 5. The activity of analyzed enzymes in blood serum in the experimental groups of rats

The mean activities of AspAT and ALAT showed the highest, statistically significant values in comparison with control group, in the blood serum of the rats intoxicated with 20% ethanol during 60 days (group II $p<0.01$; $p<0.001$) and group IV rats receiving a single dose of endotoxin after 60 days of the intoxication with 20% ethanol ($p<0.001$).

The highest statistically significant activity of GGTP was observed in the blood serum of the rats of group III receiving a single dose of endotoxin ($p<0.01$) and rats intoxicated with 20% ethanol (group II) ($p<0.01$).

Activity of ADH in blood serum reached the highest statistically significant values in comparison with control group in group II of rats receiving only 20% ethanol *ad libitum* during 60 days in pH 9.2; ($p<0.001$); pH 9.6 ($p<0.05$); pH 10.4 ($p<0.001$).

Table II

Activity of aminotransferase, AspAT, ALAT, GGTP in blood serum of rats

Group	n	AspAT IU/l	p	ALAT IU/l	p	GGTP IU/l	p
I	20	50.2±8.2		5.1±2.2		7.5±2.7	
II	20	75.5±2.1	I–II <0.01	11.9±2.5	I–II <0.001	12.5±2.6	I–II <0.01
III	20	62.2±13.2	NS	8.2±0.3	I–III <0.05 II–III <0.05	14.9±3.9	I–III <0.01
IV	20	73.2±8.7	I–IV <0.001	10.6±1.9	NS	10.8±7.3	NS

Group:
I – healthy rats
II – 20% ethanol
III – endotoxin
IV – 20% ethanol + endotoxin

AspAT – asparagine aminotransferase
ALAT – alanine aminotransferase
GGTP – gammaglutamylotranspeptidase

The highest, but similar to values obtained in control group, activity of ADH was observed in pH 7.4 in the blood serum of rats of group IV receiving ethanol and single dose of endotoxin.

The activity of ADH in blood serum of rats is presented in Fig. 6 and Table III.

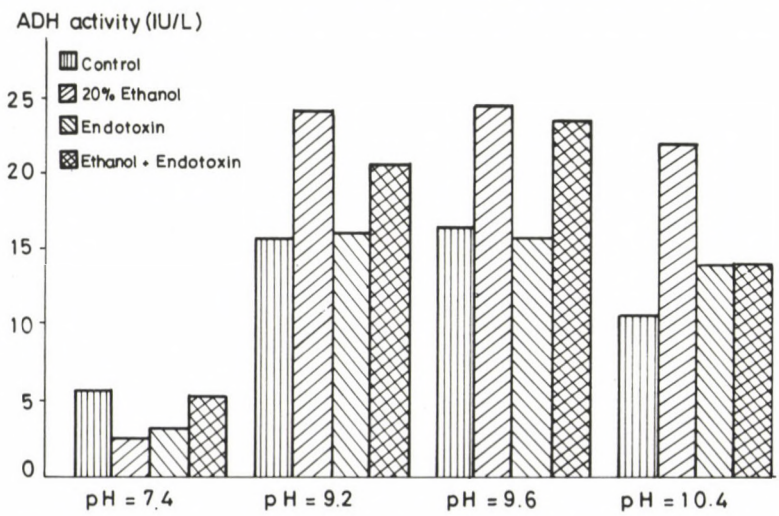

Fig. 6. The activity of analyzed ADH in blood serum in the experimental groups of rats

Table III

Activity of ADH in blood serum of different pH

Group	n	pH = 7.4	p	pH = 9.2	p	pH = 9.6	p	pH = 10.4	p
I	20	4.6 ± 1.2		14.5 ± 2.2		15.3 ± 2.1		9.5 ± 1.3	
II	20	1.4 ± 0.3	I – II < 0.001	22.8 ± 3.1	I – II < 0.001	23.1 ± 7.8	I – II < 0.05	20.6 ± 4.6	I – II < 0.001
III	20	2.1 ± 0.9	I – III < 0.001	14.8 ± 6.5	II – III < 0.01	14.5 ± 5.6	II – III < 0.05	12.7 ± 4.0	< 0.01
IV	20	4.3 ± 1.4	II – IV < 0.001	19.4 ± 7.4	NS	22.2 ± 8.9	NS	12.9 ± 8.4	NS

I – healthy rats
II – 20% ethanol
III – endotoxin
IV – 20% ethanol + endotoxin
ADH – alcohol dehydrogenase

Discussion

Liver is the main organ in which the process of biochemical and immunological degradation, taking place [3, 8].

A special role in these processes is played by Browicz-Kupffer cells and hepatocytes [8].

Mainly in chronic liver disease, disturbances of function of Browicz-Kupffer cells and hepatocytes can be accompanied by peripheral endotoxemia, the additional, serious pathogenic factor which complicates clinical courses of the basic disease [1].

The data concerning the pathomechanism of postalcoholic liver injury and endotoxin are in modern scientific literature scantly demonstrated.

The data concerning the influence of two toxic factors, i.e. ethanol and endotoxin on the development of liver injury are demonstrated relatively poor in modern scientific literature.

In our experiment, we tried to evaluate the influence of intraperitoneally given endotoxin *E. coli* 0127:B8 on morphologic and biochemical progression of experimental postalcoholic liver changes in rats.

The supply of 20% ethanol to experimental male Wistar rats during 60 days produces development of morphological liver changes according to criteria of alcoholic liver inflammation.

A single dose of endotoxin in the liver of observed animals provokes intensification of neurotic changes of the liver and inflammatory changes of hepatocytes. Browicz-Kupffer cells, these changes are accompanied by haemorrhagic focuses. It is interesting to notice that the presence of mononuclear cells concentrated mainly in the central part of lobule and surrounding necrotically changed hepatocytes were found in liver specimens of these animals receiving 20% ethanol and a single dose of endotoxin.

Inflammatory infiltration with neutrophiles was located mainly in sinusoides of the liver in the group of experimental animals, in which application of endotoxin was not proceded by the supply of ethanol (group III).

Among mediators of inflammation investigated in liver homogenate, only serotonin reached relatively high level in comparison with the control group, in group III of experimental animals receiving endotoxin.

These morphological and biochemical changes in the liver of animals receiving ethanol and endotoxin were accompanied by the increased activity of asparagine and alanine aminotransferases in the blood serum.

In accordance with the obtained results we hypothesize that endotoxin given to rats interaperitoneally, in doses which are the 30% of the LD_{50} value intensifies postalcoholic liver changes which can be stated liver morphological examination.

Further experimental observations are needed to answer what other immunological and biochemical mechanisms determine the progression and/or decease of postalcoholic liver injury with endotoxemia.

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EFFECTS OF AET AND MEA ON THE LYSOSOMAL HYDROLASE ACTIVITIES IN THE MOUSE ORGANS

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The adult male Swiss mice were injected intraperitoneally with AET (2-aminoethylisothiouronium Br.HBr) or MEA (cysteamine HCl), in a toxic dose of 400 mg/kg body weight. The acid phosphatase (E.C. 3.1.3.2) and arylsulphatase (E. C. 3.1.6.1) activities in crude homogenates of liver and kidneys were assessed every fourth hour throughout a 24-h period.

