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TARTU ÜLIKOOLI

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MOLECULAR PHARMACOLOGY OF RECEPTORS III

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MOLECULAR PHARMACOLOGY OF RECEPTORS III

Editor Lembit H. Allikmets

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Preface

This edition deals mostly with the peripheral benzodiazepine binding sites on blood cells and with changes of their affinity under tranquillizers and GABAergic compounds. The other topic concerns several new data on the role of voltage-sensitive calcium channels in the development of benzodiazepine abstinence, and also interactions between calcium channel blockers and of agonists of dopamine, serotonin and m-cholinoreceptors (Zharkovsky et al.). Some papers deal with vasoactive peptide receptors and with the pharmacology of cholecystokinin-8 agonists. There have been included also couple of reviews and methodological papers.

This year professor of our department Leo Nurmand celebrated his 60. birthday. He has been active more than 35 years in planning and developing the teaching process in pharmacology and not only in pharmacology, but also in several other topics in our Medical Faculty. This volume includes his paper on the development in teaching of pharmacology in Tartu University after WWII.

The volume is devoted to him, teacher, gentleman and humanitarian.

Lembit H. Allikmets

Chairman, Dept. of Pharmacology
Dean, Faculty of Medicine

THE EFFECT OF STRESS ON ω_3 BENZODIAZEPINE RECEPTORS IN RAT BLOOD PLATELETS AND LYMPHOCYTES: THE EFFECT OF NONBENZODIAZEPINE TRANQUILIZERS

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Abstract

The effect of several models of stress was studied on ω_3 (peripheral-type) benzodiazepine receptors in rat blood platelets and lymphocytes. Electric footshock lowered significantly ^{3}H-Diazepam binding on blood platelets whereas swimming stress and chronical injection enhanced the number of ^{3}H-Ro 5-4864 binding sites both on platelets and lymphocytes. Nonbenzodiazepine tranquilizers phenibut and buspirone after *in vivo* administration had different effects on blood cell benzodiazepine receptor. Phenibut (100 mg/kg) significantly enhanced the affinity and had a tendency to lower number of ^{3}H-Ro 5-4864 binding sites on platelets. Buspirone (5 mg/kg) was without any effect. The pretreatment of animals with phenibut (100 mg/kg) counteracted the effect of swimming stress on ω_3 benzodiazepine receptors both on platelets and lymphocytes. Buspirone (5 mg/kg) did not have any effect on the swimming stress induced changes of ^{3}H-Ro 5-4864 binding on platelets but had a tendency to lower the enhanced binding on lymphocytes. The possible mechanisms involved in the changes of ω_3 benzodiazepine receptors on blood cells after various stressful stimuli is discussed. The possibility to use blood cell benzodiazepine receptors as sensitive markers of emotional status (anxiety) in humans is suggested.

Introduction

Specific benzodiazepine (BD) binding sites in the brain and in several other tissue outside the central nervous system were discovered a decade ago using tritiated diazepam as a ligand [20]. The central BD binding sites, mostly referred now as BD receptors, are coupled to the GABA receptor -

chloride ionophore complex. The BD receptor ligands modulate the efficacy of GABA as an inhibitory transmitter. Depending on the chemical structure of the ligand the modulation is either positive (GABA action enhanced) or negative (GABA action depressed) [2]. Multiplicity of central BZ receptors has been proposed. The binding of ß-carboline derivatives has been used to demonstrate at least two different binding sites (BD_1 and BD_2) in the rat brain [2].Moreover, the so called peripheral BD receptor is also present in the central nervous system [1, 19]. Therefore, recently a new nomenclature of BD receptors that does not designate location and is defined in terms of pharmacological specificity has been proposed. The Greek letter omega, as ω_1, ω_2 and ω_3 to designate respectively the central BD_1, BD_2 and the peripheral BD receptors has been used [11].

Several lines of evidence demonstrate that central BD receptors are affected by stress [14, 15]. However, little is known about the influence of stress on the peripheral type BZ recognition sites [19]. The aim of the present study was to examine the action of various kind of stressful stimuli on ω_3 BD receptors in rat blood platelets and lymphocytes. In addition to this the effect of nonbenzodiazepine tranquilizers on stress induced changes of ω_3 BD receptors on the blood cells was studied.

Materials and methods

Animals and drugs. Male albino laboratory rats weighing 230-280 g (Rappolovo Farm, Leningrad) were used. The animals were maintained on food and water ad libitum at 20 ± 1 °C on a reversed lighting cycle with lights off from 2000 to 0800. Phenibut hydrochloride [ß-(phenyl)-GABA] and buspirone hydrochloride (Bristol-Myers, Evansville, IN, U.S.A.) were dissolved in saline. Both drugs were injected in a volume of 1 ml per kg intraperitoneally 50 and 40 min prior to experiments respectively.

Forced swimming stress. Swimming stress was carried out as described previously [14] with minor modifications. The stress was produced by forcing the rats to swimm in a water basin (50 x 40 x 25 cm) at 20 ± 1 °C for 5 min. After termination of the forced swimming the animals were immediately sacrificed by decapitation.

Foot shock stress. The foot shock stress involved a series of electrical foot shocks delivered in individual boxes with floors made of brass rods, 1 cm apart. Shocks were provided by a stimulator which delivered shocks (60HZ, 0.5mA) during 10 s with 5 s intervals during a 5 min session.

Chronical injection stress. It was proposed that chronical injection procedure itselt may be stressful. In chrcnical treatments with various drugs usually only two groups are compared: chronical vehicle and chronical drug. In this case stress-protective drug may demonstrate results that are not due to any specific action. Before carrying out chronical experiments with specific ω_3 BD receptor ligands we decided to clarify this problem. Therefore we decided to compare chronically injected animals (1 ml/kg of saline once a day during 10 days) to the group of animals receiving only one injection.

***In vitro* binding studies.** After decapitation the trunk blood of every rat was collected into a plastic tube containing 0.5 ml acid citrate dextrose (ACD) anticoagulant. Lymphocytes were isolated according to the method of Boyum [3] using Ficoll-Pague gradient and were washed twice before the binding experiment. Platelets were obtained from platelet-rich plasma by centrifugation. The pooled blood cells from at least 8 animals were for each binding assay. ^{3}H-Diazepam (spec. act. 81 Ci/mmol) and ^{3}H-Ro 5-4864 (spec. act. 84 Ci/mmol, both Amersham Radiochemicals, England) were used in eight different concentrations (0.5 - 34 nM and 0.5 - 24 nM respectively) for labelling peripheral-type BZ binding sites on blood platelets or lymphocytes. Unlabelled Ro-4864 (Hoffman - La Roche, Basel, Switzerland) was used to determine nonspecific binding. The binding experiments were performed in a total volume of 125 µl during 30 min at 0 °C. All binding experiments were carried out in a modified Hanks solution (pH 7.3). The intact blood platelets and lymphocytes were used throughout these studies.

After incubation of the tubes containing lymphocyte or platelet suspension the reaction was stopped by rapid filtration over Whatman GF/B filters. The filters were washed with 3 x 3 ml of ice cold buffer. Specific binding was calculated by subtracting the nonspecific from total binding at each given radioactivity concentration. Protein was measured by the Lowry et al [12] method.

Calculations and statistics. Maximum binding (B_{max}) and affinity constants (K_D) were calculated using the Scathard plot analysis. The Scathard plots were computed using linear regression programm. For the data presented, only the plots with a correlation coefficent of 0.85 or more were accepted.

The significance of the differences between the results was tested using Student's t-test; $P < 0.05$ was considered significant.

Results

The effect of foot shock on ω_3 BD receptors in rat blood platelets and lymphocytes. The short-lasting (5 min) electric foot shock lowered the number and decreased the affinity of ^{3}H-Diazepam binding sites on blood platelets. On lymphocytes the effect of foot shock was less evident (table 1).

Table 1

The effect of foot-shock stress on ω_3 benzodiazepine receptors in rat blood platelets and lymphocytes. ^{3}H-Diazepam was used to label benzodiazepine binding sites on intact blood cells. The data expressed are mean ± S.E.M. of at least three indipendent experiments each carried out in triplicate.

Experimental group	$_3$H-Diazepam binding	
	B_{max} (fmol/10^8 cells)	K_D (nM)
Platelets		
Control	488 ± 53	9.6 ± 1.6
Foot-shock	321 ± 41*	12.2 ± 2.1
Lymphocytes		
	B_{max} (fmol/10^6 cells)	K_D (nM)
Control	108 ± 21	8.7 ± 1.3
Foot-shock	88 ± 11	9.8 ± 1.2

* $P < 0.05$ as compared to control animals

The effect of forced swimming stress on ω_3 BD receptors in rat blood platelets and lymphocytes. Forced swimming stress increased the number of ^{3}H-Ro 5-4864 binding sites both on platelets and on lymphocytes. Those changes were statistically significant. The affinity for the ligand was slightly decreased but the changes were never statistically significant (table 2).

Table 2

The effect of swimming stress on ^{3}H-Ro 5-4864 binding in rat intact blood platelets and lymphocytes. The data expressed are mean ± S.E.M. of five experiments each carried out in triplicate.

Experimental group	^{3}H-Ro 5-4864 binding	
	B_{max} (fmol/mg)	K_D (nM)
Platelets		
Control	5895 ± 450	10.1 ± 0.8
Swimming stress	8931 ± 930*	12.3 ± 1.4
Lymphocytes		
Control	4737 ± 580	8.2 ± 1.1
Swimming stress	8016 ± 1108*	10.8 ± 1.4

* $P < 0.05$ as compared to control group

The effect of chronical injection of saline on ω_3 BD receptors in rat cerebral cortex, blood platelets and lymphocytes. The chronical injection procedure caused marked changes in ω_3 BD receptors (table 3). The number of ^{3}H-Ro 5-4864 binging sites was increased not only on platelets and lymphocytes but also in cerebral cortex. However, the changes in cerebral cortex did not reach statistically significant values. The affinity for the ligand was not considerably changed in this series of experiments (table 3).

Table 3

The effect of acute and subchronical injection of saline on ω_3 benzodiazepine receptors in rat cerebral cortex, blood platelets and lymphocytes. ^{3}H-Ro 5-4864 was used to label benzodiazepine receptors. The data expressed are mean ± S.E.M. of at least three indipendent experiments each carried out in triplicate.

Experimental group	^{3}H-Ro 5-4864 binding	
	B_{max} (fmol/mg)	K_D (nM)
I. Cerebral cortex		
Acute injection	111 ± 12	2.03 ± 0.21
Chronical injection	147 ± 15	2.15 ± 0.33
II. Platelets		
Acute injection	4860 ± 630	9.9 ± 1.1
Chronical injection	7530 ± 690*	11.3 ± 1.2
III. Lymphocytes		
Acute injection	3380 ± 310	8.2 ± 0.9
Chronical injection	4910 ± 530*	11.5 + 1.4

* $P < 0.05$ as compared to acute injection group.

The effect of phenibut and buspirone on ω_3 BD receptors in rat blood platelets. The effect of two nonbenzodiazepine tranquilizers on blood platelet ω_3 BD receptors was studied to find out if these compounds have any effects per se table 4). It was found that *in vivo* administration of phenibut (100 mg/kg) had a tendency to lower and buspirone (5 mg/kg) to enhance the number of ^{3}H-Ro 5-4864 binding sites. In addition to that only phenibut significantly increased the affinity for the ligand. In this study the calculations of the apparent number of binding sites were made paralell either per mg of protein or 10^8 platelets. Naturally, the values received were different but the tendencies observed

were similar. This and other similar results encouraged us to present the binding site data only per mg of protein (table 4).

Table 4

The effect of phenibut (100 mg/kg) and buspirone (5 mg/kg) pretreatment on ω_3 benzodiazepine receptors in rat blood platelets. ^{3}H-Ro 5-4864 was used to label ω_3 benzodiazepine binding sites. The data expressed are mean ± S.E.M. of at least three indipendent experiments each carried out in triplicate.

Experimental group mg/kg	^{3}H-Ro 5-4864 binding	
	B_{max}	K_D (nM)
Saline	6102 ± 870 fmol/mg 1269 ± 181 fmol/10^8 cells	10.7 ± 0.9
Phenibut 100	5531 ± 613 fmol/mg 1027 ± 112 fmol/10^8 cells	7.1 ± 1.2*
Buspirone	7471 ± 892 fmol/mg 1544 ± 185 fmol/10^8 cells	13.1 ± 1.4

* $P < 0.05$ as compared to saline controls.

The effect of phenibut and buspirone pretreatment on swimming stress induced changes of ω_3 BD receptors in rat blood platelets and lymphocytes. The pretreatment of rats with phenibut (100 mg/kg) effectively counteracted the swimming stress caused changes of ^{3}H-Ro 5-4864 binding on platelets and on lymphocytes (table 5). Lower doses of phenibut (25-50 mg/kg) were almost without effect. Buspirone (5 mg/kg) did not eliminate the stress induced changes of ^{3}H-Ro 5-4864 binding on platelets but like phenibut counteracted the effect of stress on lymphocytes (table 5).

Table 5

The effect of phenibut (100 mg/kg) and buspirone (5 mg/kg) pretreatment on swimming stress induced changes of ω_3 benzodiazepine receptors in rat blood platelets and lymphocytes. ^{3}H-Ro 5-4864 was used to label benzodiazepine receptors on blood cells. The data expressed are mean ± S.E.M. of at least three indipendent experiments each carried out in triplicate.

Experimental group, mg/kg	^{3}H-Ro 5-4864 binding	
	B_{max} (mg/prot)	K_D (nM)
I. Platelets		
Control	6312 ± 532	13.1 ± 0.9
Swimming stress	9578 ± 1012*	15.8 ± 1.6
Swimming stress + Phenibut 100	7038 ± 843	12.4 ± 1.3
Swimming stress + Buspirone 5	10109 ± 1434	14.3 ± 1.5
II. Lymphocytes		
Control	4987 ± 632	10.1 ± 1.1
Swimming stress	8434 ± 911*	12.8 ± 1.4
Swimming stress + Phenibut 100	611 ± 1013	11.5 ± 1.2
Swimming stress + Buspirone	6494 ± 834	13.2 ± 1.5

* $P < 0.05$ as compared to control group.

Discussion

After various stressful situations both the increase [15] and decrease [14] of the number of central BD receptors has been reported. Most BDs used so for are nonselective ligands for the central ω_1 and ω_3 (in literature often referred to as BD_1 and BD_2 respectively) BD receptors. Several ligands with nonbenzodiazepine chemical structure (CL 218872, some betacarbolines, CGS 8216 and Zolpidem) have selectivity for ω_1 receptor subtype. The role

of ω_1 and ω_2 BD receptors in the neurochemistry of stress has not been comparatively studied. The effect of stress on ω_3 (peripheral-type) BD receptors has also been poorly studied. However, recently it has been shown that foot shock can decrease the number of ^{3}H-Flunitrazepam binding sites in rat kidneys and adrenals [9]. This finding is in agreement with our results demonstrating that foot shock lowers the number of ^{3}H-Diazepam binding sites in rat blood platelets. This finding of ours is supported by the data showing that in man the binding capacity of the BD binding sites in the platelets of anxious patients has been shown to be reduced in comparison to normal controls [22]. However, in other two models of stress (forced swimming and chronical injection) the number of ^{3}H-Ro 5-4864 binding sites on platelets and lymphocytes was increased. Different ligands used in these studies cannot be the reason of the discrpancy. Similar changes in binding characteristics after swimming stress were obtained when blood platelets were labeled with ^{3}H-Diazepam (unpublished data). It is possible that different models of stress can induce different neurochemical changes.

The mechanism involved in the stress induced changes of ω_3 BD receptors on blood cells is difficult to explain. In contrary to the central-type BD recognition sites ω_3 BD receptors are not modulated by GABA or chloride ions *in vitro* [13]. However, it should be mentioned that the regulation of central (ω_1 + ω_2) and ω_3 BD receptors in several peripheral organs seems to be similar after *in vivo* pretreatment with GABA agonists [16, 17]. Moreover, recently we have demonstrated that several models of stress (foot shock, naive animals in comparison to handling-habituated animals etc.) cause similar changes both in central (cerebral cortex) and in ω_3 (kidneys and adrenals) BD receptors [9, 10]. It has been also reported that an inescapable tail shock produces a reduction of ^{3}H-Ro 5-4864 binding sites in rat heart and kidney but not in CNS [6]. The localization of relatively high concentrations of ω_3 BD receptors in the pituitary and adrenals [5] may indicate that these recognition sites are involved in the neuroendocrinological regulation of emotional behavior. The existence of at least one common endogenous modulator or endogenous ligand for all BD recognition sites may be suggested. Imidazopyridine alpidem, a

new BD receptor ligand, possesses rather similar affinity for the ^{3}H-Diazepam binding site in the cerebellar cortex and ^{3}H-Ro 4-4864 binding site in the kidney [11]. Many older generation BD (diazepam, flunitrazepam etc.) receptor ligands have also rather poor selectivities for ω_1, ω_2 and ω_3 BD receptors. DBI/(diazepam binding inhibitor) is a proposed endogenous ligand peptide for central BD receptors. Interestingly, the DBI/mRNA is expressed not only in CNS neurons, but also in glial cells, liver, kidney and heart [8].

In the present study it was demonstrated that the pretreatment of rats with phenibut increased the affinity of ^{3}H-Ro 5-4864 binding in rat blood platelets and effectively attenuated the elevation of maximum binding caused by swimming stress. It has been reported that the rise of serum prolactin levels caused either by swimming, immobilization, ether or cold stress was inhibited by pretreatment of rats with baclofen [4]. Phenibut, a drug structurally closely related to baclofen, is a weak $GABA_B$ receptor agonist [16]. Both $GABA_A$ and $GABA_B$ receptor agonists increase the affinity of ^{3}H-Flunitrazepam binding not only in cerebral cortex but also in kidneys [18]. Although these data support the findings of the current study the mechanism by which GABA receptor agonists can modulate ω_3 BD receptors remains unknown.

Buspirone pretreatment had only a tendency to enhance the number of ω_3 binding sites in rat blood platelets. Recently it was demonstrated that *in vivo* buspirone in a dose related manner increases *in vivo* labelling of central BD receptors with ^{3}H-Ro 15-1788 [7]. However, this effect of buspirone seems to be unspecific because neuroleptic drugs and apomorphine also caused similar changes of the *in vivo* ^{3}H-Ro 15-1788 binding.

Contrary to the phenibut buspirone pretreatment of animals counteracted the stress induced changes of ω_3 BD receptors only on lymphocytes. Buspirone is also known to lower increased corticosterone and prolactin levels in stressed rats [21]. It may be suggested that the stress-protective effect of phenibut and buspirone on the level of blood cell ω_3 BD receptors is an indirect one, probably mediated through hormonal changes caused by these drugs.

Two main conclusions can be made from the studies carried out here. First, it is possible to assume that the number of ω_3 BD receptors in rat blood platelets and lymphocytes is enhanced or lowered depending on the model of stress used. The stress induced changes of ω_3 BD receptors seem to be similar to those recorded in central BD receptors. Therefore the possibility to use ω_3 BD receptors on blood cells as markers of the functional activity of central BD receptors may be suggested and further studies in this direction recommended. Secondly, the nonbenzodiazepine tranquilizers phenibut and buspirone, seem to have stressprotective action on the level of blood cell ω_3 BD receptors. However, further studies to establish the role and function of ω_3 BD receptors in the neurochemical mechanisms involved in stress are necessary.

References

1. Basile A.S., Skolnick P.J. Subcellular localization of "peripheral type" binding sites for benzodiazepines in rat brain // J. Neurochem. - 1986. - Vol. 46. - P. 305-308.
2. Braestrup C., Nielsen M. Benzodiazepine receptors // Handbook of Psyhopharmacology / Eds. L.L. Iversen, S.D. Iversen and S.H. Snyder. - New York: Plenum Press, 1983. - Vol. 17. - P. 258-384.
3. Boyum A. Isolation of mononuclear cells and granulocytes from human blood // Scand. J. Clin. Lab. Invest. - 1986. - Vol. 21. - P. 77-89.
4. D'Eramo J.L., Somaza G.M., Kertesz E., Libertun C. Baclofen, a GABA derivative, inhibits stress-induced prolactin release in the rat // Eur. J. Pharmacol. - 1986. - Vol. 120. - P. 81-85
5. De Souza E.B., Anholt R.R.H., Murphy K.M.M., Snyder S.H., Kuhar M.J. Peripheral-type benzodiazepine receptors in endocrine organs: autoradiographic localization in rat pituitary, adrenals and testis // Endocrinology - 1985. - Vol. 116. - P. 567-573.

6. Drugan R.C., Basile A.S., Crawley J.N., Paul S.M., Skolnick P. Inescapable shock reduces ^{3}H-Ro 5-4864 binding to peripheral-type benzodiazepine receptors in the rat // Pharmacol. Biochem. Behav. - 1986. - Vol. 24. - P. 1673-1677.

7. Goeders N.E., Ritz M.C., Kuhar M.J. Buspirone enhances benzodiazepine receptor binding *in vivo* // Neuropharmacology. - 1988. - Vol. 27. - P. 275-280.

8. Gray P.W., Glaister D., Seeburg P.H., Guidotti A., Costa E. Cloning and expression of cDNA for human diazepam binding inhibitor, a natural ligand of an allosteric regulatory site of the γ-aminobutyric acid type A receptor // Proc. Nat. Acad. Sci. U.S.A. - 1986. - Vol. 83. - P. 7547-7551.