Different patterns of temporal changes in the acid phosphatase and arylsulphatase activities in liver and kidneys expressed in nkat per 1 mg of protein, 1 g of fresh tissue and per the whole organ weight, were found. The extent and timing of the alterations in the activity of each of the lysosomal hydrolases were dependent on the particular organ chosen and aminothiol compound given.

Keywords: aminothiols, lysosomal hydrolases, mouse organs

It has been demonstrated that the S-containing compounds such as AET (2-aminoethylisothiouronium Br. HBr) and MEA (cysteamine HCl) could modify the effects of ionizing radiation on the acid phosphatase (orthophosphoric monoester phosphohydrolase, E.C. 3.1.3.2) and arylsulphatase (arylsulphate sulphohydrolase, E.C. 3.1.6.1) activities in the animal organs [9, 11]. However, scant information is available about the influence of the aminothiol agents given alone, without subsequent irradiation, on the lysosomal hydrolase activities [6, 10].

The purpose of the present study was to determine and compare temporal changes of the acid phosphatase and arylsulphatase activities in liver and kidneys of mice treated with AET or MEA, in a toxic dose [3, 4].

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Methods

The experiments were carried out with adult male Swiss mice. All were kept under constant conditions with LD 12/12 regime (light: 08.00–20.00, dark: 20.00–08.00). They were fed standard granulated chow and given drinking water *ad libitum*.

The animals were divided into three groups. Group I, consisting of untreated mice served as a control. Group II mice were treated with AET, and those of Group III with MEA. They were injected intra-peritoneally with AET (2-aminoethylisothiouronium bromide hydrobromide, Sigma, USA), or MEA (cysteamine hydrochloride, International Enzymes Limited, England), in a dose of 400 mg/kg of body weight in 200 µl of aqua pro injection (Polfa, Poland), always at 18.45 [9–11].

The mice were killed by cervical dislocation every fourth hour throughout a 24-hour period, starting at 20.00. Then the weight of liver and kidneys, the activities of acid phosphatase and arylsulphatase as well as the concentration of protein in crude homogenates of the organs, were assessed.

The activity of acid phosphatase was measured according to Igarashi et al. [5] using p-nitrophenyl phosphate as a substrate, and the activity of arylsulphatase was determined according to Worwood et al. [12] using p-nitrocatechol sulphate as a substrate. The concentration of protein was assessed according to the method of Lowry et al. [7] with bovine serum albumin as a standard. The absorbance for the liberated p-nitrophenyl for acid phosphatase establishment was read at 400 nm, the liberated p-nitrocatechol for arylsulphatase determination at 515 nm, and the protein concentration was measured at 500 nm, using a Specord UV Vis Spectrophotometer.

The enzyme activity was expressed as product liberated in nanokatals (nkat, SI Units) related to 1 mg of protein (specific activity), 1 g of fresh tissue and to the whole organ weight. The results were evaluated by an analysis of variance and the Duncan's new multiple range test, using a Commodore C64 computer.

Results

The groups of mice showed differences (at $P<0.05$) in the acid phosphatase and arylsulphatase activities in liver (Table I) and kidneys (Table II).

Acid phosphatase activity in liver (Table I)

The specific activity of acid phosphatase in liver expressed in nkat per 1 mg of protein was, as compared with that of Group I, statistically significantly lower at 20.00[I] and 24.00 in Groups III and II.

The acid phosphatase activity in liver expressed in nkat per 1 g of fresh tissue was, in relation to that of Group II, statistically significantly lower at 04.00 in Groups III and I.

The activity of acid phosphatase expressed in nkat per the whole weight of liver was, in comparison with that of Group III, statistically significantly higher at 24.00 in Groups I and II.

Differences in the temporal changes of acid phosphatase activity in liver related to 1 mg of protein in Group I and 1 g of fresh tissue in Groups I and III, were encountered.

Arylsulphatase activity in liver (Table I)

The specific activity of arylsulphatase in liver was, in relation to that of Group I, statistically significantly lower at 20.00^{I} in Groups III and II.

The arylsulphatase activity in liver expressed in nkat per 1 g of fresh tissue was, in comparison with that of Group II, statistically significantly lower at 20.00^{I} in Groups III and I.

The activity of arylsulphatase expressed in nkat per the whole weight of liver was, as compared with that of Group III, statistically significantly higher at 16.00 in Groups I and II.

There were differences in the temporal changes of arylsulphatase activity in liver related to 1 mg of protein in Groups I and III and 1 g of fresh tissue in Groups I, II, III, as well as that calculated per the whole organ weight in Group III.

Acid phosphatase activity in kidneys (Table II)

Statistically significant differences in the activity of acid phosphatase in kidneys expressed in nkat per 1 mg of protein, 1 g of fresh tissue and per the whole organ weight between the particular groups of mice were not found.

There were differences in the temporal changes of acid phosphatase activity in kidneys calculated per 1 mg of protein and 1 g of fresh tissue in Group III.

Arylsulphatase activity in kidneys (Table II)

The specific activity of arylsulphatase in kidneys was, in comparison with that of Group III, statistically significantly lower at 08.00 in Groups II and I.

There were no statistically significant differences in the activity of arylsulphatase in kidneys expressed in nkat per both 1 g of fresh tissue and the whole organ weight between the particular groups of mice.

Differences in the temporal changes of arylsulphatase activity in kidneys related to 1 mg of protein in Groups I and II, as well as that encountered per 1 g of fresh tissue and the whole organ weight in Group II, were found.

Table I

Mean (± SD) activities of acid phosphatase and arylsulphatase in crude homogenate of liver in the particular groups of mice

	Group	Time of killing 2000[I]	2400	0400	0800	1200	1600	2000[II]
Number of mice	I	9	11	11	10	11	10	10
	II	10	11	10	10	10	10	8
	III	10	11	11	11	10	10	11
nkat per 1 mg protein x 10^{-2}	I	109.24 ± 19.57	103.87 ± 12.30	93.72 ± 13.35	96.47 ± 19.17	90.37 ± 13.49	90.80 ± 11.06	88.71 ± 13.27
	II	91.98 ± 16.19	89.37 ± 7.33	94.91 ± 13.21	94.67 ± 24.28	93.79 ± 25.00	94.03 ± 9.59	91.77 ± 8.81
	III	89.60 ± 9.34	85.55 ± 10.10	84.74 ± 10.90	91.98 ± 22.27	98.65 ± 20.17	89.72 ± 16.20	97.69 ± 12.23
	I:II:III	III II I	III II I	N.S.	N.S.	N.S.	N.S.	N.S.
	I	2000^{II} 12000	16000 0400	0800	2400	2000^{I}		
nkat per 1 g fresh tissue	I	75.27 ± 11.46	62.87 ± 5.73	59.98 ± 6.33	66.31 ± 12.17	62.56 ± 9.88	64.08 ± 5.61	64.86 ± 10.61
	II	71.56 ± 8.97	63.54 ± 7.17	64.63 ± 6.76	64.56 ± 16.91	63.15 ± 13.92	64.56 ± 4.85	65.62 ± 5.31
	III	69.64 ± 7.76	62.71 ± 6.04	54.61 ± 5.33	58.23 ± 11.80	63.82 ± 10.46	63.02 ± 10.26	66.04 ± 10.49
	I:II:III	N.S.	N.S.	III I II	N.S.	N.S.	N.S.	N.S.
	I	0400 1200	2400 1600	2000^{II} 0800	2000^{I}			
	III	0400 0800	2400 1600	1200 2000^{II}	2000^{I}			
nkat per the organ weight	I	117.94 ± 16.80	94.20 ± 8.93	101.60 ± 15.39	113.88 ± 27.11	98.89 ± 18.42	97.18 ± 8.11	97.68 ± 17.86
	II	98.56 ± 21.30	93.79 ± 12.55	98.19 ± 19.21	105.54 ± 30.30	98.12 ± 17.34	100.14 ± 11.84	99.15 ± 10.94
	III	102.25 ± 19.70	80.72 ± 7.73	84.39 ± 16.03	92.59 ± 24.70	101.03 ± 23.47	93.40 ± 21.54	98.15 ± 15.38
	I:II:III	N.S.	III II I	N.S.	N.S.	N.S.	N.S.	N.S.

Acid phosphatase activity in liver

Table I. continued

Arylsulphatase activity in liver

nkat per 1 mg protein x 10^{-2}	I	15.76 ± 1.26	16.40 ± 2.13	14.60 ± 2.51	13.24 ± 2.06	13.73 ± 2.09	13.39 ± 1.92	15.00 ± 2.06
	II	14.48 ± 2.09	14.77 ± 1.83	13.23 ± 2.38	13.24 ± 2.27	13.86 ± 2.83	13.89 ± 2.59	12.21 ± 2.48
	III	13.01 ± 1.68	14.50 ± 1.37	15.24 ± 2.07	14.30 ± 2.72	14.59 ± 2.16	11.95 ± 1.86	14.80 ± 1.68
	I:II:III	III II I	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
	I	0800 1600 1200 0400 2000^{II} 2000^{I} 2400						
	III	1600 2000^{I} 0800 2400 1200 2000^{II} 0400						
nkat per 1 g fresh tissue	I	10.93 ± 1.00	9.98 ± 1.48	9.37 ± 1.40	9.14 ± 1.49	9.14 ± 0.71	9.50 ± 1.39	10.86 ± 1.03
	II	11.29 ± 0.84	10.49 ± 1.20	9.06 ± 1.69	8.72 ± 1.04	9.31 ± 1.49	9.43 ± 1.08	9.56 ± 1.93
	III	10.10 ± 1.11	10.68 ± 0.71	9.84 ± 1.08	9.11 ± 1.53	10.05 ± 1.41	8.53 ± 0.85	9.92 ± 0.79
	I:II:III	III I II	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
	I	1200 0800 0400 1600 2400 2000^{II} 2000^{I}						
	II	0800 0400 1200 1600 2000^{II} 2400 2000^{I}						
	III	1600 0800 0400 2000^{II} 1200 2000^{I} 2400						
nkat per the organ weight	I	16.38 ± 2.03	14.92 ± 2.31	15.81 ± 2.60	15.42 ± 1.78	14.69 ± 2.75	14.71 ± 2.11	15.89 ± 2.45
	II	16.20 ± 2.32	15.42 ± 1.80	13.60 ± 2.70	14.19 ± 2.34	14.80 ± 3.03	14.60 ± 1.79	14.30 ± 2.29
	III	14.67 ± 1.79	13.69 ± 1.79	15.10 ± 2.32	14.34 ± 2.67	15.86 ± 3.31	12.23 ± 1.78	14.83 ± 1.78
	I:II:III:	N.S.	N.S.	N.S.	N.S.	N.S.	III II I	N.S.
	III	1600 2400 0800 2000^{I} 2000^{II} 0400 1200						

Groups of mice: I-control, II-treated with AET, III-treated with MEA
N.S. – non-significant, significant differences at $P < 0.05$

Table II

Mean (± SD) activities of acid phosphatase and arylsulphatase in crude homogenate of kidneys in the particular groups of mice. For explanation of sign see Table I

Acid phosphatase activity in kidneys

	Group	Time of killing 2000[I]	2400	0400	0800	1200	1600	2000[II]
Number of mice	I	11	10	10	10	11	11	12
	II	11	11	11	11	11	11	10
	III	11	11	10	10	11	10	12
nkat per 1 mg protein x 10^{-2}	I	56.16 ± 15.72	74.29 ± 16.85	61.90 ± 15.34	61.36 ± 13.62	57.96 ± 22.14	50.60 ± 22.14	51.49 ± 12.13
	II	63.90 ± 17.02	57.94 ± 14.87	66.99 ± 16.38	58.47 ± 15.34	54.39 ± 17.49	54.49 ± 15.28	54.42 ± 19.45
	III	63.66 ± 18.98	67.92 ± 10.26	63.90 ± 17.29	73.04 ± 19.38	64.80 ± 17.41	49.56 ± 16.41	46.60 ± 13.43
	I:II:III	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
	III	2000[II] 1600	2000[I] 0400	1200	2400	0800		
nkat per 1 g fresh tissue	I	25.79 ± 6.84	33.01 ± 5.55	29.21 ± 6.96	33.24 ± 8.57	27.88 ± 10.68	25.90 ± 9.65	25.51 ± 6.36
	II	31.07 ± 6.75	30.58 ± 5.64	32.19 ± 5.73	29.43 ± 7.51	25.69 ± 8.23	28.40 ± 7.76	28.28 ± 9.67
	III	28.59 ± 7.78	30.03 ± 11.37	30.84 ± 6.48	32.33 ± 7.49	30.09 ± 9.31	25.54 ± 9.05	22.35 ± 5.17
	I:II:III	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
	III	2000[II] 1600	2000[I] 1200	0400	0800	2400		
nkat per the organ weight	I	11.18 ± 2.97	14.14 ± 1.88	12.78 ± 3.45	16.01 ± 4.43	11.90 ± 4.71	11.58 ± 5.05	11.20 ± 3.10
	II	14.60 ± 3.75	13.34 ± 3.00	13.55 ± 2.76	13.27 ± 3.11	10.93 ± 2.64	11.83 ± 3.50	12.65 ± 5.02
	III	12.71 ± 3.48	14.06 ± 2.88	13.17 ± 3.33	13.76 ± 3.43	12.37 ± 4.16	10.67 ± 3.79	9.90 ± 2.34
	I:II:III	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.