9. Kiivet R.-A., Harro J., Rägo L., Põld M. Changes in GABA and benzodiazepine receptors after foot shock in the rat: influence of diazepam // Acta et Commentationes Universitatis Tartuensis N° 839: Molecular Pharmacology of Receptors / Ed. L. Allikmets. - Tartu, 1988. - Vol. 2. - P. 45-55.

10. Kiivet R.-A., Rägo L., Harro J., Zilmer M., Vihalemm T. Biochemical and behavioral characterization of naive and handling-habituated rats // Acta et Commentationes Universitatis Tartuensis N° 839: Molecular Pharmacology of Receptors / Ed. L. Allikmets. - Tartu, 1988. - Vol. 2. - P. 33-45.

11. Langer S.Z., Arbilla S. Imidazopyridines as a tool for the characterization of benzodiazepine receptors: a proposal; for a pharmacological classification as omega receptor subtypes // Pharmac. Biochem. Behav. 1988. - Vol. 29. - P. 763-766.

12. Lowry O.H., Rosenbrough N.J., Farr A.L., Randall R.J. Protein measurement with the Folia phenol reagent // J. Biol. Chem. - 1951. - Vol. 193. - P. 265-275.

13. Martini C., Lucacchini, Hrelia, S., Rossi R.J. Central- and peripheral-type benzodiazepine receptors // GABA-ergic transmission and anxiety / Eds. G. Biggio and E. Costa. - New York: Raven Press, 1986. - P. 1-11.

14. Medina J.H., Novas, M.L., De Robertis E. Changes in benzodiazepine receptors by acute stress: different effect of chronic diazepam or Ro 15-1788 treatment // Eur. J. Pharmacol. - 1983. - Vol. 96. - P. 181-185.

15. Miller L.G., Thompson M.L., Greenblatt D.J., Dentsh S.J., Shader R.J., Paul S.M. Rapid increase in brain benzodiazepine receptor binding following defeat stress in mice // Brain Res. - 1987. - Vol. 414. - P. 395-400.

16. Rägo L.K., Nurk A.M., Kornejev A.Ya., Allikmets L.H. Binding of phenibut with bicuculline insensitive GABA receptors in the rat brain // Bull. Exp. Biol. Med. - 1982. - Vol. 11. - P. 58-59.

17. Rägo L.K., Kiivet R.-A.K., Harro J.E., Allikmets L.H. Benzoduazepine binding sites in mice forebrain and kidneys: Evidence for similar regulation by GABA agonists // Pharmacol. Biochem. Behav. - 1986. - Vol. 14. - P. 1-3.

18. Rägo L.K., Kiivet R.-A.K., Allikmets L.H. Similar regulation of central and peripheral benzodiazepine binding sites by GABA agonists // Bull. Exp. Biol. Med. - 1987. - Vol. 12. - P. 685-687.

19. Saano V., Rägo L., Räty M. Perpiheral benzodiazepine binding sites // Pharmac. Ther. - 1989. - Vol. 41. - P. 503-514.

20. Squires R.F., Braestrup O. Benzodiazepine receptors in rat brain // Nature. - 1977. - Vol. 266. - P. 732-734.

21. Urban J.H., Van De Kar L.D., Lorenz S.A., Bethea C.L. Effect of the anxiolytic drug buspirone on prolactin and corticosterone secretion in stressed and unstressed rats // Pharmacol. Biochem. Behav. - 1986. - Vol. 25. - P. 457-462.

22. Weizman R., Tanne Z. Graneu M., Karp L., Golomb M., Tyano S., Gavish M. Peripheral benzodiazepine binding sites on platelet membranes are increased during diazepam treatment of anxious patients // Eur. J. Pharmac. - 1987. - Vol. 138. - P. 288-292.

PERIPHERAL-TYPE BENZODIAZEPINE BINDING SITES ON RAT BLOOD PLATELETS AND LYMPHOCYTES: EFFECT OF *IN VIVO* ADMINISTRATION OF GABA ERGIC DRUGS

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Abstract

In vitro GABA mimetics do not alter the binding characteristics of peripheral-type benzodiazepine (BZ) binding site ligands. The effect of *in vivo* administration of muscimol, dipropylacetate and phenibut on blood cell peripheral-type BZ binding sites in rat was examined. Intact blood platelets and lymphocytes, labelled either by ^{3}H-Ro 5-4864 or ^{3}H-Diazepam, were used in this study. Interestingly, not only muscimol, a $GABA_A$ receptors agonists, but also dipropyl-acetate and phenibut increased the affinity of BZ recognition sites on both types of blood cells studied. The number of BZ binding sites on blood cells was unaffected by in vivo pretreatment with the GABA-ergic drugs. The possible mechanism of in vivo modulation of peripheral-type BZ binding sites on blood cells by GABA-ergic drugs is discussed.

Introduction

The central-type benzodiazepine (BZ) binding site, often referred to as the BZ receptor, is an integral part of the $GABA_A$ receptor-chloride channel complex in the mammalian CNS [1]. Specific high affinity binding sites for benzodiazepines exist also in a variety of peripheral organs (adrenals, kidney, heart, lung etc) and on blood platelets [2], lymphocystes [3] and granulocytes [4]. It has become clear that the pharmacology and physiology of these two types of BZ binding sites is quite distinct (for review see 5).

Several lines of evidence demonstrating that classical $GABA_A$ receptor agonists enhance the affinity of benzodiazepines for central-type benzodiazepine recognition sites while diazepam increases the density of the low affinity GABA binding sites indicate the existence of coupling between $GABA_A$ and

benzodiazepine receptors [1]. In contrast to central-type BZ binding sites peripheral-type BZ binding sites are not modulated by GABA or chloride ions *in vitro* [6, 7]. However, recently we have found that the *in vivo* pretreatment of animals with $GABA_A$ and $GABA_B$ agonists is capable to modulate BZ binding sites in several peripheral organs as well [8, 9].

The aim of the present study was to examine the action of *in vivo* administration of several GABA mimetic drugs on blood cell peripheral-type BZ sites in rat. As the result of this study we present evidence indicating that BZ recognition sites on intact blood platelets and lymphocytes are regulated similarly to CNS BZ receptors by *in vivo* pretreatment with GABA-mimetic drugs.

Materials and methods

Animals and drugs. Male albino laboratory rats weighing 230-260 g of body weight (from Rappolovo Farm, Leningrad) were used. The animals were maintained on food and water ad libitum at 20 ± 1 °C on a reversed lighting cycle with lights off from 2000 to 0800. Phenibut (ß-phenyl-Gaba, Olaine Pharmaceuticals, Latvia), muscimol (Research Biochemicals Inc., Wayland, U.S.A.) and dipropylacetate (sodium valproate, Orion Pharmaceutica, Espoo, Finland) were dissolved in saline. The drugs were injected in a volume of 1 ml per kg intraperitoneally 50 min prior to decapitation.

***In vitro* bindings studies.** After decapitation the trunk blood of each rat was collected into a plastic tube containing 0.5 ml of acid citrate dextrose (ACD) anticoagulant. Lymphocytes were isolated according to the method of Boyum [10] using the Ficoll-Pague gradient and washed twice before the binding experiment. Platelets were obtained from platelet-rich plasma by centrifugation. The pooled blood cells from at least 8 animals were used for each binding assay. ^{3}H-Diazepam (spec. act. 82 Ci.mmol) and ^{3}H-Ro 5-4964 (spec. act. 84 Ci/mmol, both Amersham Radiochemicals, England) were used in eight different concentrations (0.5 - 34 nM and 0.5 - 24 nM respectively) for labelling peripheral-type BZ binding sites on blood platelets or lymphocytes. Unlabelled Ro 5-4864 (Hoffman - La Roche, Basel, Switzerland) was used to determine nonspecific binding. The binding experiments were performed in

a total volume of 125 µl during 30 min at 0 °C; all binding experiments were carried out in a modified Hanks solution (pH 7.3). Intact blood platelets and lymphocytes were used throughout these studies.

After incubation of the tubes containing either lymphocyte or platelet suspension the reaction was stopped by rapid filtration over Whatman GF/B filters. The filters were washed with 3 x 3 ml of ice cold buffer. Specific binding was calculated by subtracting the nonspecific from total binding at each given radioactivity concentration. Protein was measured by the Lowry et al. [11] method.

Calculations and statistics. Maximum binding (B_{max}) and affinity constants (KD) were calculated using the Scathard plot analysis. The Scathard plots were computed using linear regression program. For the data presented, only the plots with a correlation coefficent of 0.85 or more were accepted.

The significance of the differences between the results was tested using Student's t-test; $p < 0.05$ was considered significant.

Results

The effect of GABA agonists *in vitro* on peripheral-type BZ binding sites on intact blood platelets.

The binding studies were carried out in a modified Hanks Solution or TRIS-HCl (pH = 7.3) during 30 min at 0 °C or during 15 min at 37 °C. *In vitro* GABA (1 - 1000 µM), muscimol (0.1 - 500 µM) and phenibut (10 - 1000 µM) did not alter ^{3}H-Diazepam binding with intact blood platelets (data not shown).

The effect of *in vivo* administration of GABA agonists on ^{3}H-Diazepam and ^{3}H-Ro 5-4864 binding with intact blood platelets and lymphocytes.

In these studies muscimol (1.5 mg/kg), dipropylacetate (75 - 150 mg/kg) and phenibut 9100 mg/kg) were used in doses that were previously shown to act on ^{3}H-flunitrazepam binding in rat cerebral cortex and kidneys. The lower doses of these compounds did not produce statistically significant increase in the affinity for the ligand, neither in CNS nor in peripheral organs (data not shown, see also 14).

Muscimol (1.5 mg/kg) *in vivo* increased the affinity of ^{3}H-Ro 5-4864 binding both on platelets and lymphocytes (tables 1 and 2). Phenibut significantly increased only ^{3}H-Ro 5-4864 binding on blood platelets. Dipropylacetate, an indirect GABA agonist, increased the affinity of ^{3}H-Diazepam binding on blood platelets and lymphocytes similarly to muscimol (table 3).

Table 1

The effect of *in vivo* administration of muscimol (1.5 mg/kg), phenibut 100 mg/kg) and buspirone (5 mg/kg) on ^{3}H-Diazepam binding on intact blood platelets and lymphocytes in rats. The data expressed are ± S.E.M. of at least 3 indipendent experiments each carried out in triplicate.

Experimental group, mg/kg	^{3}H-Diazepam binding	
	B_{max} (fmol/mg prot.)	K_D (nm)
I. Platelete		
Saline	610 ± 80	14.8 ± 1.2
Muscimol 1.5	580 ± 60	12.1 ± 1.1
II. Lymphocytes		
Saline	510 ± 30	12.1 ± 0.9
Muscumol 1.5	440 ± 60	8.9 ± 0.8*
Saline	480 ± 40	16.3 ± 1.4
Phenibut	390 ± 80	19.1 ± 1.8

* $P < 0.05$ as compared to saline treated animals

Table 2

The effect of *in vivo* administration of muscimol (1.5 mg/kg), phenibut (100 mg/kg) and buspirone (5 mg/kg) on ^{3}H-Ro 5-4864 binding on intact blood platelets and lymphocytes in rats. The data expressed are mean ± S.E.M. of at least 3 independent experiments each carried out in triplicate.

Experimental group, mg/kg	^{3}H-Ro 5-4864 binding	
	B_{max} (fmol/mg prot.)	K_D (nM)
I. Platelet		
Saline	6870 ± 530	11.2 ± 0.8
Muscimol	5790 ± 810	7.5 ± 0.6*
Saline	6910 ± 720	10.7 ± 1.0
Phenibut	5030 ± 870	7.1 ± 0.8*
II. Lymphocytes		
Saline	4870 ± 660	9.4 ± 0.8
Muscimol	4250 ± 740	6.9 ± 1.1

* $P < 0.05$ as compared to saline treated animals

Table 3

The effect of *in vitro* administration of dipropylacetate (DPA; 150 mg/kg) on ^{3}H-Diazepam binding on intact blood platelets and lymphocytes in rats. The data expressed are mean ± S.E.M. of at least 3 indipendent experiments each carried out in triplicate.

Experimental group, mg/kg	^{3}H-Diazepam binding	
	B_{max} (fmol/mg prot.)	K_D (nM)
I. Platelete		
Saline	490 ± 70	16.3 ± 1.2
DPA 75	410 ± 110	13.4 ± 1.5
DPA 150	380 ± 60	10.7 ± 1.6*
II. Lymphocytes		
Saline	380 ± 50	13.4 ± 1.3
DPA 75	390 ± 80	12.3 ± 1.8
DPA 150	320 ± 40	9.1 ± 1.1*

* $P < 0.05$ as compared to saline treated control animals

Discussion

Phenibut, a structural analogue of baclofen, has been demonstrated do displace ^{3}H-GABA from $GABA_B$ binding sites and is considered to be a $GABA_B$ agonist [12]. Dipropylacetate (sodium valproate) raises the synaptic concentrations of GABA by slowing its metabolic degradation [13] and would thus be an indirect GABA agonist.

In our previous experiments it has been shown that muscimol, a $GABA_A$ receptor agonist, pretreatment significantly increased the affinity of BZ binding sites not only in cerebral cortex but also in kidneys. Similar changes were obtained also with (-)baclofen, a stereoselective $GABA_B$ agonist.

In the present study it was demonstrated that muscimol (1.5 mg/kg) increased the affinity for the ligand both on blood platelets and on lymphocytes. Phenibut (100 mg/kg) increased only the affinity of ^{3}H-Ro 5-4864 binding on platelets whereas dipropylacetate (from 150 mg/kg) effectively lowered the K_D values of ^{3}H-Diazepam binding in both cell types.

Accordings to these results it is possible to conclude that both $GABA_A$ and $GABA_B$ type agonists can increase *in vivo* the affinity of peripheral-type BZ binding sites on blood cells. This *in vivo* action of GABA agonists is difficult to explain. Several studies have revealed that there are no GABA binding sites, no GABA-ergic regulation, no barbiturate regulation and no ^{35}S-TBPS binding present in the purified peripheral-type DZ receptors [7]. However, such kind of experiments have not been actually carried out on blood cells. In spits of this our experiments showing that GABA and muscimol *in vitro* cannot modulate BZ ligand binding to its recognition sites on platelets asserts that the peripheral-type BZ binding sites on blood cells are not coupled to GABA receptor gated chloride channel. Although the problem of endogenous ligand for BZ receptors is still obscure, our present data seem to support the idea that there should be at least one common endogenous modulator (ligand?) for both types of BZ receptors. It has been shown that DBI (a neuropeptide with diazepam binding inhibiting properties) m RNA is expressed not only in neurons but also in many peripheral organs where peripheral-type BZ binding sites are present [15, 16]. Recently it was demonstrated that physiologically active porphyrines are very potent ligands for peripheral-type BZ binding sites in several species and organs [17]. However, the porphyrines are about 1000 times less potent at central-type BZ receptors [17].

On the other hand, if to consider that naive animals are always stressed by decapitation procedure [18] then the effect of GABA agonists on peripheral-type BZ binding sites may be a stress-protective action without any direct interaction with them. However, various stressful situations seem to change mainly BZ receptor density [19, 20] but in our studies GABA agonists increased the affinity.

In conclusion, the *in vivo* pretreatment of rats with GABA agonists increased the affinity of peripheral-type BZ binding sites on blood platelets and lymphocytes. The mechanism of this action of GABA agonists remains to be elucidated.

References

1. Haefely W. Allosteric modulation of neurotransmitter receptors by drugs // New Tests for New Drugs / Ed. S. Garattini. - Milano: Wichting Editore, 1987. - P. 23-48.

2. Moingeon P., Dessaux R., Fellous R., Alberici G.F., Bidart J.M., Motte P., Bohuon C. Benzodiazepine receptors on human blood platelets. // Life Sci. 1984.- Vol. 35, P. 2003-2009.

3. Moingeon P., Bidart J.M., Alberici G.F., Bohuon C. Characterization of a peripheral-type benzodiazepine binding site on human circulating lymphocytes // Eur. J. Pharmacol. - 1983. - Vol. 92. - P. 147-149.

4. Bound P.A., Cundall P.L., Rolfe B. ^{3}H-Diazepam binding to human granulocytes // Life Sci. - 1985. - Vol. 37. - P. 11-16.

5. Rägo L., Saano V., Räty M., Adojaan A. Peripheral-type benzodiazepine binding sites: functional receptors or acceptors // Acta et Commentationes Universitatis Tartuensis N° 766: Molecular Pharmacology / Ed. L. Allikmets. - Tartu, 1988. - Vol. 2. - P. 1-24.

6. Patel J., Marangos P.J. Differential effects of GABA on peripheral and central type benzodiazepine binding sites in brain // Neurosci. Lett. - 1982. - Vol. 30. - P. 157-160.

7. Martini C., Lucacchini A., Hrelia S., Rossi C.A. Central- and peripheral-type benzodiazepine receptors // GABA-ergic transmission and anxiety / Eds. G. Biggio and E. Costa. - New York: Raven Press, 1986. - P. 1-11.

8. Rägo L.K., Kiivet R.-A., Allikmets L.H. Similar regulation of central and peripheral benzodiazepine binding sites by GABA agonists // Bull. Exp. Biol. Med. - 1987. - Vol. 12. - P. 685-687.

9. Rägo, L.K., Kiivet R.-A., Harro J.E., Allikmets L.H. Benzodiazepine binding sites in mice forebrain and kidneys: Evidence for similar regulation by GABA agonists // Pharmacol. Biochem. Behav. - 1986. - Vol. 24. P. 1-3.

10. Boyum A. Isolation of mononuclear cells and granulocytes from human blood // Scand. J. Clin. Lab. Invest. - 1968. - Vol. 21. - P. 77-89.

11. Lowry O.H., Rosenbrough N.J., Farr A.L., Randall R.J. Protein measurement with the Folin phenol reagent // J. Biol. Chem. - 1951. - Vol. 193. - P. 265-275.

12. Rägo L.K., Nurk A.M., Korneyev Ya., Allikmets L.H. Binding of phenibut with bicuculline insensitive GABA receptors in the rat brain // Bull. Exp. Biol. Med. - 1982. - Vol. 11. - P. 58-59.

13. Harvej P.K.P. Some aspects of the neurochemistry of Epilin // Clinical and Pharmacological Aspects of Sodium Valproate (Epilim) in the Treatment of Epilepsy / Ed. N.J. Legg. - Tunbridge Wells: MCS Consultants, 1976. - P. 130-134.

14. Rägo L.K., Kiivet R.K., Allikmets L.H. Pharmacological and physiological regulation of central and peripheral benzodiazepine receptors // Mechanism of action of anxiolytic, anticonvulsant and hypnotic drugs / Ed. S.A. Andronati. - Kiev: Naukova Dumka, 1988. - P. 78-89 (in russ.).

15. Guidotti A., Forchetti G.M., Corda M.G., Konkel D., Bennet C.D., Costa E. Isolation, characterization and purification to homogenity of an endogenous polypeptide with agonistic action on benzodiazepine receptors. // Proc. Natl. Acad. Sci. USA. - 1983. - Vol. 80. - P. 3531-3535.

16. Costa E. Future trends of research in benzodiazepine-betacarboline-3-carboxylate ester recognition sites and their endogenous ligands // GABA-ergic transmission and anxiety / Eds. G. Biggio and E. Costa. - New York: Raven Press, 1986. - P. 239-242.

17. Verma A., Snyder S.H. Characterization of porphyrin interactions with peripheral type benzodiazepine receptors // Mol. Pharmacol. - 1988. - Vol. 34. - P. 800-805.

18. Biggio G. The action of stress, beta-carbolines, diazepam and Ro 15-1788 on GABA receptors in the rat brain // Benzodiazepine recognition site ligands: biochemistry and pharmacology / Eds. G. Biggio and E. Costa. - New York: Raven Press, 1983. - P. 105-119.

19. Medina J.H., Novans M.L. & Robert's E.De. Changes in benzodiazepine receptors by acute stress: different effect of chronic diazepam or Ro 15-1788 treatment // Eur. J. Pharmacol. - 1983. - Vol. 96. - P. 181-185.

20. Rägo L.K., Kiivet R.-A., Harro J., Põld M. Behavioral differences in an elevated plus-maze: correlation between anxiety and decreased number of GABA and benzodiazepine receptors in mouse cerebral cortex // Naunyn-Schmiedeberg's Arch. Pharmacol. - 1988. - Vol. 337. - P. 675-678.

THE ROLE OF VOLTAGE-SENSITIVE CALCIUM CHANNELS IN THE DEVELOPMENT OF ABSTINENCE AFTER CHRONIC DIAZEPAM TREATMENT

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Abstract

In experiments on male rats chronic diazepam (10 mg/kg/day; i.p., 30 days) treatment induced spontaneous abstinence 48-72 hours after termination of the drug treatment. Administration of benzodiazepine receptor antagonist CGS 8216 (5 mg/kg; i.p.) to diazepam withdrawn animals induced an additional increase in the severity of benzodiazepine symptoms. Calcium channel antagonists verapamil (10 mg/kg) and nifedipine (5 and 10 mg/kg) administered 30 min prior to CGS 8216 suppressed the signs of abstinence. In animals withdrawn from chronic diazepam decreased /^{3}H/ Nitrendipine binding and increased $^{45}Ca^{2+}$ uptake by depolarized synaptosomoses were observed. An increased synaptosomal $^{45}Ca^{2+}$ uptake was antagonized by *in vitro* addition of diazepam (1 μM) or verapamil (1 μM). In control animals these drugs did not change synaptosomal $^{45}Ca^{2+}$ uptake. It is proposed that chronic diazepam treatment induced the activation of voltage sensitive calcium channels and intraneuronal calcium accumulation which can be contributed to the development of abstinence signs.

Introduction

Because of very widespread use of benzodiazepines in medical practice, the dependence - producing liability of these drugs and the severity of symptoms associated with their withdrawal are the concerns of considerable importance. A number of withdrawal symptoms resulting from an abrupt cessation of benzodiazepines in laboratory animals have been reported [2, 3, 10, 12, 17, 18]. The abstinence syndrome in the laboratory animals chronically treated with benzodiazepines might also be induced by the administration of benzodiazepine receptor

antagonists Ro 15-1788 [13], or CGS 8216 [24, 25]. The extensive experimental evidences suggested that the structural basis of the action of benzodiazepines is represented by a hypothetical GABA-benzodiazepine-barbiturate-Cl ionophore receptor complex (for review see 5, 21]. Benzodiazepines interacting with benzodiazepine receptor enhance the affinity of GABA receptor which results in the opening of chloride channels and consequently, in an increase of the membrane conductance for chloride ions [5]. There is some evidences suggesting that the abnormalities of GBB complex develop in the course of chronic benzodiazepine treatment and these abnormalities might be contributed to the development of benzodiazepine withdrawal syndrome.

Thus, a decrease in a number of benzodiazepine receptors and diminution of the stimulatory effect of GABA benzodiazepine binding has been found in animals withdrawn from chronic benzodiazepine treatment [4, 16]. These changes were accompanied with the increased chloride conductance in response to GABA [23].

In addition, some evidence has suggested that alterations in calcium conductance might be involved in the action of benzodiazepines and consequently in the development of withdrawal syndrome after chronic benzodiazepine treatment. In our previous preliminary study, the organic calcium channel antagonists abolished the signs of benzodiazepine abstinence precipitated in rats by benzodiazepine receptor antagonist CGS 8216. The effect of acute benzodiazepines on Ca^{2+} flux in neuronal tissue remains, however, controversal. There have been reports that benzodiazepines enhance calcium entry into synaptosomal preparations and nifedipine blocked this effect [14]. Calcium channel abtagonists were also effective against abstinence induced by morphine and ethanol [1, 9]/ Chronic morphine treatment results in an increased [^{3}H]nitrendipine binding and accumulation of intrasynaptosomal Ca^{2+} [19, 22]. These observations promoted the further examination of the role of voltage sensitive calcium channels in the withdrawal syndrome following chronic benzodiazepine treatment. Also, the effects of calcium channel antagonists on the signs of benzodiazepine was further studied.

Materials and methods

Animals and drug treatment. Male Wistar rats with initial weight 200-230 g were housed in groups of 8-10 animals in standard vivarium conditions. The groups of animals were administered i.p. either diazepam (10 mg/kg) or 0.9 % NaCl saline once daily for 30 days. At various times after saline or diazepam withdrawal animals were sacrificed by the cervical dislocation and the brains were taken for the assessment of $^{45}Ca^{2+}$uptake or [^{3}H]nitrendipine binding. Remaining groups of animals were taken for behavioral study. The signs of precipitated abstinence were assessed.

Behavioral study. Animals chronically treated with diazepam were withdrawn for 48-72 hrs and then randomly divided into groups of 8-10 animals, one of which was administered i.p. benzodiazepine receptor antagonist CGS 8216 (2-phenylpyrazolo [4,3-c]quinolin-3(5H)-one, 5 mg/kg, i.p. or CGS 8216 in combination with calcium channel antagonists and the signs of abstinence were checked within 30 min after CGS 8216 administration. The most pronounced signs of abstinence were head twitches and myoclonic jerks of forepaws, therefore the number of these behavioral signs was counted within an observation period.

[^{3}H]Nitrendipine binding. The preparation of membranes and [^{3}H]nitrendipine binding were performed according to the method of Marangos et al. [11]. The rat forebrains were homogenized in 25 volumes (w/v) of 50 mM TRIS HCl buffer (pH = 7.4 at 25 °C). The resulting homogenate was centrifuged at 1000 g for 10 minutes. The supernatant was recentrifuged again at 30 000 g for 30 min. The resulting pellet was resuspended in 200 volumes (w/v) of TRIS HCl buffer. The aliquots of membrane preparation containing approximately 0.2 mg of membrane protein, were incubated in the dark with various concentrations of [^{3}H]nitrendipine (0.025 - 1.0 nM) for 90 min at 25 °C in a total volume of 1 ml. Nonspecific binding was determined using 1 μM nifedipine. Assays were terminated by vacuum filtration on Whatman GF/B filters with two 5 ml washes of ice cold buffer. The filters were counted in 8 ml of dioxine based scintillator with the counting effectiveness about 45 %.

$^{45}Ca^{2+}$ uptake by rat brain synaptosomes. Preparation of synaptosomes and $^{45}Ca^{2+}$ uptake were performed according to the method of Mendelson et al. (1984) with a slight modification. The cerebral cortices were dissected out and homogenized in 20 vol of ice-cold 0.32 M sucrose using a glass homogenizer with teflon pestle. All subsequent steps for isolation of synaptosomes were done at 0 - 4 °C. The homogenated was centrifuged at 1000 g for 10 min and the resulting supernatant was recentrifuged at 17 000 g for 20 min. This pellet (P_2) was resuspended in 0.32 M sucrose at 2-4 mg/ml of protein with teflon pestle. The aliquots (0.050 ml) of this synaptosomal suspension were added to the glass tubes containing 0.750 ml of buffer (136 mM NaCl, 5.0 mM KCl, 1.3 mM $MgCl_2$, 20 mM TRIS HCl, 11 mM glucose, pH = 7.4) and 0.1 ml of drug solutions or vehicle (NaCl buffer without glucose). The tubes were preincubated for 20 min on ice and then for 10 min at 37 °C in a shaking water bath. The uptake was initiated by adding 50 µM of $^{45}Ca^{2+}$ in 0.5 M of NaCl for non-depolarized samples and in 0.5 M of KCl for depolarized samples. Cold $CaCl_2$ (50 µM) was added consequently. The final $CaCl_2$ concentration was 100 µM. The incubation was continued for 60 sec. The uptake was terminated by rapid filtration through GF/B filters followed by four 4 ml washings with ice-cold solution containing 145 mM of KCl, 1.2 mM of $CaCl_2$ and 20 mM of TRIS HCl, pH = 7.4. All experiments were performed in triplicate. Filters were put in scientillation coctail and counted in a Beckman scintillation counter LS-6800.

Chemicals and drugs. [^{3}H]Nitrendipine (Psec. act. 81.1 Ci/mmol) was purchased from New England Nuclear plc., $^{45}CaCl_2$ (Spec.act. 0.9 Ci/mmol) from Isotop (USSR). Calcium channel antagonists: nifedipine and verapamil were generously provided by Orion Farmaceutica (Finland), diazepam - from La Roche (Basel, Switzerland), CGS 8216 was a gift from Sandoz Ltd. (Basel, Swutzerland). Nifedipine, diazepam and CGS 8216 were dissolved in saline containing a few drops of Tween-80. Verapamil was dissolved in 0.9 % NaCl solution.

Statistics. Statistical analysis was performed using Student's t-test for unpaired samples or Dunnett's test where appropriate.

Results

Effect of calcium channel blockers on CGS 8216 precipitated abstinence. Chronic treatment with diazepam (10 mg/kg) for 30 days followed by administration of CGS 8216 (5 mg/kg, i.p.) produced increased locomotor activity, head twitches, burst of myoclonic jerks of forepaws, increased emotional reactivity, poker tail, incidents of facial clonus. This behavioral syndrome developed in 5-10 min after CGS 8216 administration and lasted for 40 min. CGS 8216 when administered to control animals did not produce any behavioral abnormalities. Similar signs were seen in spontaneously withdrawn animals, however, they were less pronounced and a considerably lower number of animals exhibited those signs (data not shown). Since head twitches and myoclonic jerks of forepaws were the most prominent and could be easily measured quantitatively these signs were chosen for the assessment of the severity of abstinence in further experiments. Our previous studies [24, 25] have shown that CGS 8216 induced behavioral syndrome in diazepam withdrawn animals depended on the dose and duration of diazepam treatment, and was suppressed by the administration of diazepam.

The effect of calcium channels' antagonists of the signs of CGS 8216 precipitated abstinence is presented in table 1. When calcium channel antagonists verapamil (10 mg/kg) and nifedipine (5 mg/kg and 10 mg/kg) were administered 30 min prior to the CGS 8216 treatment, the drugs effectively suppressed the signs of abstinence in diazepam withdrawn animals (Table 1). Verapamil and nifedipine in similar doses were also effective in suppressing the signs of spontaneous abstinence (data not shown).

Table 1

The effect of calcium channel antagonists and agonists on the benzodiazepine abstinence precipitated by CGS 8216 (5 mg/kg) in rats. Drugs were administered i.p. 30 min before CGS 8216 administration. Data are means ± SEM.

Drug dose (mg/kg)	N	Abstinence signs	
		Number of	
		Head twitches	Myoclonic jerks
Control	8	6.30 ± 2.50	5.00 ± 0.8
Verapamil 10.0	8	0.12 ± 0.06**	0.43 ± 0.3**
Control	7	6.70 ± 1.70	4.00 ± 1.4
Nifedipine 5.0	8	2.90 ± 1.10	1.10 ± 0.5**
Control	13	8.20 ± 1.50	4.10 ± 1.0
Nifedipine 10.0	8	3.80 ± 0.70**	1.40 ± 0.5**

* - $P < 0.05$; ** - $P < 0.01$ (Dunnett's t-test).

[^{3}H]Nitrendipine binding in diazepam-withdrawn rats. [^{3}H]Nitrendipine bound with high affinity to membranes from rat forebrain. The saturation analysis in co-ordinates of Scatchard revealed only single population of binding sites with the density of 140 ± 20 fmol/mg protein. Chronic treatment with diazepam resulted in a decrease in the density of binding sites 48 hours after the termination of drug treatment, whereas binding affinity was not changed in comparison with control (fig. 1).

Calcium uptake in diazepam withdrawn rats. Acute or chronic afministration in a dose of 10 mg/kg i.p. did not induce any changes in $^{45}Ca^{2+}$ uptake in low K^+ medium (resting synaptosomes) or in high K^+ medium (depolarized synaptosomes) 1 hour after withdrawal. However, 48-72 hours after the termination of chronic diazepam treatment a significant increase in $^{45}Ca^{2+}$ uptake was observed (Table 2). This increase in $^{45}Ca^{2+}$ uptake returned to the control level 120 hours after the termination of chronic diazepam treatment.

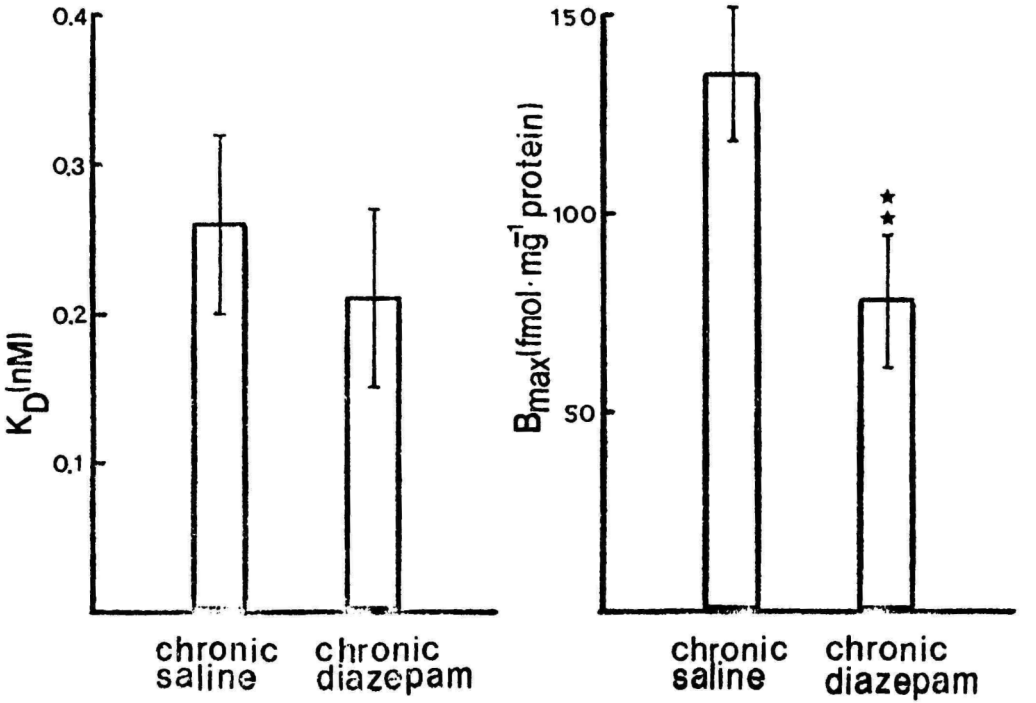

Fig. 1. [^{3}H]Nitrendipine binding to the membranes from the rat forebrain 48 hrs after chronic saline or diazepam (10 mg/kg/day; 30 days) treatment. The data are means ± SEM of 4 separate experiments. * - $P < 0.05$.

Table 2

$^{45}Ca^{2+}$ uptake by rat brain synaptosomes in control animals and animals withdrawn from chronic diazepam (10 mg/kg/day, 30 days) treatment. Results presented are means ± SEM.

	Time (hrs) after withdrawal	N	$^{45}Ca^{2+}$ uptake (nmol/mg protein/min)		
			5 mM K^+	55 mM K^+	Netto uptake
Control: chronic saline		12	0.94 ± 0.08	2.14 ± 0.16	1.21 ± 0.12
Acute diazepam	1	4	0.96 ± 0.22	1.96 ± 0.40	1.0 ± 0.18
Chronic diazepam	1	3	0.73 ± 0.12	1.74 ± 0.24	1.01 ± 0.16
	24	2	1.04 ± 0.07	2.49 ± 0.14	1.46 ± 0.08
	48	4	0.84 ± 0.12	2.56 ± 0.10*	1.72 ± 0.06*
	72	5	1.21 ± 0.17	2.95 ± 0.26*	1.74 ± 0.11*
	120	3	0.75 ± 0.17	1.96 ± 0.22	1.21 ± 0.12

* - $P < 0.05$ (Student's t-test).

Table 3

Effect of diazepam on $^{45}Ca^{2+}$ uptake by synaptosomes from the rat forebrain 72 hrs after termination of chronic saline or chronic diazepam (10 mg/kg/day, 30 days). Calcium uptake by rat brain synaptosomes was tested in low potassium medium (5 mM K^+) or in high potassium medium (55 mM K^+). Results represent means ± SEM.

Group, drug concentration	N	$^{45}Ca^{2+}$ uptake (nmol/mg protein/min)		
		5 mM K^+	55 mM K^+	Netto uptake
Chronic saline	12	0.94 ± 0.08	2.14 ± 0.16	1.21 ± 0.12
+ diazepam 1 μM	5	0.82 ± 0.07	1.91 ± 0.19	1.10 ± 0.12
10 μM	2	0.91 ± 0.27	2.13 ± 0.25	1.22 ± 0.17
Chronic Diazepam	5	1.21 ± 0.17	2.95 ± 0.26*	1.74 ± 0.11*
+ Diazepam 1 μM	2	1.14 ± 0.14	1.85 ± 0.11	0.7 ± 0.09*
10 μM	4	0.84 ± 0.12	1.48 ± 0.14	0.64 ± 0.07**

* - $P < 0.05$; ** $P < 0.01$ (Student's t-test).

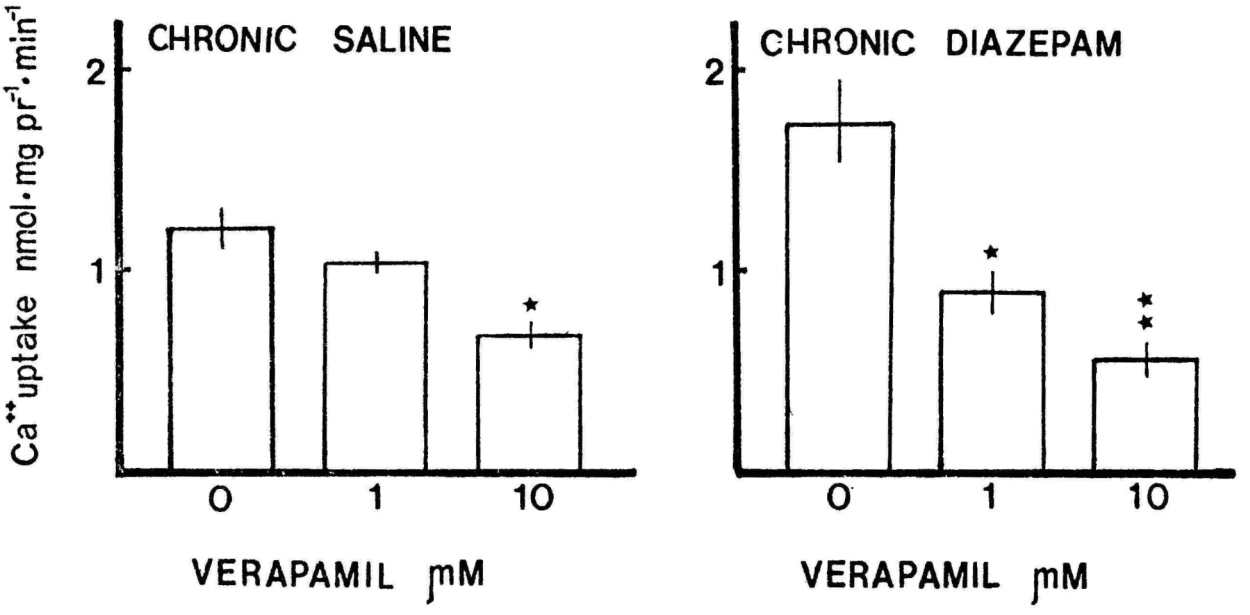

Fig. 2. Effect of verapamil on $^{45}Ca^{2+}$ uptake by depolarized synaptosomes (55 mM K^+) 72 hrs after chronic saline or diazepam (10 mg/kg/day; 30 days) treatment. The data are means ± SEM of three separate experiments. * - $P < 0.05$; ** - $P < 0.01$.

The diazepam added to the incubation medium containing depolarized synaptosomes in concentration 1 and 10 μM from the brains of diazepam withdrawn rats prevented the increase in $^{45}Ca^{2+}$ uptake, whereas it did not change $^{45}Ca^{2+}$ uptake by synaptosomes from control animals (Table 3).

Verapamil in concentration of 1 μM did not change $^{45}Ca^{2+}$ uptake by depolarized synaptosomes from control animals, but reduced an increased $^{45}Ca^{2+}$ uptake by synaptosomes from diazepam withdrawn rats (Fig. 2). Verapamil in a concentration of 10 μM decreased $^{45}Ca^{2+}$ uptake by synaptosomes from both control and diazepam-withdrawn animals.

Discussion

A number of studies have shown that the increase of Ca^{2+} influx via voltage sensitive calcium channels might be involved in the development of withdrawal syndrome after chronic morphine [19, 22] or chronic alcohol [9] treatment. Calcium channel antagonists were effective in suppressing the abstinence signs induced by chronic morphine or ethanol [1, 9].

The results of the present study also show that the calcium channel antagonists verapamil and nifedipine are effective in the suppression of the signs of diazepam-induced abstinence. Similarly, during withdrawal from chronic diazepam treatment an increased Ca^{2+} influx via voltage sensitive calcium channels was also observed. The increased influx of calcium was found within 48–72 hours which coincided with the peak of severity of the spontaneous or CGS 8216 precipitated abstinence. Diazepam and calcium channel antagonist verapamil added *in vitro* reduced an increased $^{45}Ca^{2+}$ uptake by synaptosomes from diazepam withdrawn animals. Taken together these data suggest that an increase in Ca^{2+} influx via voltage sensitive channels might be involved in the development of diazepam withdrawal syndrome. However, the increase in Ca^{2+} uptake in diazepam withdrawn animals was accompanied by the decreased of [^{3}H]nitrendipine binding. These dindings are difficult to explain since in the previous studies with morphine withdrawn animals an increased Ca^{2+} uptake by rat brain synaptosomes simultaneously with elevated [^{3}H]nitrendipine binding has been observed in these animals [19, 22].

Some studies, however, suggested that [^{3}H]nitrendipine binds only to the closed calcium channel.The activation of channel induced a rapid fall in the affinity of dihydropyridine site for [^{3}H]nitrendipine [6]. In our experiments, however, only a decrease in the density of dihydropyridine binding sites without any changes in affinity constant has been observed. It might be proposed that the observed decrease in the density of [^{3}H]nitrendipine binding sites might represent a compensatory reaction developing in response to an increased Ca^{2+} uptake by synaptosomes from the brain of diazepam withdrawn animals. In our experiments, diazepam added to incubation medium did not change $^{45}Ca^{2+}$ uptake by synaptosomes from control animals but, suppressed an increased $^{45}Ca^{2+}$ uptake by synaptosomes from diazepam withdrawn animals. These data indicate that chronic diazepam treatment may increase calcium channels sensitivity. This proposition is further supported by the fact that verapamil in relatively low concentration (1 μM) was able to suppress synaptosomal $^{45}Ca^{2+}$ uptake only in diazepam withdrawn animals. The accumulation of intrasynaptosomal calcium after diazepam withdrawal might alter many neuronal functions including the increase of intrasynaptosomal protein phosphorylation and amine release, which in turn might be involved in the appearance of the abstinence signs. In conclusion, our data suggest that benzodiazepine-induced abstinence may have similar mechanisms with morphine or alcohol induced abstinence and that an accumulation of intraneuronal calcium may also be contributed for the development of abstinence signs in diazepam withdrawn animals.