Table II continued

Arylsulphatase activity in kidneys

nkat per 1 mg protein x 10^{-2}	I	78.96 ± 15.89	77.00 ± 12.62	70.54 ± 13.04	59.75 ± 7.72	67.85 ± 10.30	62.23 ± 14.62	66.37 ± 8.50
	II	75.77 ± 17.72	69.23 ± 17.08	69.15 ± 11.31	55.46 ± 9.38	69.15 ± 17.16	62.24 ± 8.05	58.30 ± 11.70
	III	79.77 ± 23.36	72.07 ± 14.86	68.85 ± 19.20	70.03 ± 11.43	63.32 ± 15.20	60.38 ± 6.22	60.07 ± 11.20
	I:II:III	N.S.	N.S.	N.S.	II I III	N.S.	N.S.	N.S.
	I	0800 1600 2000^{II} 1200 0400 2400 2000^{I}						
	II	0800 2000^{II} 1600 0400 1200 2400 2000^{I}						
nkat per 1 g fresh tissue	I	36.03 ± 5.10	33.31 ± 2.82	32.51 ± 5.05	31.25 ± 5.52	32.80 ± 5.24	31.92 ± 5.46	32.69 ± 3.12
	II	36.87 ± 6.30	36.79 ± 6.21	32.78 ± 3.31	27.76 ± 2.79	32.29 ± 6.33	32.61 ± 4.53	30.42 ± 5.40
	III	35.53 ± 7.75	33.84 ± 5.12	33.70 ± 7.78	30.95 ± 2.39	30.10 ± 4.36	31.16 ± 4.28	28.93 ± 2.97
	I:II:III	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	
	II	0800 2000^{II} 1200 1600 0400 2400 2000^{I}						
nkat per the organ weight	I	15.78 ± 3.15	14.77 ± 1.33	14.23 ± 3.16	14.97 ± 2.85	14.10 ± 2.64	14.17 ± 3.48	14.36 ± 2.16
	II	17.14 ± 3.32	16.04 ± 3.33	13.93 ± 1.97	12.64 ± 2.25	14.35 ± 3.74	13.57 ± 2.09	13.64 ± 3.44
	III	16.49 ± 4.87	14.73 ± 2.50	14.33 ± 4.36	13.34 ± 1.78	12.67 ± 2.72	13.06 ± 2.15	12.84 ± 1.98
	I:II:III:	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
	II	0800 1600 2000^{II} 0400 1200 2400 2000^{I}						

Discussion

Treatment of adult male mice with AET and MEA, in a toxic dose of 400 mg/kg body weight influenced the acid phosphatase and arylsulphatase activities in liver and kidneys. It could be recorded that the extent and timing of the changes in the lysosomal hydrolase activities in the organs expressed in nkat per 1 mg of protein 1 g of fresh tissue and the whole organ weight, analyzed throughout the 24-h period, starting one hour and a quarter after AET and MEA injection was dependent on the particular organ chosen and the aminothiol agent given.

Administration of the thiols into the organism of mice caused greater changes in the lysosomal hydrolase activities in the liver than in kidneys, moreover the two sulphhydryl substances AET and MEA affected the activity of each enzyme analyzed, in a different way. Considering the results of the present report and also Mazur et al. [10] it was concluded that alterations in the acid phosphatase and arylsulphatase activities in liver, kidneys and testes obtained after AET and MEA treatment of mice could be connected with the various nature of influence of the S-containing compounds on biochemical processes in the organs. However, mechanisms of action of the aminothiols on the cell metabolism and especially on the lysosomal enzyme activities have not yet been well known and clarified [1, 2, 6, 8].

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NEUROCHEMICAL MECHANISMS OF MEMORY CONTROL

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Modulation of memory trace retrieval in emotiogenic brain structures, cortex and brainstem reticular formation by postsynaptic noradrenergic and dopaminergic drugs was found. At the initial stage of latent inhibition – a most significant mechanism of information selection – memory trace retrieval is retarded in all structures and in the cortex and the zona incerta later on. A haloperidol model of latent inhibition was obtained. Most important role of dopaminergic system in latent inhibition was shown.

Inhibition of the GABA-benzodiazepine-ionophore complex by the blockade of GABA-receptors induced by bicuculline, the chloride channels by picrotoxin, the benzodiazepine receptors by flumazenil (R015–1788) and R015–3505 facilitates the memory trace retrieval damaged by amnesic agent. The dopaminergic activation enhances the dominant state and developes conditions for switching on the interferential GABA-ergic inhibition.

Keywords: latent inhibition, amnesia, memory, dopamine, noradrenaline, benzodiazepine-GABA-ionophore complex

So far investigators have been aiming at elucidating the participation of certain neurochemical systems in memory mechanisms. It is shown that all classical neurotransmitter and peptidergic systems modify the memory [3, 19, 21]. The cholinergic mechanism determines the process of memory trace fixation. Monoaminergic (dopamine, noradrenaline, serotonin), GABA- and peptidergic systems participate in the modulation of memory trace reproduction. The participation of dopaminergic system in memory mechanisms is well understood, but the role of synaptic mechanisms and pre- and postsynaptic receptors in the memory modulation is not clear. In the behavioral and electrophysiological studies reported here, we have characterized the role of catecholaminergic systems in latent inhibition. We also examined the antiamnesic effects of the blockade of GABA-benzodiazepine-ionophore complex.

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Methods

Behavioral experiments. Adult male Wistar rats (160–200 g) and adult male BALB/c mice (18–20 g) were used. All animals were trained to obtain the conditioned passive avoidance response [13] in a chamber consisting of the compartments: an illuminated (safe) and a dark one (punishment) where the animal received footshock (0.75–1 mA, 2 s). The animal was immediately placed in its home cage after one. Step-through latency was registered. Retention of passive avoidance was tested 24 h later and different intervals during 2 weeks.

"Psychogenic" amnesia was evoked by 5 min detention of the animals in the dark compartment after the footshock [24]. ECS amnesia after 0.5 s of 50 mA was used as well.

The effect of environmental preexposure was used for observing the latent inhibition (LI) [17].

Electrophysiological experiments. Acute experiments were carried out in anesthetized, diplazine immobilized cats weighing 2–3 kg. Conditioned evoked potentials (CEP) were used as electrographic correlates of the memory trace retrieval in different brain structures [1]. CEP arises during the temporal interval of the supporting stimulus when the latter is omitted. The conditioned neurographic response (CNR) of the radial nerve and the conditioned skin galvanic reaction (SGR) were registered as well [10]. The evoked potentials of the auditory cortex, the zona incerta, the preoptic area, the amygdaloid complex, the dorsal hippocampus, the mesencephalic reticular formation, the central gray were registered monopolarly by nychromic electrodes. The evoked potentials were processed by ART–1000 analyzers ("Saip", France).

Drugs. Apomorphine (Serva, Germany) was solved in saline, 3 mg/kg i.v.; haloperidol (Gedeon Richter, Hungary) was solved in saline, 0.75 mg/kg i.v. (for cats) or 0.5 mg/kg i.p. (for rats); amantadine (USSR), was solved in saline, 50 mg/kg i.p.; bupropion (Burroughs Wellcome Co., USA) was solved in saline, 5 mg/kg i.v. (for cats), or 30 mg/kg i.p. (for rats); (+) and (-) 3–PPP (Astra Lakemedel, Sweden) were solved in saline with the addition of 1–2 drops of 1 N HCl in doses 2 and 10 mg/kg i.p. (for mice); clonidine (USSR) was solved in saline, 1.5 mg/kg i.v. (for cats); bicucullin (Serva, Germany) was solved in saline with the addition of 0.1 N HCL, 1 mg/kg i.p.; picrotoxin (Serva, Germany) was solved in saline, 1 mg/kg i.p.; R015–1788 (flumazenil) and R015–3505 (Hoffman La Roche, Switzerland) was solved in distilled water with addition of 1–2 drops of Tween-80, 10 mg/kg i.p. and 3 mg/kg i.p., respectively. All drugs were administered before 30 min of the conditioning or testing. Analysis of behavioural and biochemical effects of 3PPP enantiomers has shown, that (+) 3PPP possesses stimulating properties for pre- (low dose) and postsynaptic (high dose) dopaminergic (DA) receptors; (-) 3PPP was considered as an agonist of presynaptic (low) receptors and an antagonist of postsynaptic (high) ones [4, 5].

Statistical analysis. Significance of the difference within groups was analyzed by Student's *t* test and ANOVA.

Results

The role of emotiogenic brain systems in memory modulation by dopaminergic and noradrenergic mechanisms.

The experiments show that both postsynaptic and presynaptic dopamine receptors are involved in the control of retrieval of amnesic memory trace. The effects of (+) 3PPP and (-) 3PPP on retrieval of passive avoidance are shown in

Table I

Retrieval of the memory amnesic trace by changing the pre- and postsynaptic DA receptor activity

Drug	Dose	Weak amnesia				Strong amnesia			
	mg/kg	testing				days			
		0	30 min	1	2	0	30 min	1	2
+3 PPP	2	46+6	89+20*	42+10	39+7	21+2	20+3	32+10	21+2
−3 PPP	2	44+7	94+21*	123+22*	50+8	23+2	22+6	29+7	20+2
+3 PPP	10	44+44	74+14*	84+16*	26+5	25+3	100+18*	90+20*	42+17
−3 PPP	10	42+5	102+14*	120+12*	36+10	29+3	98+15*	85+19*	40+12

0 – testing before drug treatment

* – $P<0.05$ – difference from 0 day, Student's t test.

Avoidance latency was registered, s, Mean + S.E.M.

Table I where the data are presented for two doses of drugs and for two kinds of "psychogenic" amnesia. It is clear that 3PPP enantiomers at the dose of 10 mg/kg have antiamnesic properties against both strong and weak amnesia. The action of (+) 3PPP and (-) 3PPP at dose of 2 mg/kg, which changes the activity of presynaptic DA receptors, consisted in the reestablishment of conditioned response only under weak amnesic influence (Table I).

Activation of noradrenergic (NA) system by postsynaptic doses of clonidine (1.5 mg/kg, i.v.) facilitates the CEP in the reticular formation, the hippocampus and in the preoptic area along with derivation of CNR. Apomorphine activation (3 mg/kg, i.v.) of DA system contributes to the display of CEP in the hippocampus, the amygdaloid complex and in the central gray and enhances the reproduction of SGR (Fig. 1). It has been shown that complete retrieval of motor and vegetative components of memory trace under activation of NA and DA systems is determined by the possibility of memory trace retrieval in the cortex and in the emotiogenic structures of the memory control system.

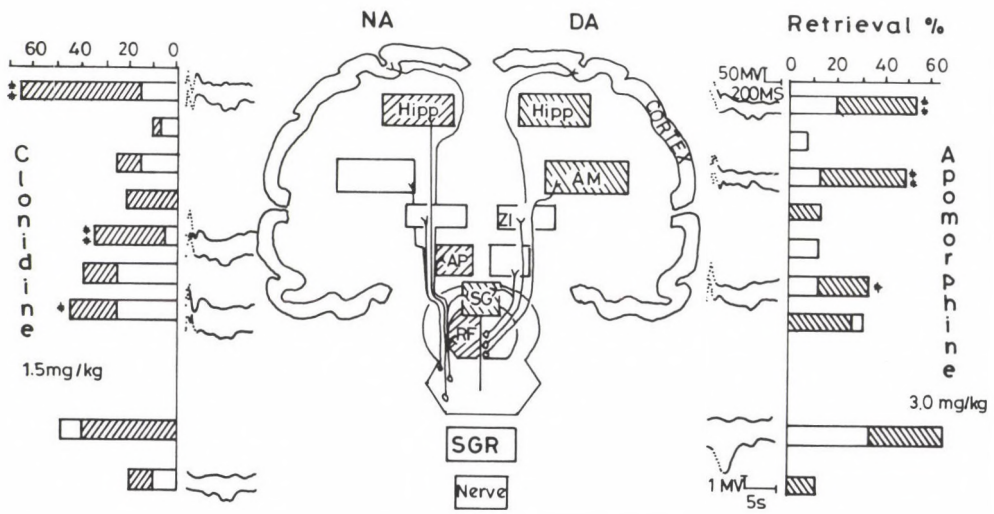

Fig. 1. Retrieval of the memory trace electrographic correlates during noradrenergic and dopaminergic system activation by their agonists. Columns – % retrieval of the conditioned evoked potential, conditioned neurographic response and conditioned skin galvanic reaction (coloured ones after the drug administration), next to them examples of enhancement of the conditioned reaction electrographic correlates are shown. Parallelograms in the centre – the structures studied: Hipp – hippocampus, AM – amygdaloid complex, ZI – zona incerta, AP – area preoptica, SG – central gray, RF – reticular formation. * – $P<0.05$; ** – $P<0.01$

Inhibition of NA synthesis in the brain by diethyldithiocarbamate predetermines the impairment of the CEP retrieval in the hippocampus and the central gray as well as the manifestation of the CNR. Simultaneous decrease of both DA and NA levels in the brain by methyltyrosine distributes CEP retrieval in the reticular formation, the hippocampus, the amygdaloid complex, the zona incerta, the central gray and in the auditory cortex. Clonidine activation of postsynaptic NA receptors induces CEP in the reticular formation, the hippocampus and the preoptic area and promotes CNR retrieval. Antiamnestic effect of apomorphine and clonidine activation of postsynaptic DA receptors and presynaptic NA receptors correspondingly is displayed in the CEP retrieval in the amygdaloid complex and central gray.

Dopaminergic mechanism of the latent inhibition. In the acute experiment the retrieval of the memory trace in a number of brain structures during LI was investigated in cats (Fig. 2). It was shown that the preliminary presentation of isolated nonreinforced conditioned stimuli caused the initial delay of memory trace retrieval in all brain structures. A more prolonged training showed that blockade of memory trace retrieval in the cortex and zona incerta is the most significant condition of the absence of the complete conditioned response reproduction. The retrieval of the vegetative component of the conditioned response (conditioned SGR) is strongly weakened.