References

1. Bongiani F., Carla V., Morono F., Pellegrini-Giempieto D.E. Calcium channel inhibitors suppress the morphine withdrawal syndrome in rats // Br. J. Pharmacol. - 1986. - Vol. 88. - P. 561-567.

2. Emmlett-Oglesky M.W., Mathis D.A., Lal H. Diazepam tolerance and withdrawal assessed in an animal model of subjective drug effects // Drug Develop. Res. - 1987. - Vol. 11. - P. 145-156.

3. File S., Pellow S. Chlordiazepoxide enhances the anxiogenic action of CGS 8216 in the racial interaction test: evidence for benzodiazepine withdrawal? // Pharmacol. Biochem. Behav. - 1985. - Vol. 23. - P. 33-36.

4. Gallager D.W., Lahoski J.M., Gonsalves S.F., Rauch S.L. Chronic benzodiazepine treatment decreases postsynaptic GABA sensitivity // Nature. - 1984. - Vol. 308. - P. 74-77.

5. Haefely W. The biological basis of benzodiazepine actions // J. Psychoact. Drugs. - 1983. - Vol. 15, N. 1-2. - P. 19-39.

6. Hagiwara S., Byerly L. Calcium channel // Acta Rev. Neurosci. - 1981. - Vol. 4. - P. 69-125.

7. Harris R.A., Loh H.H., Way E.L. Effects of divalent cations, cation chelatots and an ionophore on morphine analgesia and tolerance // J. Pharmacol. Exp. Ther. - 1975. - Vol. 195. - P. 488-498.

8. Harris R.A., Yamamoto H., Loh H.H., Way E.L. Discrete changes in brain calcium with morphine analgesia tolerance-dependence and abstinence // Life Sci. - 1977. - Vol. 20. - P. 501-506.

9. Little H.J., Dolin S.J., Halsey M.J. Calcium channel antagonists decrease the ethanol withdrawal syndrome // Life Sci. - 1096. - Vol. 39. - P. 2059-2065.

10. Lockard J.S., Levy R.H., Congdon L.L., Du Charme L.L., Salonen L.D. Clonazepam in a focal-motor monkey model: efficacy, tolerance, toxicity, withdrawal and management // Epilepsia. - 1979. - Vol. 20. - P. 683-695.

11. Marangos P.J., Patel J., Miller C., Martino A. Specific calcium antagonist binding sites in brain // Life Sci. - 1982. - Vol. 31. - P. 1575-1585.

12. Martin W.R., McNicholas L.F., Cherian S. Diazepam and pentobarbital dependence in the rat // Life Sci. - 1982. - Vol. 31. - P. 721-730.

13. McNicholas L.F., Martin W.R. The effect of benzodiazepine antagonist, Ro 15-1788, in diazepam dependent rats // Life Sci. - 1982. - Vol. 31. - P. 731-737.

14. Mendelson W.B., Skolnic P., Martin J.V., Luu M.D., Wagner R., Paul S.M. Diazepam-stimulated increases in the synaptosomal uptake $^{45}Ca^{2+}$ reversal by dihydropyridine calcium channel antagonists // Eur. J. Pharmacol. - 1984. - Vol. 104. - P. 181-183.

15. Pellegrini-Giampieto D.E., Bacciottini L., Carla V., Moroni F. Morphine withdrawal in cortical slices suppression by Ca^{2+} channel inhibitors of abstinence induced [^{3}H]noradrenaline release // Br. J. Pharmacol. - 1988. - Vol. 93. - P. 535-540.

16. Rosenberg H.C., Chiu T.H. Decreased [^{3}H]diazepam binding is a specific response to chronic benzodiazepine treatment // Life Sci. - 1979. - Vol. 24. - P. 803-808.

17. Rosenberg H.C., Chiu T.H. Time course for the development of benzodiazepine tolerance and physical dependence // Neurosci. Behav. Rev. - 1985. - Vol. 9. - P. 123-131.

18. Ryan G.P., Boisse N.R. Experimental induction of benzodiazepine tolerance and physical dependence // J. Pharmacol. Exp. Ther. - 1983. - Vol. 226, N 1. - P. 100-107.

19. Saito K., Ishii K., Fujita N., Nahahiro M., Inoki R. Selective enhancement in striatal [^{3}H]nitrendipine binding following chronic treatment with morphine // Neurochem. Int. - 1985. - Vol. 7, N. 106. - P. 1033-1036.

20. Schramur M., Towart R. Modulation of calcium channel function by drugs // Life Sci. - 1985. - Vol. 37. - P. 1843-1860.

21. Tallman J.F., Gallager D.W. The GABA-ergic system: a locus of benzodiazepine actions // Ann. Rev. Neurosci. - 1985. - Vol. 8. - P. 21-44.

22. Yamamato H., Harris R.A., Loh H.H., Way E.L. Effect of acute and chonic treatments on calcium localization and binding in brain // J. Pharmacol. Exp. Ther. - 1978. - Vol. 205. - P. 255-264.

23. Yu O., Chiu T.H., Rosenberg H.C. Modulation of GABA-gated chloride ion flux in rat brain by acute and chronic benzodiazepine administration // J. Exp. Pharmacol. - 1988. - Vol. 246. - P. 107-113.

24. Zharkovsky A.M., Zharkovsky T.A. Antiabstinence effect of phenibut and baclofen on the abstinence induced by benzodiazepine receptor antagonist CGA 8216 in rats chronically treated with diazepam // Bull. Exp. Biol. Med. (Russ.) - 1987a. - N. 6. - P. 627-678.

25. Zharkovsky A.M., Zharkovsky T.A. Abstinence syndrom induced by benzodiazepine receptor antagonist CGS 8216 in rats after chronic diazepam treatment // Bull. Exp. Biol. Med. - 1987b. - N. 9. - P. 318-321.

CALCIUM CHANNEL ANTAGONISTS INDUCED YAWNING BEHAVIOUR IN RATS

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Abstract

Calcium channel antagonist, of various chemical structure nifedipine, flunnarizine, verapamil and diltiazem after systemic administration dose dependently induced yawning behaviour in rats. The rank order potency for these drugs = flunnarizine > verapamil = diltiazem. This effect seems mediated centrally, since flunarizine and verapamil induced yawning after intracerebroventricular as well as after systemic administrations. Combined treatment of calcium channel antagonists with dopamine receptor agonists apomorphine and quinpirole or muscarinic cholinoceptor agonist pilocarpine resulted in an enhancement of yawning behaviour. Dopamine D1 and D2 receptor subtypes' antagonists sulpiride, haloperidol and SCH 23 390 or muscarinic cholinoceptor antagonist scopolamine dose dependetly inhibited nifedipine-induced yawning. Also, phosphodiesterase inhibitor milrinone supressed nifedipineinduced yawning. It is proposed that calcium flux via voltage gated channels may be involved in the expression of yawning behaviour.

Introduction

Organic calcium channel antagonists represent a large number of compounds of various chemical structure affecting calcium movement across membranes via voltage dependent calcium channels. The pharmacological actions of these drugs on the cardiovascular system are extensively studied since they have beneficial effects in the treatment of several cardiovascular disorders. However, less is know about central effects of calcium channel antagonists. Recently, specific calcium channel antagonists binding sites have been shown to be present in the central nervous system [5, 18, 23].

The functional sequences of these sites occupation remained unknown because in neuronal tissues it has been difficult to demonstrate the functional effect of organic calcium channel antagonists, since in pharmacologically meaningful concentrations these drugs have no marked effect on the calcium mediated processes [21, 29]. However, recent behavioral studies on laboratory animals have shown, that calcium channel antagonists may possess some psychopharmacological actions independently of their cardiovascular effects. It has been reported that dihydropyridines reduced immobility time in the mouse behavioral dispair test [20], antagonized reserpine induced catalepsy [11], prevented the behavioral manifestations of the morphine abstinence in rat [1, 30] and benzodiazepine abstinence in rat [30]. In addition to that, some calcium channel blockers possessed anticonvulsive activities in several models of epilepsy [3, 28, 19].

In the present study we report that calcium channel antagonists in addition to their known psychopharmacological effects induce yawning behaviour in rats. Since yawning is a readily quantified behavioral response which can be induced by various agents including dopamine receptor agonists and muscarinic cholinceptor agonists the effect of drugs interacting with these receptors on calcium channel antagonists induced yawning was studied.

Materials and method

Animals. Male Wistar rats weighing 250-300 g were used. They were housed 8 per cage in a well illuminated room at an ambient temperature of 21-22 °C and kept on a standard diet and tap water ad libitum. All experiments were carried out between 3 p.m. and 8 p.m. in a diffiusely illuminated room.

Behavioral testing. Testing was conducted in the individual plexiglaas boxes of 20 x 20 x 20 cm. The animals were habituated to test cages for 30 min prior to drug injection. The number of yawning episodes was counted during a 60 min test using hand held counter.

Intracerebroventricular injections. Rats were anesthetized with ethyl ether. After incision of the skin of the head, a hole was made in the skull at the following coordinates: L = 1.5 mm, 2 mm

posterior to bregma. Six hours later, intracerebroventricular injections (25 µl) were made freehand in the right lateral ventricle with a microsyringe connected to a needle whose medial part of the bevel protruded only 5 mm from a guard limiting its penetration into the brain.

Drugs. The drugs used were: nifedipine, flunnarizine HCl, verapamil HCl and diltiazem HCl (all were generously donated by Orion Farmaceutica, Finland), milrirone was a gift of the Institute of Organic Syntheris (Riga, USSR). quinpirole HCl (Ely Lilly, Indianopolis, USA), apomorphine HCl, pilocarpine HCl (USSR), 2-(+)-8-chloro-2,3,4,5-tetrahydro-3-methyl-5-phenyl-1H-3-benzazepine-7-ol hemimaleate (SCH 23 390; Schering, New Jersey, USA), sulpiride (Commercial Ampoules, de Lagrange, Paris, France), haloperidol (Janssen Farmaceutica, Beerse, Belgium), scopolamine (USSR).

Nifedipine, flunnarizine, diltiazem and SCH 23 390 were suspended in solution containing 1% of Tween-80, haloperidol was dissolved in a minimal volume of glacial acetic acid and the solution was then brought to pH = 5.0 with 0.1 N NaOH and finally diluted with 0.9% NaCl solution. Apomorphine and quinpirole were dissolved in 0.9% NaCl solution containing 0.05% ascorbic acid to prevent oxydation. All other drugs were dissolved in 0.9% NaCl solution. All drug solutions and suspensions were prepared freshly before the experiments. Apomorphine and quinpirole were administered subcutaneously. All other drugs - intraperitoneally.

Statistical analysis. Individual group differences were determined by appropriate Dunnett's t-test. A probability of $P < 0.05$ was regarded as significant.

Results

Effect of clacium channel antagonists after i.p. administration. Calcium channel blockers of various chemical structure: nifedipine, flunnarizine, verapamil, diltiazem after i.p. administration to rats elicited yawning behaviour (Fig. 1). This effect was dosedependent, with maximalum effect at the dose of 10^{-20} mg/kg. However, the dose response curves were relatively shallow. No further increase in the

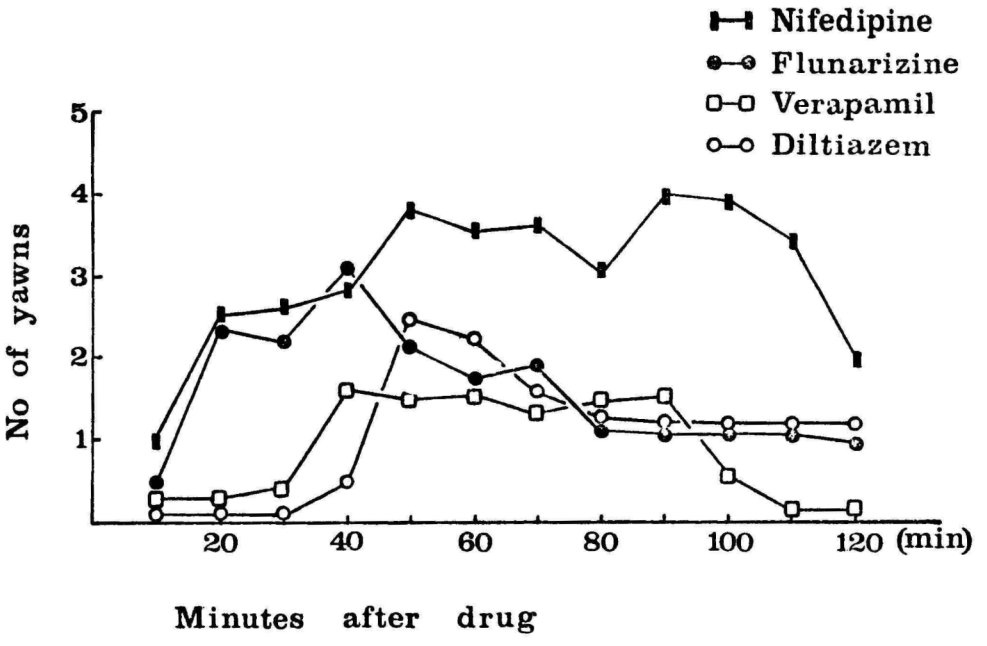

Fig. 1. Dose responses of yawning induced by calcium channel antagonists after their i.p. administration. Yawns were counted for 60 min and 10 min after nifedipine and flunnarizine. Each column represents mean ± SEM of 8 animals. * - $P < 0.05$; ** $P < 0.01$ (Dunnett's test).

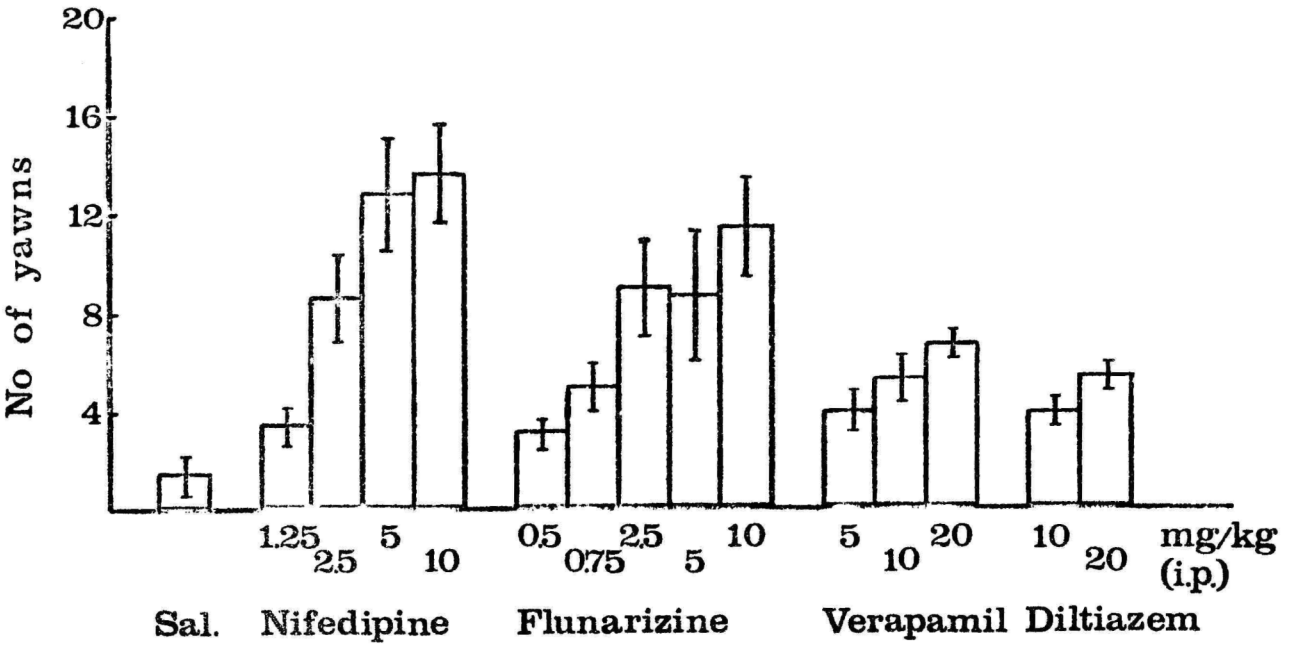

Fig. 2. Time course of yawning elicited by calcium channel antagonists after their i.p. administration. Each point is a mean number of yawns per 10 min (SE omitted for clarity) of 8-10 animals.

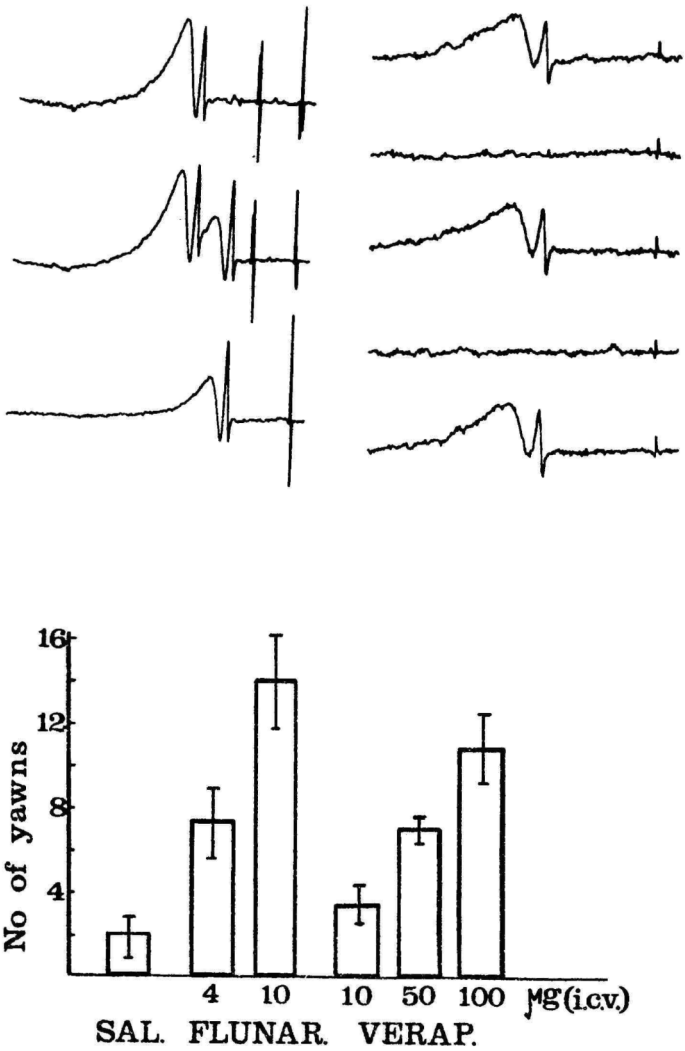

Fig. 3. Incidence of yawning in rats after intracerebroventricular (i.c.v.) injection of flunnarizine and verapamil. Each column represents mean ± SEM of 8 animals. * - $P < 0.05$; ** - $P < 0.01$ as compared to saline injected group.

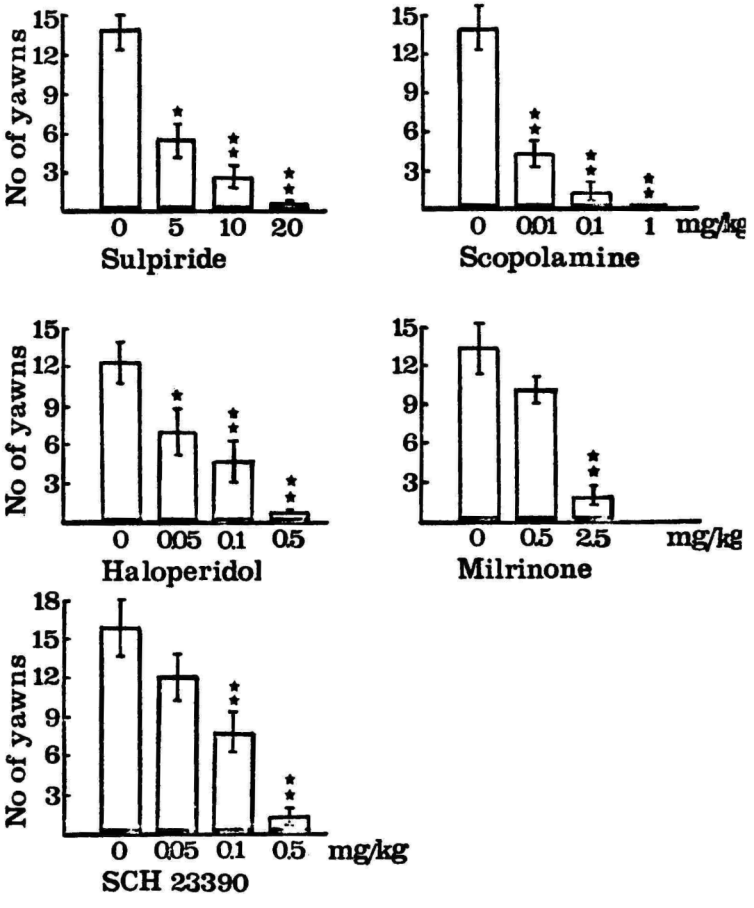

Fig. 4. Effect of selected drugs on nifedipine (10 mg/kg i.p.) induced yawning. Each column represent mean ± SEM of 8-10 animals. * - P < 0.05; ** - P < 0.01 (Dunnett's test).

Table 1

Effect of Combined treatment of dopamine receptor agonists and muscarinic cholinoceptor agonist pilocarpine with calcium channel antagonists on yawning behaviour in rats. Data are means ± SEM of groups from 8-10 animals.

Drug, dose (mg/kg)	Number of yawns in 60 min		
	Apomorphine (0.1 mg/kg)	Quinpirole (0.01 mg/kg)	Pilocarpine (1.0 mg/kg)
Control: saline	12.5 ± 0.5	11.0 ± 2.0	15.0 ± 3.6
Nifedipine 10.0	38.0±3.4**	36.0±6.0**	27.5 ± 5.0*
Flunnarizine 10.0	27.0 ± 3.1*	NT	23.8 ± 4.6*
Verapamil 10.0	21.5 ± 2.0*	14.0 ± 5.0	NT
Diltiazem 10.0	22.4 ± 4.1*	NT	16.0 ± 4.0

* - $P < 0.05$: ** - $P < 0.01$ (Dunnett's test). NT - not tested.

Milrinone which is known as potent adenosine$_1$, receptor antagonist and adenylate cyclase activator and phospodiesterase activity inhibitor [22] inhibited yawning.

Discussion

The results obtained in the present study indicate that calcium channel antagonists of various chemical ctructure and somewhat different mechanism of action at calcium channel elicit yawning behaviour in rats. This effect of calcium channel antagonists seems to be mediated centrally since this effect could be induced after intracerebroventricular as well as after intraperitoneal administration of these drugs. Among the calcium channel antagonists used in the

present study nifedipine and flunnarizine were the most potent whereas verapamil and diltiazem produced a considerably weaker effect on yawning behaviour.

These data are consistent with those obtained by the other authors who showed that flunnarizine and dihydropyridines were more powerful against sound induced convulsions in mice than diltiazem or verapamil [2].

Binding studies revealed that dihydropyridines and flunarizine are potent competitive displacers of [^{3}H]nitrendipine from its binding sites, whereas verapamil and diltiazem affect [^{3}H]nitrendipine binding via an allosteric mechanism [5, 18, 7, 23]. On the basis of these findings it may be proposed that dihydropyridine binding sites may have a more important role in the regulation of yawning behaviour.

Also, differences in the pharmacokinetics of various calcium channel antagonists should probably be taken into consideration. Thus, dihydropyridines more readily penetrate blood-brains barrier than verapamil [14, 24] that also may contribute to their properties of inducing more intense yawning behaviour.

A number of investigations have pointed out the involvement of dopamine receptors and muscarinic acetylcholine receptors in the regulation of yawning [27, 12, 10]. In confirmation to the previous studies dopamine D2 receptor agonist quinpirole or mixed D1/D2 receptor agonist apomorfine and muscarinic cholinoceptor agonist pilocarpine induced intence yawning in rats. The combined treatment of nifedipine with quinpirole, apomorfine or pilocarpine resulted in an enhancement of yawning. This enhancement, however, was additive rather than potentiating. The D2 receptor antagonist sulpiride and haloperidol as well as muscarinic cholinoceptor antagonist scopolamine were highly effective against nifedipine-induced yawnings. These data indicate the existence of interaction between dihydropyridine binding sites and dopamine and muscarine receptors in the regulation of yawning behaviour. Some recent evidence suggested the existence of direct interaction of calcium channel antagonists with dopaminergic and cholinergic systems. Organic calcium channel antagonists inhibit the specific binding of [^{3}H]spiperone to D2 receptor [4]. Similarly, muscarinic receptor binding in the channel also inhibited by calcium [6]. However, it is unlikely that direct interaction of calcium channel antagonists

with these receptors is contributed to the appearance of yawnings, because the most powerful drugs in induction of yawning nifedipine and flunnarizine were rather weak or ineffective inhibitors of binding to dopamine or muscarinic receptors. In contrast, verapamil potently inhibited binding but possessed a rather weak ability to induce yawning.

The previous studies have shown that dopamine receptor agonist quinpirole in nanomolar concentrations is able to decrease the level of intraneuronal free calcium concentration [9]. Although calcium channel antagonists are considered to be rather weak inhibitors of calcium uptake by brain synaptosomes [21, 29], some recent evidence suggest that in certain cell lines such as cerebellar granule cells, there may be powerful inhibitors of calcium uptake [15]. It appears that a reduction of intraneuronal free calcium concentration induced via different mechanisms might be an important factor contributing to the induction of yawning. Among the various intraneuronal processes mediated by calcium a particular attention has been paid to the regulation of adenylate cyclase activity [8]. Activation of adenylate cyclase activity by calcium might lead to an accumulation of cyclic adenosine monophosphate. In our previous study D1 receptor agonist SKF 38 393 which induces an increase of dopamine sensitive adenylate cyclase activity [25], had an inhibitory effect on the yawning induced by apomorphine [31]. In this study milrinone which is known to inhibit phosphodiesterase activity and thereby induces an increase in cyclic adenosine monophosphate level [22] possessed also an inhibitory action against nifedipine-induced yawning. Muscarinic cholinoreceptor agonists and dopamine D2 receptor agonists also inhibit adenylate cyclase activity [13, 26]. It is not excluded that the cyclic adenosine monophosphate level may be an important factor involved in the induction of yawnings. However this proposition remains highly speculative and needs further investigation. In conclusion the data of our experiment, indicate that the calcium channel antagonist-induced yawning may represent a convenient tool for studying psychotropic activity of these drugs.

References

1. Bongianni F., Carla V., Maoni F., Pellegrini-Giampietro D.E. Calcium channel inhibitors suppress the morphine withdrawal syndrome in rats. // Br. J. Pharmacol. - 1986. - Vol. 88. - P. 561-657.

2. De Sarro G.B., Meldrum B.S., Nistico G. Anticonvulsant effects of some calcium entry blockers in DBA 2 mice. // Br. J. Pharmacol. - 1988. - Vol. 93. - P. 247-256.

3. Desmendt L.K.C., Niemegeers C.I.E., Janssen P.A.J. Anticonvulsive properties of cinnarizine and flunnarizine in rats and mice // Arzneimittel-Forsch. - 1975. - Bd. 25. - S. 1408-1413.

4. DeVries D., Beart P.M. Competitive inhibition of [^{3}H]spiperone binding to D2 dopamine receptors in striatal homogenates by organic channel antagonists and polyvalent cations // Eur. J. Pharmacol. - 1985. - Vol. 106. - P. 133-139.

5. Ehlert F.J., Itoga E., Roeske W.R., Yamamura H.I. The interaction of [^{3}H]nitrendipine with receptors for calcium antagonists in the cerebral cortex and heart of rats // Biochem. Biophys. Res. Commun. - 1982. - Vol. 104. - P. 937-943.

6. Fairhurst A.S., Whittaher M.L., Ehlert F.J. Interactions of D600 (methoxyverapamil) and local anesteticswith rat brain α-adrenergic and muscarinic receptors // Biochem. Pharmacol. - 1980. - Vol. 29. - P. 155-157.

7. Ferry D.R., Glossman H. Evidence for multiple receptor sites within the putative calcium channel // Naunyn-Schmiedeberg's Arc. Pharmacol. - 1982. - Vol. 321. - P. 80-83.

8. Forn J. Integrated actions of cyclic nucleotides, calcium and protein phosphorilation in the nervous system // Adv. in Syclic Nucleotide and Protein Phosphorylation Research / Ed. P. Greengard. - New York: Raven Press, 1984. - Vol. 17. - P. 473-482.

9. Fujiwara H., Kato N., Shuntzoh H., Tenaka C. D_2-dopamine receptor-mediated inhibition of interacellular Ca^{2+} mobilization and release of

acetylcholine from quinea-pig neostriatal slices // Br. J. Pharmacol. - 1987. - Vol. 91. - P. 287-297.

10. Gower A.J., Berendson H.H.G., Princen M.M., Broekkamp C.L.E. The yawning and penile erection syndrome as a model for putative dopamine autoreceptor acitivity // Eur. J. Pharmacol. - 1984. - Vol. 103. - P. 81-89.

11. Hoffmeister F., Benz U., Heise A., Krause H.P., Nenser V. Behavioural effects of nimodipine in animals // Drug Res. - 1982. - Vol. 32 (I). - N. 4. - P. 347-360.

12. Holmgren B., Urba-Holmgren R. Interactions of cholinergic and dopaminergic influences on yawning behaviour // Acta Neurobiol. Exp. - 1980. - Vol. 40. - P. 633-642.

13. Hess W., Ellis J. Muscarinic receptor subtypes in the central nervous system // Int. Rev. Neurobiol. - 1985. - Vol. 26. - P. 151-199.

14. Janicki P.K., Siembab D., Paulo E.A., Krasci K.P. Single-dose kinetics of nifedipine in rats plasma and brain // Pharmacology. - 1988. - Vol. 36. - P. 183-187.

15. Kingsbury A., Balazs R. Effect of calcium agonists and antagonists on cerebellar granule cells // Eur. J. Pharmacol. - 1987. - Vol. 140. - P. 275-283.

16. Kohlhardt M., Fleckenstein A. Inhibition of the slow inward current by nifedipine in mammalian ventricular myocardium // Naunyn. Schmiedeberg's Arch Pharmacol. - 1977. - Vol. 298. - P. 267-272.

17. Little H.J., Dolin S.J., Halsey M.J. Calcium channel antagonists decrease the ethanol withdrawal syndrome // Life Sci. - 1986. - Vol. 39. - P. 2059-2065.

18. Marangos P.J., Patel J., Miller C., Martino A.M. Specific calcium antagonist binding sites in brain // Life Sci. - 1982. - Vol. 31. - P. 1575-1585.

19. Meyer F.B., Tally P.W., Anderson R.E., Syndt T.M.Jr., Yaksh T.L., Sharbrough F.W. Inhibitions of electrically induced seizures by dihydropyridine calcium channel blocker // Brain Res. - 1986. - Vol. 384. - P. 180-183.

20. Mogilinska E., Czyrak A., Maj J. Dihydropyridine calcium channel antagonists reduce immobility in the mouse behavioural despair test; antidepressants facilitate nifedipine action // Eur. J. Pharmacol. - 1987. - Vol. 138. - P. 413-416.

21. Nachshen D.A., Blaustein M.P. The effect of some organic "calcium antagonists" on calcium influx in presynaptic nerve terminals // Molec. Pharmacol. - 1979. - Vol. 16. - P. 579-586.

22. Paton D.M., Mannel J.M. Mechanisms of action of the new cardiotonic drugs // TIPS. - 1988. - Vol. 9. - P. 431-432.

23. Peroutka S.J., Allen G.S. Calcium channel antagonist binding sites labelled by [^{3}H]nimodipine in human brain // J. Neu. - 1983. - Vol. 59. - P. 933-937.

24. Schoemaker H., Lee H.R., Roeske W.R., Yamamura H. *In vivo* identification of calcium antagonist binding sites using [^{3}H]nitrendipine // Eur. J. Pharmacol. - 1983. - Vol. 88. - P. 275-276.

25. Setler P., Sarau H., Zerkle C.L., Saunders H.L. The central effect of a novel dopamine agonist // Eur. J. Pharmacol. - Vol. 50. - P.413-430.

26. Stoof J.C., Kebabian J.W. Opposing roles of D-1 and D-2 receptors in efflux of cyclic AMP from rat neostriatum // Nature. - 1981. - Vol. 294. - P. 366-368.

27. Urba-Holmgren R., Gonzales R.M., Holmgren B. Is yawning a cholinergic response? // Nature. - 1977. - Vol. 267. - P. 261-262.

28. Vezzani A., Wu U.Q., Stasi M.A., Angelico P., Samarin R. Effect of various calcium channel blockers on three different models of limbic seizures in rats // Neuropharmacology. - 1988. - Vol. 27. - P. 451-458.

29. Wei J.-W., Chiang D.-H. Studies of [^{3}H]nitrendipine binding and KCl-induced calcium uptake in rat cortical synaptosomes // Gen. Pharmacol. - 1985. - Vol. 16. - N. 3. - P. 211-216.

30. Zharkovsky A.M., Zharkovsky T.A. $GABA_B$ receptor agonists and calcium channel antagonists abolish signs of benzodiazepine and morphine abstinence in rats // I Meeting of Baltic Pharmacologists: Abstracts. - 1988. - P. 28.

31. Zharkovsky A.M., Cereska K.S. Effect of the D1 receptor agonist SKF 38 393 on some behavioural effects of apomorphine in rats // Naunyn-Schmiedeberg's Arch Pharmacol. - 1989. - Vol. 339. - P. 383-386.

SOME CHARACTERISTIC FEATURES OF THE MECHANISM OF HYPOTENSIVE ACTION OF ANGIOTENSIN II AND BRADYKININ CYCLOANALOGUES

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Abstract

Modified cyclic analogues of the peptide angiotensin II and bradykinin, unlike the natural linear precursors characterized by short-term hypertension and short-term hypotension, respectively, exert a long-term dose-dependent hypotensive action in nembutal anaesthetized rats upon intravenous administration. Our results suggest that histamine, serotonin and other endogenous vasoactive substances released from mast cells provide the hypotensive action of these analogues.

Introduction

Total semi-empirical conformational calculations carried out for many oligopeptides hawe shown the presence of energetically preferable quasi-cyclic structures stabilised by electrostatic interactions between ionic molecular groups [5]. It has been proposed that the synthesis of cyclic analogues with limited conformational mobility may open up new vistas in designing drugs with enhanced affinity to the receptor [5].

A pharmacological study of the newly synthesized modified cyclopeptides (angiotensin II and bradykinin) revealed the appearance of new properties of these compounds as compared to their linear precursors. Thus, the cyclic analogue of angiotensin II and the cyclic analogue of bradykinin exert a similar long-lasting dose-dependent hypotensive action in anaesthetized rats, whereas the natural linear kinins - angiotensin II is a typical short-term hypertensive agent but bradykinin induces a short-term hypotensive effect [1, 8, 9]. Previous studies have shown that the influence of angiotensin II and bradykinin cycloanalogues on blood pressure

is not realized through angiotensin II or bradykinin receptors [4, 11]. It is noteworthy that prior administration of $CaCl_2$ (100 mg/kg^{-1}) considerably reduced the duration of the hypotensive effect caused by the cyclic peptides [2, 4]. Nevertheless, the mechanism of hypotensive action on these cyclopeptides remains obscure.

The recent studies of cyclic analogues of bradykinin and kallidin demonstrating involvement of histamine in several vascular permeability models of inflammatory responses [17] lead infer that the long-lasting hypotensive effect of these cycloanalogues may be realized via the liberation of endogenous vasoactive substances from depot cells. This type of mechanism has been established at least for the hypotensive action of vasoactive peptide neurotensin [15].

Evidence gained in various laboratories indicates that histamine and serotonin appear to be involved in other biological manifestations of neurotensin in rat, e.g. myotropic effect, vasodilatation and increase in vascular permeability [7, 12, 15].

The present study was conducted to determine the role of histamine and serotonin in the hypotensive effect of angiotensin II (cyclo-AT) and bradykinin (cyclo-BK) cycloanalogues:

Asp-Arg-Val-Tyr-Val-His-Pro-Phe angiotensin II
Lys-Ala-Val-Tyr-Ile-His-Pro-Phe-Arg cyclo-AT
Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg bradykinin
Lys-Pro-Pro-Gly-Phe-Gly-Pro-Phe-Arg cyclo-BK

Methods

The effect of cyclo-AT (2,10 μg/kg^{-1}) and cyclo-BK (10, 50 μg/kg^{-1}) on mean arterial pressure upon intravenous administration was measured in randomly-bred rats of both sexes weighing 180-220 g. Nembutal 40 mg/kg^{-1}) injected intraperitoneally was used for anaesthesia. Corotid arterial pressure was registrered with the aid of a Bentley Trantec Physiological Pressure Transducer and a Gemini 2-channel recorder (Italy). Peptides dissolved in 0,9 % NaCl were injected into the femoral vein (0,1 ml/200 g of body weight). Intal (disodium cromoglycate, 10 mg/kg^{-1}), stabilizer of mast cell membranes; diphenhydramine (5 mg/kg) - H_1-receptor antagoginst; cimetidine (10 mg/kg^{-1}) - H_2-receptor

antagonist and methysergide (200 $\mu g/kg^{-1}$) - serotonin receptor antagonist were injected intravenously 5-10 min. prior to cyclopeptide administration. Parachlorophenylalanine, a serotonin synthesis inhibitor (400 mg/kg^{-1}), used as a suspension with TWEEN-80, was injected intraperitoneally 24 hours prior to cyclo-BK administration.

The histamine liberation assay was performed as described [10], using peritoneal mast cells from female rats (200-300 g). Histamine content was assayed fluorometrically [13]. Intal was added in the incubation media simultaneously with cyclo-BK.

The following drugs were used: serotonin-creatinin sulfat (Reneal), histamine diphosphate (Fluka), methysergide maleate (Sandoz), diphenhydramine (Sigma), cimetidine (Sigma), Intal (dinatrium cromoglycate)(Leck).

The measured values are means ± SEM. Comparison of treatment effects was made by Student's test; $p < 0.05$.

Results and Discussion

According to our hypothesis postulating that histamine and serotonin belong to factors responsible for the hypotensive action of cyclic kinins, we blocked the histamine component of arterial pressure regulation by pretreament with the H_1-, H_2-antagonists diphenhydramine and cimetidine, respectivly. The serotonin component was examined using the classical antagonist methysergide and the serotonin synthesis inhibitor parachlorophenylalanine.

As can be seen from Table I, the H_1-antagonist diphenhydramine (5 mg/kg^{-1}) diminished the hypotensive effect of cyclo-BK (10 $\mu g/kg^{-1}$) by 32 %, thus indicating with reasonable plausibility the histaminergic portion of the hypotensive action. The H_2-antagonist cimetidine failed to influence the cyclo-BK action. Cimetidine also failed to alter the effect of diphenhydramine (Table 1). Similar characteristics are also typical for exogenous histamine (Table 1) and the results obtained are in good agreement with the literature data suggesting the prevailing participation of H_1-receptors in histamine-induced hypotensive avtion [16]. Attempts to block the hypotensive effect of cyclo-BK by using the classical serotonin antagonist methysergide were

Table 1

Comparative influence of the test-drugs pretreament on the changes of the blood pressure induced by intravenous injection of cyclic bradykinin, histamine and serotonin.

Drugs	Experimental conditions	Changes of blood pressure (mm Hg)
Cyclic bradykinin (10 $\mu g/kg^{-1}$)	Control	-62,0 ± 4,6
	After diphenhydramine 5 mg/kg^{-1}	-42,8 ± 6,2*
	After diphenhydramine 25 mg/kg^{-1}	-15,2 ± 3,4*
	After cimetidine 10 mg/kg^{-1}	-69,3 ± 8,4
	After cimetidine 10 mg/kg^{-1} + + diphenhydramine 5 mg/kg^{-1}	-41,8 ± 5,6*
	After methysergide 200 mg/kg^{-1}	61,1 ± 4,4
	After methysergyde 200 mg/kg^{-1} + + diphenhydramine 5 mg/kg^{-1}	-35,8 ± 6,5*
	After parachlorophenylalanine 400 mg/kg^{-1}	-36,2 ± 5,6*
	After parachlorophenylalanine + diphenhydramine 5 mg/kg^{-1}	-29,5 ± 5,9*
Histamine (20 $\mu g/kg^{-1}$)	Control	-62,3 ± 7,7
	After diphenhydramine 5 mg/kg^{-1}	-5,5 ± 3,8*
	After cimetidine 10 mg/kg^{-1}	-64,9 ± 8,7
Serotonin (80 $\mu g/kg^{-1}$)	Control	-36,0 ± 4,4
	After methysergide 200 mg/kg^{-1}	-15,9 ± 3,1*

The results are expressed as M ± SEM (n = 6 - 8), * $p < 0.05$ vs control

unsuccessful. However, one should not attach too much significance to the failure of these antagonists to suppress the responses to cyclo-BK, since the hypotensive effect of serotonin could be completely (only by 42 %) abolished by methysergide (Table 1). Combined administration of methysergide and diphenhydramine did not alter the inhibiting influence of diphenhydramine on the hypotensive effect of cyclo-BK (Table 1). Parachlorophenylalanine, a serotonin synthesis inhibitor, abolished the hypotensive action of cyclo-BK by 42 % (Table 1).

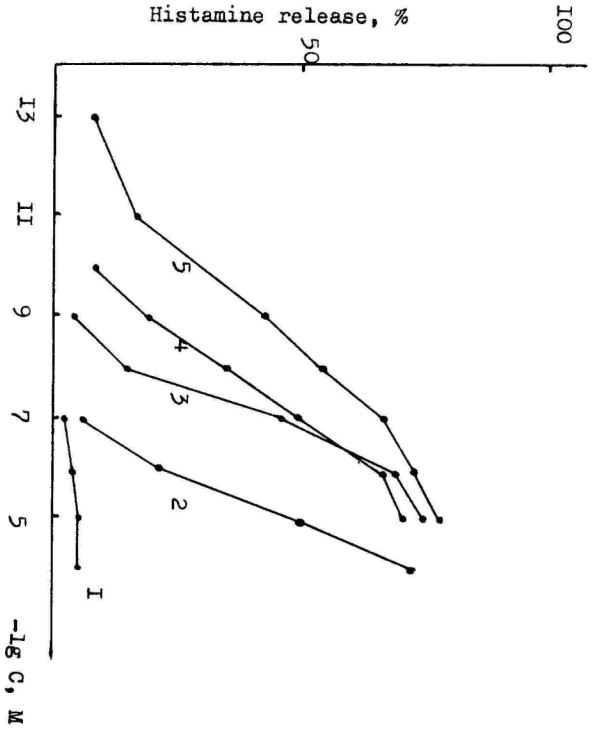

Fig. 1. Effects of angiotensin II (1), bradykinin (2), polymixine B (3), cyclobradykinin (4) and cycloangiotensin (5) on the histamine release from the rat peritoneal mast cells.