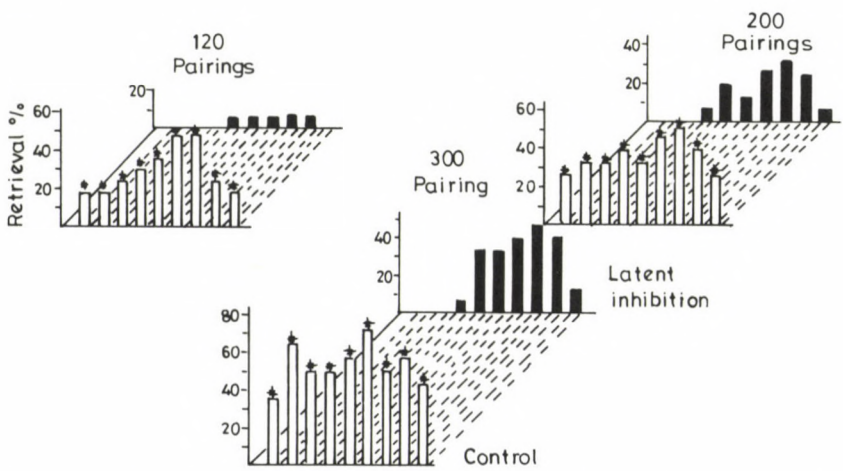

Fig. 2. Diminution of the electrographic correlates of the conditioned reaction retrieval of the background of the latent inhibition after 120, 200, 300 pairings of the conditioned stimulus and unconditioned one. The x-axis represents: the nervus radialis, auditory cortex, zona incerta, central gray, reticular formation, amygdaloid complex, hippocampus, preoptic area and skin galvanic reaction correspondingly. □ learning without preexposure with the conditioning stimulus; ■ learning on the background of the latent inhibition; * – $P<0.05$

The analysis of latent inhibition phenomenon in the one-trial inhibitory passive avoidance task in rats indicated the prevention of amnesia development and the prolongation of extinction time of conditioned response. As shown in Fig. 3 the group of rats that received 20 preexposure stimulus was significantly different from the control group at test-day 1 ($P<0.05$). The results of day 14 revealed an extinction of passive avoidance in the control group ($P>0.05$ between day 14 and day 0). An analysis of mean step-through latencies indicated a significant effect of preexposure on memory retention ($P<0.05$).

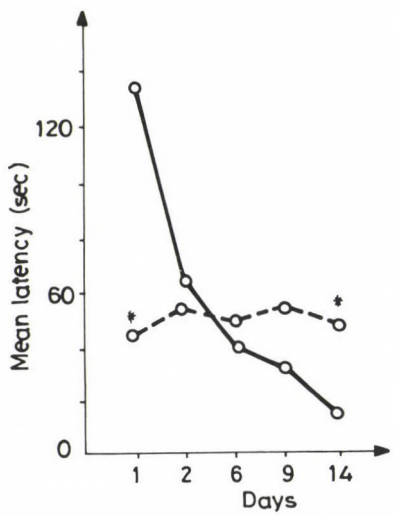

Fig. 3. Weakening of retrieval and extinction of passive avoidance response in rats after 20 preexposure stimulus. Solid line – control of learning, dashed line – latent inhibition
* $P<0.05$ in comparison with non preexposed control

Amnesia induced in naive rats by electroconvulsive shock or detention in the punishment section of the experimental chamber was prevented by preexposure to a conditioning stimulus (Fig. 4). The resistance of memory to different kinds of amnesia is a new sign of LI.

One of the most surprising findings of this study was apparent when the haloperidol-treated animals revealed LI without preexposure of the stimulus (Fig. 5). These data make it possible to create pharmacological models by using them in experimental studies on neurochemical mechanisms of LI.

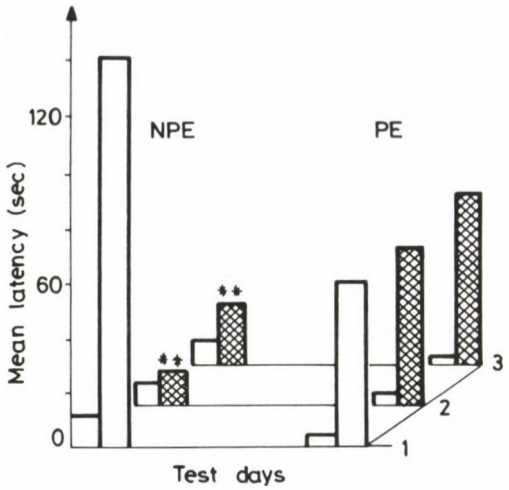

Fig. 4. Prevention of the different genesis amnesia after 20 preexposed stimuli (sign of latent inhibition). Left – control of learning, right – latent inhibition; 1 – learning, 2 – "psychogenic" amnesia, 3 – ECS amnesia, 05 in comparison with non preexposed control, ** $P<0.01$ in comparison with own control

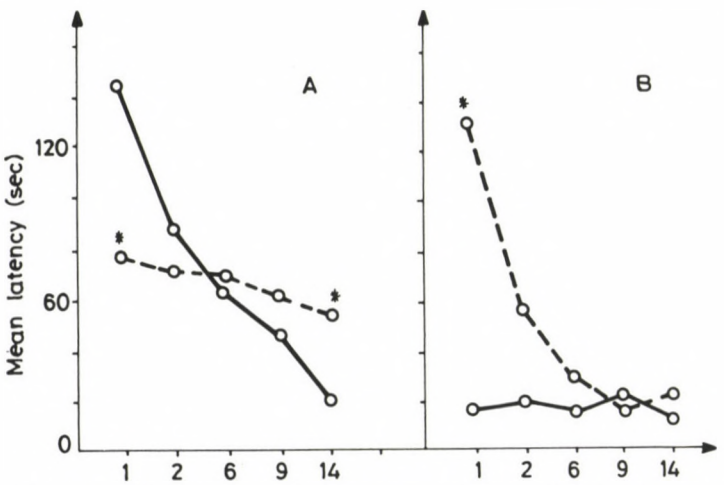

Fig. 5. Pharmacological model of the latent inhibition. A – weekening of the retrieval and prolongation of the extinction after haloperidol administration (0.5 mg/kg, 30 min before learning), B – antiamnesic effect of haloperidol. Solid line – control of learning (A) and amnesia (B), dashed line – haloperidol. * $P<0.05$ in comparison with non treated rats

In electrophysiological experiments we also showed that both in the group without (haloperidol-treated animals) and in the group with preexposure of a conditioning stimulus, the CEP was not retrieved in the cortex and the zona incerta, i.e. in the structures where simultaneous CEP retrieval provides 100% reproduction

of CNR. It causes to the failure of CNR realization in animals, in other words the conditioned connection is not realized in the motor component of the conditioned response. Thus, when the conditioned response is disturbed by haloperidol after the initial phase of CEP inhibition in all brain structures the destruction of the conditioned motor response is determined by the absence of trace retrieval in the cortex and zona incerta.

The next series of experiment was carried out in rats treated with DA agonists (amantadine, nomifensine and bupropion) 30 min before conditioning of the preexposed stimulus. The purpose of these experiments was to test effects of DA system activation on LI development. Table II indicates the mean latency of the preexposed control and preexposed treated groups in condition learning (I) and "psychogenic" amnesia (II). As can be seen, all the three parameters of LI has been disturbed by three drugs but in a different degree. The high-selective DA agonist bupropion is a more effective drug for changing of LI.

Table II

The disturbance of latent inhibition in rats by dopaminergic agonists

Groups	Subgroups	Testing days			
	I-learning II-amnesia	0	1	7	14
Preexposed	I (12)	6.5+0.8	42.2+8.2	48.2+14.7	40.4+11.1
Control	II (11)	7.0+0.9	47.3+12.6	41.8+16.5	32.3+9.6
amantadine	I (14)	11.5+2.7	82.7+12.5*	30.5+14.4	19.7+6.2*
50 mg/kg	II (12)	9.2+3.0	12.1+2.3*	10.6+3.2*	12.8+4.0*
nomifensine	I (9)	8.2+0.8	108.6+20.3*	39.0+18.6	20.3+7.4*
10 mg/kg	II (12)	8.9+1.2	14.0+6.2*	10.9+4.2*	13.8+7.5*
bupropion	I (14)	10.2+4.1	136.1+20.6*	42.2+16.7	21.7+11.4
30 mg/kg	II (14)	9.4+2.0	12.8+3.4*	14.7+6.8*	10.2+5.5

* P = 0.05 – difference from control. Other details as in Table I

Considering the surmised participation of DA brain system and the decisive role of the cortex in LI realization, in our electrophysiological experiment we tried to prevent the impairment of information selection at the expense of amplification of DA release by bupropion (Fig. 6) and by local application of 1% amantadine solution upon the suprasylvian gyrus. Amantadine application reactivated CEP retrieval in the

auditory cortex, in the zona incerta, in the preoptic area and the motor component of the conditioned reaction, the number of CEP retrieval in the amygdaloid complex increased.

The role of the benzodiazepine-GABA-ionophore complex in the memory modulation mechanisms

A comparative analysis of the effectiveness of the blockade of components of benzodiazepine-GABA-ionophore complex for amnesia prevention and retrieval of amnesic memory trace has been carried out. It is shown that the blockade of GABA-receptors by bicuculline, benzodiazepine receptors by flumazenil (R015 – 1788) and by R015 – 3505, chloride channels by picrotoxin considerably weakens the influence of amnesic agent (Table III). After presenting administration of the drugs a considerable improvement of the memory amnesic trace retrieval took place.

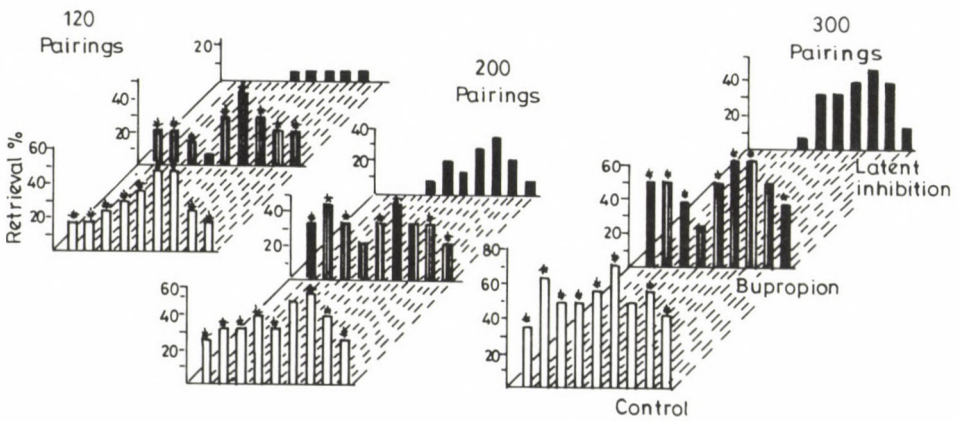

Fig. 6. Retrieval of the conditioned evoked potentials during learning in the presence of the latent inhibition on the background of bupropion administration. * – $P<0.05$ in comparison with the latent inhibition. Other details as in Fig. 2

Table III

Antiamnesic effect of the benzodiazepine- GABA- ionophore complex blockade

Groups	Testing day				
	0	1	2	3	6
Learning (control)	13+1	161+9*	149+11*	153+11*	125+13*
Amnesia (control)	16+2	28+3	21+3	21+3	17+2
Bicuculline	15+2	65+9*	30+6	–	–
Picrotoxin	20+2	62+10*	26+5	–	–
Flumazenil (R015-1788)	17+2	59+11*	51+11*	42+12	16+3
R015-3505	17+2	59+11*	58+16*	38+14	36+12

0 – day before learning.

* $P < 0.05$ – difference from control amnesia. Other details as in Table I

Discussion

The conducted experiments showed that NA and DA systems regulate the memory trace retrieval through the emotiogenic regulatory system and mesencephalic reticular formation. The involvement of the emotiogenic regulatory system into the memory trace retrieval is realized in different ways [22]. The analysis of the electrical signals of memory trace retrieval in brain structures has shown that DA system modulates memory trace retrieval via the central gray, the amygdaloid complex and the hippocampus and NA system – via the reticular formation, the preoptic area and the hippocampus.

Thus, there is sufficient evidence concerning the participation of DA system in mechanisms of alteration of memory processes. However, the DA system is not directly involved in memory trace fixation. The animals are capable to acquisite information, to fixate the memory trace after treatment of DA receptor blockers [9]. A number of hypotheses is proposed concerning the participation of DA system in behaviour which might define its role in memory control. The role of DA system in learning is explained by its contribution in anhedonia reinforcement mechanisms. This hypothesis might be competent but the explanation of the action of neuroleptics only by the anhedonic effect narrows the range of their action. Moreover, the anhedonic effect is not always proved and not for every model [15]. For example, apomorphine caused both positive and aversive reinforcing effects with different localization of its action [6, 11, 27]. DA system participation is associated by some authors with the type of memory formation, acquisition of motivation properties by a neutral signal when combined with biologically meaningful stimulus [2]. Inhibition of

the memory trace retrieval under the blockade of DA receptor and the improvement of one after the termination of the drug effect show that the DA receptor blockade does not prevent the trace fixation but retards associative learning. It is sufficient to reinforce the stimulus and learning will take place in spite of the DA receptor blockade or 6-OHDA DA pathways distruction [6, 15].

At present it becomes apparent that synaptic processes play the important role in the mechanisms of memory modulation by the DA system. The participation of presynaptic and postsynaptic DA receptors has not become clear so far. There is a hypothesis that the autoreceptor modulation contributes to stabilization of neuronal circuits repeatedly being activated during learning [25]. However, DA autoreceptor activation by selective agonists (TL-99, 3-PPP) and by low doses of apomorphine does not influence the memory processes. Our experiments have shown the effect of low doses of (+) and (-) 3-PPP only to weak amnesia, whereas high doses affecting the D1 and D2 postsynaptic receptors clearly prevent amnesia.

In the process of vital activity every organism is subjected to a great flow of information via different sensory channels. The selection of the signals for fixation and for the possible subsequent retrieval has been determined by the selection of information using parameters of novelty and emotiogeneity. The activity of neurochemical modulatory systems provides the selective reinforcement of the definite links of the regulatory memory system during the fixation and the retrieval of the information.