Unfortunately, no additive action could be noted when the two drugs - diphenhydramine and parachlorophenylalanine were administered together (Table 1). These results strongly suggest that histamine

and serotonin contribute substantially to the hypotensive action of cyclo-BK, and, possibly, cyclo-AT in anaesthetized rats. This conclusion is based mainly on the fact that the hypotensive effect of cyclo-BK becomes significantly reduced in rats pretreated with the antihistamine drug diphenhydramine and the serotonin synthesis inhibitor parachlorophenylalaline.

Effective liberation of histamine from rat peritoneal mast cells by cyclo-BK and cyclo-AT in the 10^{-13} - 10^{-5} M range of concentration provides convincing evidence for histamine involvement in manifestation of long-lasting hypotensive effect of cyclo-BK and cyclo-AT (fig. 1). Linear angiotensin II does not liberate histamine from rat mast cells in the 10^{-6} - 10^{-4} M concentration range. According to their ability to liberate histamine the studied cyclopeptides and the well-known histamine liberator polymixin B can be ranged in the sequence cyclo-AT > cyclo-BK > polymixin B > bradykinin.

Cross-tachyphylaxis observed with the cyclopeptides *in vivo* is indicative of interaction either with the same receptor sites or with different receptors but via a common cellular mechanism - in our case, the liberation of endogenous substances from mast cells. The results of radioligand assay have shown that cyclo-BK and polymixin B inhibit the binding of labelled cyclo-AT with receptors in a competitive manner [6].

Recently, it has been claimed that the mast cells triggering effect may be dependent on certain structural elements: the hydrophobic chain and the hydrophilic moiety with positively charged amino acids associated with the negatively charged component of the membrane surface, thus resulting in their decreased mobility which might be initial event of mast cells activation similar to the action of polycations [5]. It is possible that synthetic cyclopeptides containing positively charged amino acids (-Lys - Arg -) realize their action via the negatively charged component of the mast cell membranes. A similar mechanism might also explain the abolishment of hypotensive effect of cyclopeptides by pretreatment with $CaCl_2$, which, being positively charged, binds to the negatively charged components of the mast cell membranes, stabilizes them and makes them resistant to the action of cyclopeptides. The involvement of membrane process

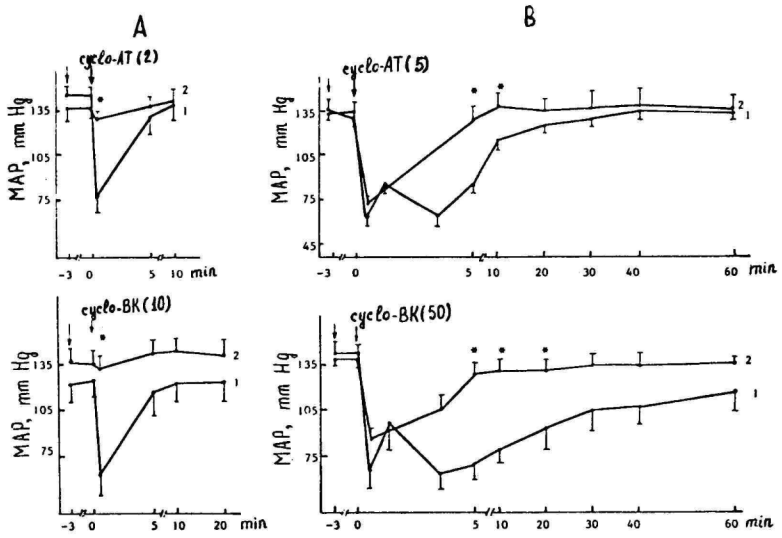

Fig. 2. The influence of Intal pretreatment on the cyclopeptides-induced changes in mean arterial pressure (MAP) of anaesthetized rats (n = 5-7).
1 - saline + cyclopeptides; 2 - Intal + cyclopeptides;
arrow indicates saline or Intal administration; * $p < 0.05$ vs control

in the liberation of endogenous substances triggered by cyclopeptides is also confirmed by data obtained with Intal, a drug used in the treatment of allergic bronchial asthma and for mast cell membranes stabilization. As shown in Fig. 2, prior administration of Intal completely reduced the short-term hypotensive effect of small doses of cyclo-BK and cyclo-AT (Fig. 2A) and shortened the duration of the long-term effects of their large doses (Fig. 2B). Intal abolished the liberation of histamine from rat perioteneal mast cells by cycloBK (10^{-6} M) in a dose-response dependent manner (Fig. 3).

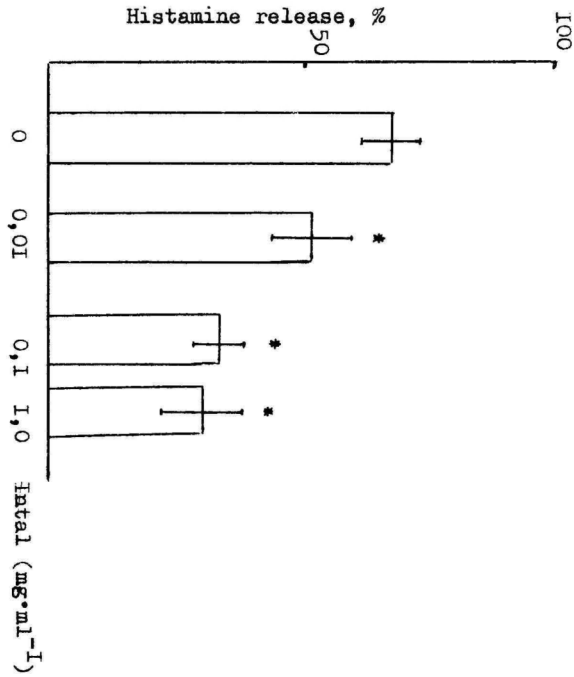

Fig. 3. Effect of Intal on the histamine release induced by cyclobradykinin (10^{-6} M) from the rat peritoneal mast cells.
* $p < 0.05$ vs control

This inhibition reaches only 50 % at the maximum Intal dose. The present findings demonstrate the expedience of further research in this class of vasoactive cyclopeptides possesing unusual properities based on their ability to liberate endogenous hypotensive substances responsible for the observed long-term decrease in blood pressure. This may serve as a guideline in the search for endogenous ligands and in the development of hypotensive drugs with a new type of action.

Conclusion

1. The hypotensive action of angiotensin II and bradykinin cycloanalogues in partially mediated by the release of histamine and serotonin from rat mast cells.
2. Angiotensin II and bradykinin cycloanalogues trigger the release of histamine from rat peritoneal mast cells.
3. The hypotensive action of cyclo-AT and cyclo-BK *in vivo* liberation of histamine from peritoneal mast cells *in vitro* are significantly reduced by prior administration of Intal, a drug capable of stabilizing mast cell membranes.

References

1. Анцанс Ю.Е., Бисениеце Д.А., Мышлякова Н.В. и др. Циклические аналоги ангиотензина с депрессорной и гистаминвысвобождающей активностью // Биоорган. химия. - 1986. - Т. 12, № 8. - С. 1118-1120.

2. Бисениеце Д.А., Анцанс Ю.Е., Мышлякова Н.В. и др. Синтез и исследование циклических аналогов ангиотензина с изучением спектра биологического действия // Биоорган. химия. - 1987. - Т. 13, № 2. - С. 149-159.

3. Быкова В.П. О происхождении клеток, накапливающих биогенезе амины // Всесоюзн. симпозиум по проблемам гистофизиологии соединительной ткани, 4-ый: Тезисы докладов. - Новосибирск, 1972. - Т. 1. - С. 23-26.

4. Мышлякова Н.В., Витолиня Р.О., Голубева В.В. и др. О механизме действия циклокининов // Бюл. экспер. биологии и медицины. - 1983. - Т. XCVI, № 11. - С. 1-128.

5. Никифоровыч Г.В., Галактионов С.Г., Чипенс Г.И. Конференция пептидных биорегуляторов. - М.: Медицина, 1983. - С. 1-192.

6. Порункевич Е.А., Раткевич М.П., Кублис Г.Г. и др. Общие принципы активности рецепторов тучных клеток пептидами и фрагментами белков // Сов.-Швейцарский симпозиум "Биологические мембраны: структура и функции: Тез. докл. - Рига, 1988. - С. 100.

7. Carraway R., Cochrane D.E., Lansman J.B. et al. Neurotenzin stimulate exocytotic histamine secretion from rat mast cells and elevates plasma histamine levels // J. Physiol. - 1982. - Vol. 323. - P. 403-414.

8. Chipens G.I., Mutulis F., Galaktinov S. Recognition of peptide and kinins: molecular aspects of the problem // Frontiers of bioorganic chemistry and molecular biology. - Oxford, 1980. - P. 99-103.

9. Chipens G.I., Mutulis F.K., Mishlyakova et al. Cyclokinins // Kinins. - New-York, London, 1981. - Vol. 3. - P. 591-595.

10. Deviller P., Renoux M., Giroud Y.-P., Regoli D. Peptides and histamine release from

peritoneal mast cells of rats. // Europ. Y. Pharmacol. - 1985. - Vol. 117. - P. 89-96.

11. Golubeva V., Klusha V. Mechanism of action of hypotensive cyclic kinins in anasthetized rats // Intern. Symposium of Physiology and Pharmacology of mooth Muscle, 5th: Abstracts. - Varna, 1988. - P. 59.

12. Kerouac R., St-Pierre S., Rioux F. Influence of various drugs on the variation of blood pressure, hematocrit and plasma histamine caused by neurotensine and compound 48/80 in rats // Neuropeptides. - 1982. - Vol. 3. - P. 145-157.

13. May C.D., Lyman M., Alberto R., Cheug Y. Procedures for immunochemical study of histamine release from leucocytes with small volume of blood // Y. Allergy. - Vol. 46, N 1. - P. 12-20.

14. Quirion R., Rioux F., Regoli D., St-Pierre S. Compaund 48.80 inhibits neurotensin-induced hypotension in rats // Life Sci. - 1980. - Vol. 221, N. 2. - P. 236-240.

15. Repke H., Bienert H. Mast cell activation - a receptor-independent mode of substance P action? // FEBS. - 1987. - Vol. 221, N. 2. - P. 236-240.

16. Van de Voorde J., Leuson J. Effect of histamine on aorta preparations of different species // Arch. Int. Pharmacodyn. Ther. - Vol. 268, N. 1. - P. 95-105.

17. Walley E.T. Receptors mediating the increase in vascular perme ability to kinins: comparative studies in rats, quinea-pig and rabbit // Arch. Pharmacol. - 1987. - Vol. 336. - P. 99-104.

THE EFFECT OF SYSTEMIC ADMINISTRATION OF CCK-8 AGONISTS ON CNS RECEPTOR BINDING PARAMETERS

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Abstract

The effect of systemic administration of caerulein and pentagastrin on rat brain benzodiazepine, opiate, and GABA receptor characteristics was examined in radioligand binding analysis. It was shown that the CCK-8 agonists can induce certain alterations in CNS receptor parameters. Namely, the treatment of rats with both caerulein and pentagastrin decreases the number of benzodiazepine binding sites in frontal cortex and increases it in hippocampus. The binding of ^{3}H-GABA in hippocampal membranes was also increased. The administration of pentagastrin induced some changes also in opiate binding, but these phenomena were observable only if the higher (sedative) doses of the drug were used. The fact that peripherally administered neuropeptides may influence the binding characteristics of CNS receptors helps to understand the behavioral effects of these compounds which are believed to penetrate poorly the blood-brain-barrier.

Introduction

It is well documented that the administration of anxiogenic drugs may induce severe emotional, hormonal, and vegetative reactions both in man and in experimental animals [5, 18, 19]. Earlier we have observed in behavioral tests that drugs acting at cholecystokinin octapeptide (CCK-8) receptors have certain effects on the anxiety rate of mice and rats. Caerulein and pentagastrin, the CCK-8 receptor agonists, reduce the exploratory activity of rodents, whereas proglumide, the CCK-8 antagonist, inhibits not only the effect of CCK-8 agonists but

also the reduction in exploration caused by anxiogenics acting at GABA receptor complex [9]. There is some preliminary evidence about possible anxiogenic action of CCK-related peptides in man [4]. The ability of these peptides to penetrate the blood-brain-barrier is expected but not documented insofar. However, it seems that several neuropeptides exert their behavioral effects through a primary target in periphery [14, 17]. Therefore, we have examined the effect of systemic administration of CCK-8 agonists on the *in vitro* radioligand binding to CNS receptors. The key role of GABA receptor - chloride channel complex in the neurochemistry of stress and anxiety [2, 7, 20] was the reason for studying benzodiazepine and GABA low affinity binding sites. As the endogenous opiates are suggested to be of importance in some behavioral effects of CCK-8 [10] the opiate receptor binding was also measured.

Materials and methods

Female albino rats of no particular strain (Rappolovo Farm, Leningrad) were used in this study. The animals were taken from their home cage, injected intraperitoneally with saline or the drug in a randomized order and placed into individual chambers separated from each other with plexiglass walls. Each control or treatment group consisted of 4 rats. The doses of peptides administered were selected according to previous behavioral experiments. The lowest dose of caerulein or pentagastrin used has anxiogenic-like effect on exploratory behavior of rats with no evidence for sedative action. The highest challenge dose of both peptides has clear sedative effect as measured in open field test. 15 min later animals were killed by decapitation. Brain regions were dissected according to Glowinski and Iversen [6]. Homogenized brain tissue was washed twice in TRIS-HCL buffer (pH 7.4) by centrifugation (48 000 g for 20 min) and resuspension. *In vitro* binding studies to label benzodiazepine binding sites were carried out at the presence of 0.2-8 nM of ^{3}H-flunitrazepam (spec.act. 85.0 Ci/mmol, Amersham Radiochemicals) in a total incubation volume of 500 µl, containing about 0.1 mg protein. 10 µmol/l of flunitrazepam was used to determine nonspecific binding. After 60 min incubation on ice the reaction was stopped by rapid filtration over Whatman GF/B

filters. The filters were washed with 4 x 3 ml of ice-cold buffer and dropped into scintillation vials. The ^{3}H-diprenorphine binding assay was carried out in polypropylene tubes at the presence of 0.125-4 nM of radiolabelled ligand (spec.act. 32 Ci/mmol, Amersham). The membranes were incubated at room temperature for 45 min. Nonspecific binding was determined by adding naloxone (10 μmol/l). The samples were centrifuged in Beckman Microfuge 12 for 3 min at 11 000 rpm. The supernataent was discarded and the tips of tubes were cut into vials. Na^{+} - independent ^{3}H-GABA binding was measured as described by Guarneri et al [8] with some modifications. The homogenized brain tissue was washed twice in TRIS-HCL buffer and frozen overnight. Just before binding assay, the membrane preparations were thawed and washed in the same buffer for four times and then suspended in the buffer at a final concentration of 0.4 μg protein for sample. The membranes were incubated at 0° C in a total volume of 250 μl with 100 nM ^{3}H-GABA (spec. act. 71.5 Ci/mmol, Amersham). After 15 min the reaction was terminated by rapid centrifugation for 3 min at 11 000 rpm in microfuge. The supernatant was discarded, pellets washed twice with ice-cold buffer and cut into vials. The radioactivity was counted using a Beckman LS - 6800 liquid scintillation counter. The analysis of saturation curves was performed using Scatchard plots and nonlinear least squares regression analysis. Student's t-test for paired observations was used to determine statistical significance. Protein was measured according to the procedure of Lowry et al [13].

Results

As shown in Table 1, systemic administration of caerulein resulted in a decrease in the apparent number of cortical benzodiazepine binding sites. Similar effect was observed after the lowest dose of pentagastrin. In hippocampus, the lowest doses of both peptides induced an increase in the number of benzodiazepine binding sites. In certain doses (see Table 1), caerulein and pentagastrin increased the affinity of benzodiazepine binding sites for radioligand in mesolimbic area. The ^{3}H-flunitrazepam binding in corpus striatum was not influenced.

Table 1

Scatchard analysis of saturation data of ^{3}H-flunitrazepam binding in rat brain membranes after acute treatment with CCK-8 agonists. Results are from pooled tissue of 4 animals per group.

Treatment	Dose (μg/kg)	B_{max} (fmol/mg prot.)	K_D (nmol/l)
1	2	3	4
FRONTAL CORTEX			
Saline		1604 ± 110	1.59 ± 0.39
Caerulein	0.1	1311 ± 53	1.13 ± 0.12
Caerulein	1	1068 ± 66*	0.91 ± 0.11
Caerulein	10	1106 ± 86*	1.18 ± 0.22
Saline		1185 ± 93	1.19 ± 0.31
Pentagastrin	0.5	909 ± 76*	0.79 ± 0.22
Pentagastrin	5	1204 ± 14	0.73 ± 0.03
Pentagastrin	50	1300 ± 75	1.20 ± 0.17
HIPPOCAMPUS			
Saline		1011 ± 53	0.98 ± 0.15
Caerulein	0.1	1172 ± 31*	1.19 ± 0.06
Caerulein	1	966 ± 69	1.28 ± 0.25
Caerulein	10	1038 ± 72	0.92 ± 0.14
Saline		777 ± 56	0.77 ± 0.16
Pentagastrin	0.5	992 ± 48*	0.79 ± 0.13
Pentagastrin	5	1122 ± 21*	1.14 ± 0.05
Pentagastrin	50	875 ± 12	0.87 ± 0.03
MESOLIMBIC AREA			
Saline		897 ± 45	1.24 ± 0.10
Caerulein	0.1	992 ± 60	0.96 ± 0.10
Caerulein	1	1011 ± 53	0.85±0.08*
Caerulein	10	915 ± 91	0.87 ± 0.16

Continued

Table 1 continued

1	2	3	4
Saline		1127 ± 111	1.10 ± 0.20
Pentagastrin	0.5	935 ± 27	0.72 ± 0.07
Pentagastrin	5	866 ± 126	0.50±0.10*
Pentagastrin	50	986 ± 97	0.74 ± 0.25
CORPUS STRIATUM			
Saline		665 ± 74	0.77 ± 0.18
Caerulein	0.1	879 ± 54	0.96 ± 0.11
Caerulein	1	711 ± 46	1.06 ± 0.12
Caerulein	10	677 ± 41	0.70 ± 0.09
Saline		646 ± 155	0.80 ± 0.37
Pentagastrin	0.5	840 ± 148	0.87 ± 0.29
Pentagastrin	5	699 ± 57	0.81 ± 0.26
Pentagastrin	50	652 ± 46	0.70 ± 0.21

* - $P < 0.05$ as compared to saline group.

Scatchard analysis of ^{3}H-diprenorphine binding revealed that pentagastrin treatment of rats increased the number of binding sites in frontal cortex and decreased it in hippocampus and mesolimbic area (Table 2). However, these effects were observable after higher doses of the CCK-8 agonist.

The binding of ^{3}H-GABA was also influenced by the administration of caerulein and pentagastrin to rats. Both CCK-8 agonists failed to modify ^{3}H-GABA binding in the membranes of frontal cortex but increased it in hippocampus (Fig. 1).

Table 2

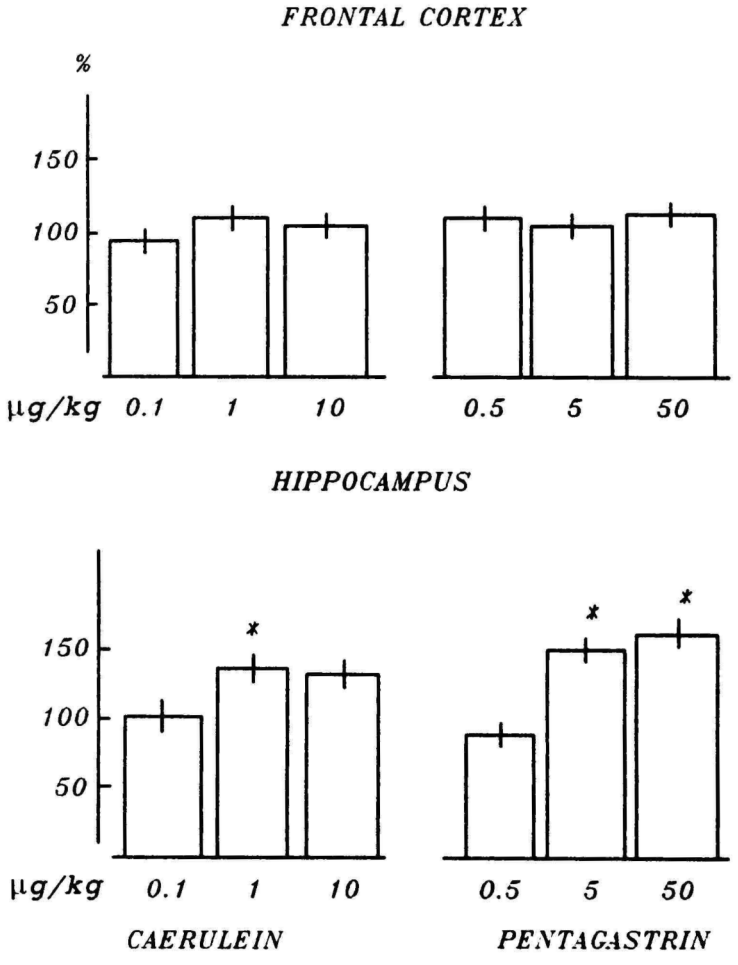
Scatchard analysis of saturation data of ^{3}H-diprenorphine binding in rat brain membranes after acute treatment with pentagastrin. Results are from pooled tissue of 4 animals per group

Brain region	Dose (μg/kg)	B_{max} (fmol/mg prot.)	K_D (nmol/l)
Frontal cortex	0	226 ± 25	1.69 ± 0.29
	0.5	227 ± 40	1.80 ± 0.52
	5	313 ± 18*	2.03 ± 0.18
	50	318 ± 37	1.99 ± 0.36
Hippocampus	0	240 ± 25	3.07 ± 0.46
	0.5	221 ± 7	2.39 ± 0.12
	5	133 ± 31	1.19 ± 0.50
	50	114 ± 11*	0.79 ± 0.15*
Mesolimbic area	0	262 ± 43	1.64 ± 0.40
	0.5	204 ± 12	1.33 ± 0.14
	5	239 ± 11	1.49 ± 0.11
	50	108 ± 15*	1.46 ± 0.35
Corpus striatum	0	365 ± 43	2.03 ± 0.38
	0.5	250 ± 9	1.55 ± 0.10
	5	450 ± 19	2.00 ± 0.12
	50	355 ± 49	2.00 ± 0.38

* - $P < 0.05$ as compared to control group

Figure 1

The effect of administration of CCK-8 agonists on ^{3}H-GABA (100 nmol/l) binding in rat brain membranes. Results are from pooled tissue of animals per group and are expressed as per cents from control ± SEM. 100 % corresponds to about 800 fmol/mg protein or about 500 fmol/mg protein in frontal cortex and hippocampus respectively.