LI refers to the finding that nonreinforced preexposure to a stimulus retards subsequent conditioning to that stimulus when it is paired reinforcement [18]. Latent inhibition is a most important mechanism of information selection. What is underlying the mechanism of LI? The decisive factor in habituation development is the assessment the biological significance of a stimulus. It appears that preexposure to a stimulus induced functional elimination of certain pathways in the central nervous system by different ways: by modification of pre- and postsynaptic structures, activation of a peculiar type of synapsis, by means of homosynaptic depression, by hippocampal inhibition of the activating system and by amplification of synchronizing system action [3, 7, 23, 25, 26].

There are data about the role of DA brain neurons for attention processes. Sensory inattention is observed after degeneration of the ascending DA axons by 6-OHDA. Denervation of the mesolimbicocortical DA terminals induced by bilateral 6-OHDA administration in the hypothalamus impaired the investigation of new objects and diminished the number of approaches to them. The apomorphine activation of DA receptors recovered the normal explorative behaviour in these animals.

Our data show that under LI memory trace is being fixed, but in certain brain structures it is not retrieved. Under activation of cortical DA synapsis the retrieval is

enhanced not only in the cortex but also in a number of subcortical structures – the amygdaloid complex and the zona incerta. The repeated reinforcement of the preexposed stimulus gradually weakens LI in the reticulate formation and the emotiogenic structures. However, the manifestation of CEP in these structures is not sufficient for the retrieval of the motor component of the conditioned reaction since there is no trace retrieval in the brain cortex. The analysis of the interaction of different brain structures during the conditioned reaction has indicated that CNR retrieval in 100% of the cases is a simultaneously manifested CEP retrieval in the auditory cortex, the zona incerta and the preoptic area. The emergence of the CEP in the auditory cortex with a large latent period testifies about the secondary delivery of impulses from the subcortical structures, primarily from the limbic system to the projection site. The synthesis of physiological and signal stimulus properties of information [12] from a complex evaluation of signals occur in cortical centers and it is the basis for the purposeful behaviour.

Under the LI the trace retrieval is deteriorated as the conditioned stimulus used repeatedly without reinforcement is fixed as the irrelevant one. The blockade of switching of the nervous conducting of the sensory pathways in the cortex to the subcortical structures and to the motor neurons might be a decisive factor. This can be conditioned by decreasing the activity of the modulating DA mechanism. The obtained data makes it possible to surmise the physiological mechanism of the LI development. It is possible that during the preexposure with the stimulus the lowering of the activity of DA mechanism takes place.

The possibility of elimination of LI effects by an increase of synaptic DA concentration in the brain cortex by bupropion and amantadine [10] maintain the importance of the cortical DA mechanism for LI. It is probable that the blockade of the DA mechanism of brain cortex sensory areas is a determining factor for LI development.

Our findings rather suggest that the failure of the retrieval mechanism underlying the LI in consequence of DA activity decrease. Decrease of the activity of the DA synaptic transmission by haloperidol before learning of non-preexposed animals blicks the significance of information supresses attention to novelty elements, produces, analogously to LI, spatial-temporal distribution of the memory trace in brain structures. Although the LI model as presented here, is related to the DA hypothesis, it can and should be used for studying the involvements of other neurochemical systems in process of attention.

The inhibition of various components of the benzodiazepine-GABA-ionophore complex improves the memory trace retrieval damaged by amnesic influences. It has been suggested, that flumazenil has negligible positive intrinsic efficacy and exerts no consistent intrinsic pharmacological effects except a mild anticonvulsant activity [8]. However, other results clearly show that the benzodiazepine antagonist flumazenil

fails to reverse the effects of chlordiazepoxide on habituation and on the amnesic action of benzodiazepines [16, 20]. Furthermore, flumazenil prevents the occurrence of amnesia induced by the cholinergic receptor antagonist scopolamine [14]. Therefore, it is likely that the antagonist of benzodiazepine receptor has, at least in part, intrinsic pharmacological efficacy on memory.

The pattern of the results from the different tests supports the hypothesis that activation of the dopaminergic brain system facilitates the development of the spacial-temporal construction of structures participating in the memory control. Moreover, the finding of this study provides additional support for the proposition that disruption of LI constitutes a valid animal model of schizophrenic attention deficit.

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INTERNATIONAL SOCIETY FOR PATHOPHYSIOLOGY HAS BEEN SET UP

The International Society for Pathophysiology has been set up at the International Constituent Congress for Pathophysiology (Moscow, 1991). The establishment of the Society reflects the modern trends of biomedical science development.

Pathophysiology, as a biomedical science of the mechanisms of development and elimination of pathological processes, began due to a necessity of experimental analysis of pathological process.

Pathophysiology originated independently in various countries which, testifies the objective necessity for appearance of pathophysiology in the development of medicine.

Modern pathophysiology ceased to be just experimental pathology, it has also acquired a new quality, and has become an integrative biomedical science. This feature is due to the growing differentiation of medicine, and to an ever increasing body of diverse information about various pathological processes. All this has brought about the necessity for the birth of an integrative biomedical science. Pathophysiology has become such a science.

The misfortunes afflicting the human race present medicine with new, and more and more serious and urgent problems. Pathophysiology has to play an important role in solving these problems. In these conditions, a paramount importance become consolidation and unification of the efforts of scientists of various profiles who are, somehow or other, concerned with a wide-range of pathophysiological problems throughout the world.

Therefore, the past Constituent Congress of the International Society for Pathophysiology was of special importance. At its numerous sections (about 20) and symposia (about 60) the problems of pathophysiology of cells, organs and systems, were discussed united by one main, characteristic of pathophysiology, topic: "Dysregulation and Its Correction".

The International Society for Pathophysiology, which was established at the Congress, is open to specialists of any profile, experimentalists and clinicians, to all concerned with pathophysiological problems.

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The Society will have the following sections: pathophysiology of most important systems and organs, typical pathological processes (inflammation, hypoxia, adaptation, microcirculation, metabolic disorders, shock), cellular pathophysiology, ecological pathophysiology, space flight pathophysiology, clinical pathophysiology, veterinary pathophysiology and teaching of pathophysiology. The sections of the Society will co-operate with the corresponding international scientific associations. Such cooperative aims and activities of the Society reflect the peculiarities of modern pathophysiology as an integrative biomedical science.

Professor Georgy Kryzhanovsky, a prominent Russian pathophysiologist was elected the first President of the International Society for Pathophysiology (ISP). Professors Yutaka Oomura, Japan, Osmo Hänninen, Finland and Aubrey Taylor, USA, were elected as Vice Presidents. Dr. Vladimir Shinkarenko, Russia, was elected for the as Secretary General, Dr. J.-M. Mencia-Huerta, France, was elected post of Treasurer.

The International Union of Physiological Sciences (IUPS) has set up and approved in its framework a commission for pathophysiology. The ISP is expected to become an associate member of the IUPS.

The ISP will publish the International Journal of Pathophysiology.

The next congresses are to be convened in Japan in 20–26 November, 1994, in Kyoto, and then in Finland and Hungary.

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