Discussion

The fact that any exposure to stressful situations induces certain alterations in benzodiazepine receptor binding characteristics is not a new one. However, there is no general agreement in the direction of changes of these characteristics. Stressful manipulations are reported to increase [16, 22] and to decrease [11, 12, 15] the number of brain benzodiazepine binding sites. Unfortunately there is little evidence about the emotional state of "stressed" animals in these studies. In a recent study of Bodnoff et al [3] a reduction in novelty-induced fear was paralleled by an increase in the number of benzodiazepine binding sites in rat brain membranes. However, no attempt was made in this study to clarify the brain regions where such alterations are present. Our results from this study clearly indicate that the administration of anxiogenic drugs may induce changes of distinct direction in different brain regions.

Several authors have suggested that stressful manipulations may increase opiate receptor binding [1, 21]. In our experiments an increase in the number of opiate binding sites after pentagastrin treatment was observed in frontal cortex, but in hippocampal and mesolimbic membranes this measure was decreased. The combination of labelled and unlabelled ligand used by us makes it possible to determine only the total opiate binding. Therefore the effects of anxiogenic drug treatment on separate subtypes of opiate receptor remain to be elucidated. However, it should be kept in mind that the changes in ^{3}H-diprenorphine binding occurred only in case the CCK-8 agonist was administered in sedative doses.

The administration of both caerulein and pentagastrin had no effect on low affinity ^{3}H-GABA binding in frontal cortex but increased it in the hippocampus of rats. These results seem to be in conflict with the experiments of Biggio [2] who has found that stress induces downregulation of low affinity ^{3}H-GABA binding in rat cerebral cortex. However, his group has used a quite different experimental paradigm involving chronic exposure of control animals to stressful situations.

In conclusion, the results of present study show that previous systemic administration of anxiogenic (and sedative) doses of CCK-8 agonists may influence the CNS receptor binding parameters in certain brain

regions. This fact should be considered in discussions about central versus peripheral origin of the behavioral effects of systemically administered neuropeptides.

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References

1. Adams J.U., Andrews J.S., Hiller J.M., Simon E.J., Holtzman S.G. Effects of stress and ß-funaltrexamide pretreatment on morphine analgesia and opioid binding in rats // Life Sci. - 1987. - Vol. 41. - P. 2835-2844.

2. Biggio G. The action of stress, ß-carbolines, diazepam, and Ro 15-1788 on GABA receptors in the rat brain // Benzodiazepine Recognition Site Ligands: Biochemistry and Pharmacology / Ed. by G. Biggio and E. Costa. - New York: Raven Press, 1983. - P. 105-119.

3. Bodnoff S.R., Suranyi-Cadotte B., Quirion R., Meaney M.J. Postnatal handling reduces novelty-induced fear and increases [^{3}H]-flunitrazepam binding in rat brain // Eur. J. Pharmacol. - 1987. - Vol. 144. - P. 105-107.

4. Bradwejn J., Meterissian G., Koszycki D. The effects of cholecystokinin-tetrapeptide in panic disorder // 16th CINP Congr. Munich 1988 Abstr. Psychopharmacology. - 1988. - Vol. 96. - P. 184.

5. Dorow R., Horowski R., Paschelke G., Amin M., Braestrup C. Severe anxiety induced by FG 7142, a ß-carboline ligand for benzodiazepine receptors // Lancet. - 1983. - Vol. 2. - P. 98-99.

6. Glowinski J., Iversen L.L. Regional studies of catecholamines in the rat brain - I. The disposition of (^{3}H)-norepinephrine, (^{3}H)-dopamine and (^{3}H)-dopa in various regions in the brain // J. Neurochem. - 1966. - Vol. 13. - P. 655-669.

7. Gray JA, Quintero S, Mellanby J, Buckland C, Fillenz M, Fung SC. Some biochemical, behavioral, and electrophysiological test of the GABA hypothesis of anti-anxiety drug action // Actions and Interactions of GABA and Benzodiazepines / Ed. by N.G. Bowery. - Raven Press, 1984. - P. 239-262.

8. Guarneri P., Guarneri R., La Bella V., Scondotto S., Scoppa F., Piccoli F. Lateral differences in GABA binding sites in rat brain // Neurochem. Res. - 1988. - Vol. 13. - P. 209-211.

9. Harro J., Vasar E., Allikmets L. The role of CCK-8-ergic mechanisms in the genesis of anxiety in mice // Neurotransmitters and Behavior, All-Union Conference, Abstr. - Novosibirsk, 1988. - P. 113-114.

10. Itoh S., Katsuura G., Takashima A. Interactions of cholecystokinin, ß-endorphin, and their antagonists on passive avoidance behavior in rats. // Can. J. Physiol. Pharmacol. - 1987. - Vol. 65. - P. 2260-2264.

11. Kiivet R.-A., Harro J., Rägo L., Põld M. Changes in GABA and benzodiazepine receptors after foot-shock in the rat: influence of diazepam // Molecular Pharmacology of Receptors II / Ed. by L. Allikmets. - Tartu, 1988. - P. 45-54.

12. Lippa A.S., Kleifner C.A., Yunger L., Sano C.M., Smith W.V., Beer B. Relationship between benzodiazepine receptors and experimental anxiety in rats // Pharmacol. Biochem. Behav. - 1978. - Vol. 9. - P. 853-856.

13. Lowry O.H., Rosenbrough N.I., Farr A.L., Randall R.I. Protein measurement with the folin phenol reagent // J. Biol. Chem. - 1951. - Vol. 193. - P. 265-272.

14. Martinez J.L., Weinberger S.B., Schulteis G. Enkephalins and learning and memory: a review of evidence for a site of action outside the blood-brain barrier // Behav. Neurol. Biol. - 1988. - Vol. 49. - P. 192-221.

15. Medina J.H., Novas M.L., Wolfman C.N.V., Levi de Stein M., De Robertis E. Benzodiazepine receptors in rat cerebral cortex and hippocampus undergo rapid and reversible changes after acute stress // Neuroscience. - 1983. - Vol. 9. - P. 331-335.

16. Miller L.G., Thompson M.L., Greenblatt D.J., Deutsch S.I., Shader R.I., Paul S.M. Rapid increase in brain benzodiazepine receptor binding following defeat stress in mice // Brain Res. - 1987. - Vol. 414. - P. 395-400.

17. Morley J.E. Behavioral effects of administered cholecystokinin // ISI Atlas Pharmacol. - 1985. - Vol. 1. - P. 49-51.

18. Pellow S., File S.E. The effects of putative anxiogenic compounds (FG 7142, CGS 8216 and Ro 15-1788) on the rat corticosterone response // Physiol Behav. - 1985. - Vol. 35. - P. 587-590.

19. Petersen E.N., Jensen L.H. Proconflict effect of benzodiazepine receptor inverse agonists and other inhibitors of GABA function // Eur. J. Pharmacol. - 1984. - Vol. 103. - P. 91-97.

20. Shephard R.A. Neurotransmitters, anxiety and benzodiazepines: a behavioral review // Neurosci. Biobehav. Rev. - 1986. - Vol. 10. - P. 449-461.

21. Sirakova I., Panova D., Georgiev P., Sirakov L.M. Opiate receptor binding in the brain of rat during stress // Pav. J. Biol. Sci. - 1988. - Vol. 23. - P. 54-56.

22. Soubrie P., Thiebot M.H., Jobert A., Montrastruc S.L., Hery F., Hamon M. Decreased convulsant potency of picrotoxin and pentylenetetrazol and enhanced ^{3}H-flunitrazepam cortical binding following stressful manipulations in rats // Brain Res. - 1980. - Vol. 189. - P. 505-517.

NMDA-RECEPTOR: THE BASIC RESULTS OF STUDY AND PERSPECTIVES OF CLINICAL USE OF ANTAGONISTS

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Abstract

Amino acids mediate about a half of the synaptic transmission in the mammalian brain. NMDA-receptor is the most examined type of excitatory amino acids receptors. Structure of NMDA receptor-channel complex, agonists and antagonists, their effects in animals are reviewed. Use of NMDA-antagonists for treatment of epilepsy, hypoxia (including perinatal), stroke and neurodegenerative disorders is predicted. Involvement of NMDA-receptors in mechanisms of psychoses and drug addiction is suggested. Bibliography 44 ref.

Introduction

History of the study of the excitatory amino acids (EAA) has begun in the early 1950s when the convulsive action of glutamate and aspartate was shown [42]. In the past three decades significant advances have been made in understanding the function of EAA. The current state of the problem was presented at the International symposium "Excitatory Amino Acids '88" (Brazil, Manaus, March 28 - April 2, 1988). Its materials are taken as a basis for this review. Abstracts from the symposium are published in a special issue of **Neurochemistry International** (1988, v. 12, suppl. I.)

The demonstration of the direct depolarizing action of l-glutamate on spinal neurones in 1958 led to the speculation that one or more endogenous amino acids may act as exctitatory transmitter substances in the mammalian central nervous system [42]. It is known now that amino acid mediated neuronal communication represents at least 50 % of the synaptic activity of mammalian brain and practically every neuron receives information coded through the release of an EAA transmitter [8]. EAA

are now considered to play key roles as neurotransmitters in epilepsy, spasticity, neurodegenerative disorders, anxiety, learning and, probably, psychoses.

The name of amino acids "excitatory" is relevant both to cellular and behavioral levels. The effect of EAA is considered to be linked with opening of the cationic channels in neuronal membrane. Side by side with ionotropic mechanism there is a metabolotropic one including the activation of specific enzymes [8]. L-glutamate is the common ligand for EAA receptors. According to their specific ligands, glutamate receptors are divided into three types: N-methyl-d-aspartate (NMDA), kainate and quisqualate [36]. It is suggested that metabolotropic effect is mediated via a specific fourth type of glutamate receptor related to inositol phosphatase [39].

EAA receptors are distributed throughout the brain [44]. At many central synapses the postsynaptic membrane contains a mixture of NMDA and non-NMDA receptors [9]. The distribution and properties of these receptors are dynamic, with changes in receptor population during normal aging and in response to pathological processes such as epilepsy, trauma, Alzheimer's disease [9] and hypoglycemic states [43].

Because of a restricted space of the publication I have to dwell on the NMDA-receptors only. On the one hand this type of EAA receptors is best studied, on the other hand namely NMDA-receptor is in the focus of our interests because it is the site of action of kynurenines - endogenous metabolites of tryptophan - whose neuroactivity has been discovered and is now intensively studied in our laboratory [18-21].

Molecular organization and structure of NMDA receptor complex

NMDA receptor and related ionic channel are included in the complex which appears to be of protein nature and consists of multiple subunits (Fig. 1) [11, 32]. NMDA receptor-coupled ionic channel is permeable for cations Na^+, K^+ and Ca^{++}, meanwhile ionic channels related to other EAA receptors are permeable for Na^+ and K^+ only [7]. At least five distinct sites of binding within NMDA-receptor complex are:

1) for agonists and competitive antagonists
2) for allosteric modulators

3) for non-competitive antagonists, three different sites for a) Mg^{++}, b) Zn^{++}, c) PCP-like drugs.

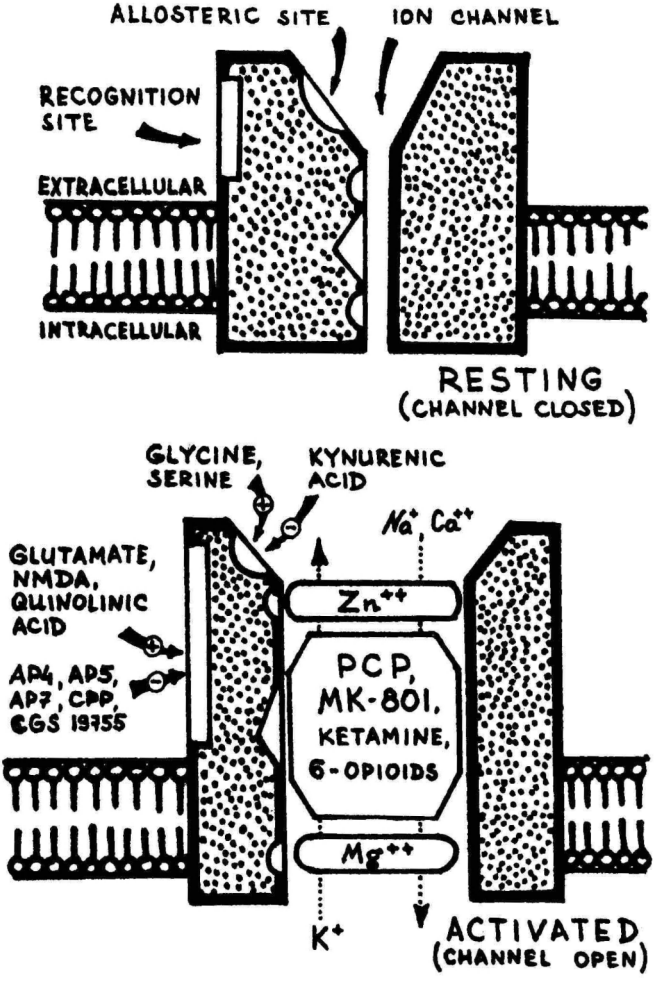

Fig. 1. NMDA receptor-channel complex (according to A.C. Foster and G.E. Fagg (1987), modified). For legends see the text.

NMDA-receptor agonists. NMDA-receptor received its name from N-methyl-D-aspartate (NMDA) - a first specific synthetic agonist [36]. Non-specific endogenous agonists are l-glutamate and l-aspartate. Cyclic analog of NMDA, quinolinic acid, which is the normal constituent in a mammalian brain [31] is considered to be an endogenous specific agonist [37].

Competitive antagonists bind to the agonist recognition binding site but do not open the ionic channel. This group includes phosphonic derivatives of amino acids: 2-amino-4-phosphonobutyrate (APB, AP4), 2-amino-5-phosphonovalerate (APV, AP5), 2-amino-7-phosphonoheptanoate (APH, AP7), 3-(2-carboxypiperazin-4-yl)propyl-I-phosphonic acid (CPP) [11, 36, 37] and cis-4-phosphonomethyl-2-piperidine carboxylic acid (CGS 19 755).

Allosteric modulators. NMDA receptor exists in agonist and antagonist preferring conformations. Glycine and D-serine allosterically convert antagonist-preferring conformation into agonist-preferring one and reduce antagonist binding while enhancing agonist binding [15, 29]. Kynurenic acid has an opposite effect [15, 24, 28]. It is worth noting that two functional antagonists, i.e. quinolinic acid and kynurenic acid, are metabolically closely interrelated because both are endogenous metabolites of tryptophan at the kynurenine pathway [4, 19. 31, 35, 38].

Noncompetitive antagonists. Binding sites for noncompetitive antagonists are located inside the open state of NMDA receptor-copuled ionic channel. They act by direct channel blockade ("like a cork"). Mg^{++} cation blocks the channel in a voltage-dependent manner [1]. Its binding site is located apparently near to the internal surface of the membrane. Another cation - Zn^{++} - has its own binding site. There is evidence that tricyclic antidepressants (organic molecules!), most effectively desmethylimipramine, bind to the Zn^{++}-recognition site [34].

PCP-like drugs recognition site is the third binding site inside the channel. This group includes phencyclidine (PCP; 1-(1-phenylcyclohexyl)-piperidine), ketamine (±))-2-o-chlorophenyl-2-methylaminocyclohexanone), MK-801 ((+)-5-methyl-10,11-dihydro-5-dibenzo(a,d)cyclohepten-5,10-iminomaleate) and sigma-opioids: cyclazocine, pentazocine and N-allyl-normetazocine (NANM, SKF 10,047). According to their activity they are rated in an such order: MK-801 > PCP > cyclazocine > ketamine > SKF 10,047

> pentazocine [24]. The presence of an endogenous ligand for PCP-binding site - so called alfa-endopsychosine - is also suggested [8].

Thus, the NMDA receptor system is a remarkable complex entity, the normal function of which depends on a dynamic equilibrium among multiple facilitative and inhibitory factors.

Effects of NMDA agonists and antagonists

NMDA-agonists induce membrane depolarization and excitation of neurons. This is manifested dose-dependently in locomotor hyperactivity [33] and seizures on electrophysiological [17] and behavioral [18, 20, 33] levels. Excessive excitation is followed by neurodegeneration through rapid Ca^{++}-independent or slow Ca^{++}-dependent mechanisms [7]. NMDA agonist-induced neurodegeneration is characterized by destruction of only neuronal bodies associated with the safety of sparing axons and glial proliferation [3].

Data about synergism between glycine and NMDA-agonists are true for *in vitro* experiments. *In vivo* administration of glycine in rats antagonized excitatory and convulsive effects of quinolinic acid, NMDA receptor agonist [20].

NMDA-antagonists effects are presented in the literature more widely. Besides the effects opposite to the mentioned effects of agonists, i.e. anticonvulsant [6, 16, 20, 28, 37] and neuroprotective in agonist-induced neurodegeneration [26, 37] NMDA-antagonists possess the following effects:

- anxiolytic (for competitive antagonists only) [23],
- impairment of certain kinds of learning and memory [10, 30],
- muscle relaxation (in genetically spastic rats) [40],
- catalepsy (in pigeons), locomotor activation, stereotyped sniffing, head movements, loss of motor coordination, ataxia (in rodents), anesthesia, nystagmus and profuse salivation (in rhesus monkeys) [16],
- neuroprotection in hypoxia [12], including hypobaric [14], and in hypoglycemia [41],
- preventig of edema and restoring of magnesium homeostasis after traumatic brain injury [27].

Known competitive NMDA-antagonists do not penetrate through the blood brain barrier and are effective only under intracerebroventricular (i.c.v.) administration. Noncompetitive antagonists including the most active one - MK-801 - may be administred systemically. There is the following order of NMDA-antagonists potency according to their behavioral effects: CPP (i.c.v.) > MK-801 (i.c.v.) > PCP (i.c.v.) > MK-801 (i.p.) > AP5 (i.c.v.) > kynurenic acid (i.c.v.) > PCP (i.p.) > CPP (i.p.) > ketamine (i.p.) [16].

To the effects observed in the experiments on laboratory animals we can add some clinical effects of PCP and ketamine which have begun to be used in humans long time before the discovery of their relation to the NMDA-receptors. These drugs belong to the group of the so-called "dissociative anesthetics". They induce analgesia and short-time general anesthesia. On the other hand, after emergence they induce hallucinations, changes in perceived body image, feeling of detachment from the enviroment, deficits in cognitive ability, sometimes aggressive behavior [2, 13]. In case of PCP these side effects are so severe that a PCP-model of schizophrenia has been suggested [5].

Although tricyclic antidepressants are also used in humans, their effects cannot be considered as the effects of the NMDA-receptor blocade because their NMDA-inhibitory potency is relatively weak in comparison with their well-established effects on the monoamine uptake system [34].

Clinical use of NMDA-antagonists: perspectives

There is evidence that the anticonvulsant and neuroprotective action of NMDA-antagonists can be used in the case of epilepsy and some neurodegenerative disorders such as Alzheimer's disease, Huntington's chorea, lateral amiotrophic sclerosis because of a great similarity between morphological changes in these disorders and the NMDA agonist-induced (e.g. quinolinic acid-induced) neurodestruction.

So far as NMDA-antagonists are potent against neurodegeneration in perinatal hypoxia, ketamine, a clinically used anesthetic has certain advantages over other analgesic drugs for use during the delivery. Its neuroprotective action would be of great use against hypoxia. On the other hand, because of psychotic side effects of ketamine the after-effects

of even single ketamine administration on the fetus must be thoroughly examined. It cannot be excluded also that insignificant addictive properties of ketamine in adults (occasional observations) may be dangerous in the earliest age.

PCP, a more active analog of ketamine, was not introduced in wide clinical practice due to its more severe psychotic side effects and significant addictive potency. Thus, the effects of PCP-like drugs may be tentatively classified into two groups:

1) "positive" - analgesic (clinical data), anticonvulsant and neuroprotective (experimental data),

2) "negative" - psychotomimetic and addictive (clinical data).

While the experimental "positive" effects are proved to be related to the blockade of NMDA receptor-coupled cationic channel, the involvement of EAA system in "negative" ones and analgesia is still unknown. The following questions have to be answered:

1) Does the EAA system participate in mechanisms of analgesia, psychoses and drug addiction?

2) If yes, what is the ratio of NMDA-antagonist and sigma-opioid agonist properties in these effects of PCP-like drugs?

Answers to these questions may lead to new approaches in treatment of psychotic states and drug addiction.

References

1. Ascher P. Magnesium block of the NMDA receptor channel // Frontiers in excitatory amino acids research / Eds. E.A. Cavalheiro, J. Lehmann, L. Turski. - New York: Alan R. Liss Inc., 1988. - P. 151-157.

2. Balster R.L. The behavioral pharmacology of phencyclidine // Psychopharmacology: the third generation of progress / Ed. H.Y. Meltzer. - New York: Raven Press, 1987. - P. 1573-1579.

3. Björklund H., Olson L., Dahl D., Schwarcz R. Short- and long-term consequences of intracranial injections of the excitotoxin quinolinic acid as evidenced by GFA immunohistochemistry of astrocytes // Brain Res. - 1986. - Vol. 371. - P. 267-277.

4. Carla V., Lombardi G., Beni M., Russi P., Moneti G., Moroni F. Identification and measurement of kynurenic acid in the rat brain and other organs // Anal. Biochem. - 1988. - Vol. 169. - P. 89-94.

5. Castellani S., Bupp S.J. Molecular mechanisms in phencyclidine-induced psychosis and its treatment // Pharm. Biochem. Behav. - 1987. - Vol. 28. - N 1. - P. 119.

6. Chapman A.G. Anticonvulsant activity of excitatory amino acids antagonists // Frontiers in excitatory amino acids research / Eds. E.A. Cavelheiro, J. Lehmann, L. Turski. - New York: Alan R. Liss Inc., 1988. - P. 203-210.

7. Choi D.W. Glutamate neurotoxicity and diseases of the nervous system // Neuron. - 1988. - Vol. 1. - P. 623-634.

8. Costa E., Guidotti A., Manev H., Szekely A.M., Wroblewski J.T. Signal transduction at excitatory amino acid receptors: modulation by gangliosides // Frontiers in excitatory amino acids research / Eds. E.A. Cavalheiro, J. Lehmann, L. Turski. - New York: Alan R. Liss Inc., 1988. - P. 29-38.

9. Cotman C.W., Stevens D.R. Excitatory amino acid receptors: anatomical organisation and functional implications // Ibid. - P. 117-123.

10. Davis S., Butcher S.P., Morris R.G.M. The role of NMDA receptor in certain kinds of learning // Ibid. - P. 385-392.

11. Foster A.C., Fagg G.E. Taking apart NMDA receptors // Nature. - 1987. - Vol. 329. - P. 395-396.

12. Foster A.C., Gill R., Woodruff G.N., Iversen L.L. Noncompetitive NMDA receptor antagonists and ischaemia-induced neuronal degeneration // Frontiers in excitatory amino acids research / Eds. E.A. Cavalheiro, J. Lehmann, L. Turski. - New York: Alan R. Liss Inc., 1988. - P. 707-714.

13. Greenberg B.D., Segal D.S., Jacobs B.L. Hallucinogens: phencyclidine // Psychopharmacology / Ed. D.G. Grahame-Smith. - Elsevier Science Publishers B.V., 1985. - Vol. 2, Part I: Preclinical Psychopharmacology. - P. 343-363.

14. Ikonomidou C., Frierdrich G., Salles K.S., Labruyere J., Price M.T., Olney J.W. Glutamate-like damage in infant rat brain produced by hypobaric-ischemic conditions // Frontiers in excitatory amino acids research / Eds. E.A. Cavelheiro, J. Lehmann, L. Turski. - New York: Alan R. Liss Inc., 1988. - P. 657-660.

15. Johnson K.M., Snell L.D., Jones S.M., Qi H.-Q. Glycine antagonist activity of simple glycine analogues and N-methyl-D-aspartate receptor antagonists // Ibid. - P. 551-558.

16. Koek W., Woods J.H. N-methyl-D-aspartate antagonism and phencyclidine-like activity: behavioral studies // Ibid. - P. 535-542.

17. Kozlovsky V.L., Prakhye I.V. Electroenchephalographic manifestations of rat convulsive response to intraventricular administration of quinolinic acid and kynurenine // Pharmacology and Toxicology (in Russian). - 1988. - N 6. - P. 26-29.

18. Lapin I.P. Kynurenines and seizures // Epilepsia. - 1981. - Vol. 22. - P. 257-265.

19. Lapin I.P. Kynurenines and behavior // Frontiers in excitatory amino acids research / Eds. E.A. Cavelheiro, J. Lehmann, L. Turski. - New York, Alan R. Liss Inc., 1988. - P. 605-611.

20. Lapin I.P., Prakhie I.B., Kiseleva I.P. Antagonism of seizures induced by the administration of the endogenous convulsant quinolinic acid into rat brain ventricles // J. Neur. Transm. - 1986. - Vol. 65. - P. 177-185.

21. Lapin I.P, Ryzhov I.V. Molecular mechanisms of the neuroactivity of kynurenines // Molecular Mechanism of Action of psychotropic drugs (in Russian) / Ed. L. Allikmets. - Tartu, 1987. - P. 73 - 83.

22. Lehmann J., Sills M.A., Tsai C., Loo P.S., Bennett D.A. Dextromethorphan modulates the NMDA-type receptor-associated ion channel by binding to its closed state // Frontiers in excitatory amino acids research / Eds. E.A. Cavalheiro, J. Lehmann, L. Turski. - New York: Alan R. Liss Inc., 1988. - P. 571-578.

23. Liebman J.M., Bennett D.A. Anxiolytic action of competitive N-methyl-D-aspartate receptor antagonists: a comparison with benzodiazepine modulators and dissociative anesthetics // Ibid. - P. 301-308.

24. Lodge D. Modulation of N-methylaspartate receptor-channel complexes: an overview // Ibid. - P. 527-534.

25. Marcoux F.W., Probert A.W., Goodrich J.E., Dominick H.A. The NMDA-antagonist, ketamine, blocks hypoxia-induced calcium accumulation in cultured cortical neurons and prevents ischemic hippocampal cell injury // Ibid. - P. 683-686.

26. McDonald J.W., Silverstein F.S., Johnston M.V. Comparison of neuroprotective effects of competitive and non-competitive NMDA antagonists against NMDA-mediated neurotoxicity in an *in vivo* perinatal rat model // Ibid. 601-604.

27. McIntosh T., Soares H., Heyes R., Simon R. The NMDA-receptor antagonist MK-801 prevents edema and restores magnesium homeostasis after traumatic brain injury in rats // Ibid. - P. 653-656.

28. Meldrum B.S. What are the future prospects for agents decreasing excitatory neurotransmission as anti-epileptic agents? // Ibid. - P. 195-202.

29. Monaghan D.T., Cotman C.W., Olverman H.J., Watkins J.C. Two classes of NMDA-recognititon sites: differential distribution and regulation by glycine // Ibid. - P. 543-550.

30. Mondadori, C., Ortmann R., Petschke F., Buerki H., D'Amato F., Meisburger J.-G., Fagg G.E. Task-dependent impairment or facilitation of learning by NMDA receptor blockade // Ibid. - P. 419-426.

31. Moroni F., Russi P., Lombardi G., Beni M., Carla V. Kynurenic acid in the mammalian brain increases during the aging process or after administration of its precursors // Ibid. - P. 629-636.

32. Olney J.W. Revelation in excitotoxicology: what next? // Ibid. - P. 589-596.

33. Prakhie I.B., Kisseleva I.P., Lapin I.P. Excitatory effects of kynurenines, amino acids

and convulsants administered into brain ventricles: differences between rats and mice // Sechenov Physiol. J. USSR. - 1982. - Vol. LXVIII. - N. 11. - P. 1516-1521. (in Russian).

34. Reynolds I.J., Miller R.J. Desmethylimipramine is a zinc-like ion-competitive antagonist of N-methyl-D-aspartate (NMDA) receptors // Frontiers in excitatory amino acids research / Eds. E.A. Cavalheiro, J. Lehmann, L. Turski. - New York: Alan R. Liss Inc., 1988. - P. 559-562.

35. Schwarcz R., Speciale C., Gramsbergen J.B.P., Turski W.A. Kynurenines in the mammalian brain // Ibid. - P. 613-620.

36. Sharif N.A. Multiple synaptic receptors for neuroactive amino acid transmitters - new vistas // International Review of Neurobiology. - 1985. - Vol. 26. - P. 85-150.

37. Stone T.W., Burton N.R. NMDA Receptors and ligands in the vertebrate CNS // Progress in Neurobiology. - 1988. - Vol. 30. - P. 333-368.

38. Stone T.W., Connick J.H. Quinolinic acid and other kynurenines in the CNS // Neuroscience. - 1985. - Vol. 15. - P. 597-617.

39. Sugiyama H. Functional and Pharmacological properties of glutamate receptors linked to inositol phospholipid metabolism // Neurochemistry International. - 1988. - Vol. 12. - Suppl. 1. - P. 3.

40. Turski L., Klockgether T., Turski W.A., Ikonomidou C., Schwartz M., Sontag K.-H. Muscle relaxant action excitatory amino acid antagonists: sites of action // Frontiers in excitatory amino acids research / Eds. E.A. Cavalheiro, J. Lehmann, L. Turski. - New York: Alan R. Liss Inc., 1988. - P. 343-350.

41. Wieloch T., Gustaffson I., Westenberg E. Effects of non-competitive NMDA receptor antagonist MK-801 on ischemic and hypoglycemic brain damage // Ibid. - P. 715-722.

42. Watkins J.C. Thirty years of excitatory amino acid research // Ibid. - P. 3-10.

43. Westenberg E., Wieloch T. Excitatory amino acid receptors and hypoglycemic brain damage // Ibid. - P. 645-652.

44. Young A.B., Penney J.B., Cha J.-H., Olson J.M.M., Greenamyre J.T., Albin R.L., Richfield E. Neuronal and anatomical localization of excitatory amino acid receptor subtypes in vertebrate brain // Ibid. - P. 109-117.

OLIGOMERIZATION LEVEL - A REGULATORY FACTOR IN FUNCTIONING OF TRANSPORT (AND RECEPTOR) PROTEINS

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Abstract

This mini-review has been dedicated to some regulation aspects of the oligomeric systems (transport and receptor proteins). It has been shown, that the brain Na, K-ATPase cooperativity for Na^+ and K^+ depends on enzyme's oligomeric state and that lipid-protein interactions participate in maintaining enzyme oligomerization levels. Experimental data suggest that one of biochemical mechanisms in genesis of some pathological processes with increased intracellular Na^+ (tumorous diseases and hypertension) involves (through several ways) disturbances at the higher oligomerization levels (allosteric properties) of Na, K-ATPase, that leads to a decrease in positive cooperative binding of Na^+. Disturbanses of oligomerization level of the transport and receptor proteins are really associated with genesis of pathological states.

In the past decade there has been collected a valuable information about Na, K-ATPase subunits (α and β) and their primary structure [1, 4], nevertheless there is little known as yet about the mechanisms by which the regulation of the Na-pump would take place. We can claim without doubt that decoding of this enzyme-system regulation mechanism is, however, a very important problem, which considerably develops our conceptions about oligomeric systems in general.

In the present mini-review we make an attempt to analyze some aspects of regulation of the brain Na, K-ATPase with main attention concentrated to the following questions: 1) Does the cooperativity for Na^+ and K^+ depend on the degree of enzyme protomerization (oligomerization)? 2) Which role do the

lipids play on the occasion of activating (or cooperative) effects of essential cations? 3) Do any regulatory properties of the Na, K-ATPase alter genesis in some pathological states?

Brain Na-pump is integrated in the plasmatic membrane protein, the protomer ($\alpha\beta$) of which consists α- and β-subunits [8]. Na, K-ATPase is characterized by cooperative interactions with Na^+ and K^+ [2, 13], at the same time the membrane lipids have been evidently engaged in regulation of the functioning of the Na, K-ATPase [8]. It has also been shown, that under several pathological states Na, K-ATPase activity undergoes certain alterations [5].

It is very logical that the regulation of this oligomeric enzyme system (Na-pump) realizes through the interactions at several structural levels of enzyme protein (subunit, protomer etc.). Therefore, it is clear that a more constructive approach to the regulation of the intact Na, K-ATPase system needs generalized information, obtained from the experiments with normal and desensitized enzyme preparations (EP) and with EP, treated with phospholipase or modifiers (including pharmacological agents) or isolated from the pathological brain tissue. By desensitization of the enzyme oligomeric structure with several factors (urea, DS-Na, digitonin, CHAPS) under a variety of conditions without and with substrates (ATP, pNPP) and after following determination of different kinetic characteristics (17, 14) it should be suggested that in the usual activation process of Na, ATPase by Na^+ and K^+ the noninteracting (noncooperative) cation-binding sites play a desicive role, while their affinity to Na^+ and K^+ differs from the affinity of the cooperative cation-binding sites. Another assumption is also possible - the same cation-binding sites participate in the both processes (activation and cooperativity), but the cooperativity of these sites appears definitely, only in the case of interactions of the components of the enzyme system. This possibility is supported by the observation that at the level of noninteracting (individual) subunits the binding of Na^+ and K^+ to Na, K-ATPase was noncooperative, while enzyme activity was not practically altered [14]. Accordingly, in order to reveal the cooperativity of Na^+ and K^+ at all, it is necessary (as a minimum), to reach the protomeric level of the enzyme. In other words, the quintessence

of the cooperativity lies in protein-protein (interprotein) interactions. However, for the manifestation of complete cooperativity towards Na^+ and K^+ the existence of cessary protomer-protomer (interprotomer) interactions [14] necessary. At the same time, it should be specially emphasized that a complete cooperativity for Na^+, unlike that for K^+, can be maintained only under conditions of nondestroyed interprotomer interactions. Consequently, the cooperativity for Na^+ (or enzyme allosteric properties for Na^+) disapears more easily than the cooperativity for K^+. These suggestions were supported by desensitization experiments [14] and by the facts, that in EP from a tumorous brain tissue (TBT) the positive cooperative binding of Na^+ to the enzyme was absent while the binding of K^+ was quite normal [3]. Besides, by inhibition of enzyme activity with Mg^{2+} [11], alongside the decrease in intersubunit interactions, there is always a remarkeble change in the Na, K-ATPase affinity and cooperativity for Na^+. In the case of K^+ this feature is less pronounced.

The above-mentioned assumptions have been supported by the experiments with psychotropic drugs (PD). For example, it has been established that PD compete with cations for the regulatory cation-binding sites of oligomeric complex [16]. Another finding is that in the presence of both Na^+ and K^+m when the enzyme works at Na, K-regime, most of PD (being found to exert positive cooperative effect) compete with Na^+. AT the same time only single PD showed competition (rise the $K_{0.5}$) with K^+ [13]. The desensitization experiments are also a good evidence for the presumption that PD cooperative binding with Na^+- and K^+-sites requires certain oligomerization levels of the enzyme protein. Thus, DS-Na treatment of EP diminished the Hill coefficients for the antidepressant imipramine and psychostimulator ritaline to the unit [13, 16].

Generalization of the above-mentioned data allows to answer the first question in the following way: Na, K-ATPase cooperativity for Na^+ and K^+ depends in fact on the levels of enzyme protomerization (oligomerization).

Among the physico-chemical interactions (for example, interprotein interactions), which guarantee a synchronous functioning of the Na, K-ATPase during the Na^+ and K^+ transmembrane transport, attention has recently been called to membrane lipids because of their regulator role for the activity

and functioning of the Na, K-ATPase (see for example, 9). According to the current conceptions [9], lipids controlling role in the regulation of the Na, K-ATPase realizes through two interdependent ways: by changes in membrane microviscosity (a feature of phasic transitions) and hydrophobic volume of plasmatic membrane. Let us analyze the brain Na-pump experimental material on the basis of these conceptions. Nowadays it is considered that the typical break on the Arrhenius plot (at 19-22 °C), obtained by membrane EP, reflects phasic transitions in the lipid bilayer [8]. This break has been obtained also on the occasion of the brain Na, K-ATPase, whereas after the treatment with phospholipase A_2 this break disappears, i.e. the curve becomes linear [15]. At the same time there there has been found an interesting feature: after treatment of EP with the desensitazers (urea, digitonin), which causes the most significant alterations in the amounts of sulfhydryl groups [14], the break on Arrhenius plot at 27.5 - 30.5 °C was detected [6].

The above-mentioned features could be explained with the help of two suggestions: first, the temperature interval of 27.5 - 30.5 °C is evidently linked to certain alterations linked to certain alterations (apparently local) in the enzyme protein conformation leading to some changes in the SH-groups packing. This viewpoint is supported by different protective effects of the ATP against the modificator (pCMB) of SH-groups at 37 °C and 28.5 - 29.5 °C and in the fact that after treatment of EP with urea and digitonin the break manifests itself at 28.5 - 29.5 °C with and without ATP [15]. In favour of the latter fact is the evidence that high concentrations of ATP can release the enzyme but of the control by membrane lipid moiety [9]. The fact that urea and digitonin (but not Ds-Na and CHAPS) did not remarkably affect the phospholipid content of membrane fragments speaks also in support of this presumption (our unpublished data).

Secondly: it may well be that the break on Arrhenius plot at 27.5 - 30.5 °C is linked to a membrane lipid state, while this break was particularly pronounced on the occasion of EP from TBT [6]. It must be added that membrane lipids in normal brain tissue (NBT) EP differ from those in TBI (see below). In connection with the latter it is interesting to mention the fact that in case of TBI no cooperative Na^+ binding to NA, K-ATPase has been established [3].

Membrane lipids effects on the enzyme protein moiety conformation are realized also through the alterations in the membrane hydrophobic volume. Proceeding from this viewpoint, it is interesting to know how the hydrophobic biological modulators would effect the brain Na, K-ATPase.

It was demonstrated that prostaglandin E_2 (PGE_2) inhibits allosterically both the whole Na, K-ATPase reaction (n_H = 1.4) and the partial K-phosphatase reaction (n_H = 1.3) [16]. Since the PGE_2 is significantly hydrophobic and the break on the Arrhenius plot (at the same time the activation energy in the low-temperature region increases), we can suppose that PGE_2's cooperative effects on the Na, K-ATPase include both an immediate effect on the phase-structural transitions of membrane lipids and changes in membranes hydrophobity. Obviously, the PGE_2-induced alterations of Na, K-ATPase lipid surroundings are transferred via the reciprocal lipid-protein contacts to the oligomeric protein. Through this mechanism the allosteric regulation by the abovmentioned biological modulator is realized.

Summarizing the facts and assumptions presented above we can give the following answer to the second question. Lipid-protein interaction play certain role in regulation of the Na-pump of the brain, whereas without doubt the lipid-protein contacts take part through the interdependent ways (experiments with prostaglandin are a good evidence for this interconnection) in the guarantee of the cooperativity for Na^+ and K^+ with oligomeric enzyme. However, it would be emphasized that in case of more deep disturbances of lipid-protein interactions (for example, in case of various pathologies) besides changes in microviscosity and hydrophobity also the products of lipid metabolism, especially, the products of lipid peroxidation (LP) can play a decisive role in regulation of Na, K-ATPase.

The literature cites numerous facts that in various pathological states Na, K-ATPase activity is altered. However, there is not much known yet about concrete alterations of the Na-pump in these conditions. In the present paper we analyze the Na, K-ATPase from TBT on the ground of this aspect.

Thus, it appears that the common biochemical parameters (optimal concentrations of Na^+, K^+, Mg^2, ATP and pH-optimum) for EP from TBI and from NBT were nearly identical. At the same time, it was established that in case of TBT the break on the Arrhenius plot is not located at 20-22 °C, but is

detectable at 27.5 - 30.5 °C [6]. Besides, there was no positive cooperative Na^+ binding with the enzyme from TBT, while the cooperativity for K^+ was normal [3].

It is clear, that the above-mentioned evidence produces several considerations. For example, if one suggests, that a typical break on the Arrhenius plot at 19-22 °C reflects the membrane lipids microviscosity and the hydrophobic volume changes, then the break in the higher temperature region (at 27.5 - 30.5 °C), would be linked mainly to certain local changes in the conformation of Na, K-ATPase oligomeric protein and, on the other hand, also to some alterations in the composition of proteinic components in case of TBT [3]. However, it can not be excluded, that in the above-mentioned differences of Na, K-ATPase from TBT and NBT, changes at lipid-protein interactions level play a certain role. In favour of the latter conception is the evidence, that there is a considerable distinction between the nonsaturability index of phospholipids in membrane fragments from TBT and NBT with an essential difference in the total content of the mentioned phospolipids (our unpublished data). It is interesting that the nonsaturability of phospholipids is in good agreement with the higher content of LP products in tissue and EP-s from tumours brain compared with that of normal brain [6].

On the basis of the above-mentioned facts and conceptions it should be summarized, that the pathologies with disturbances of interconnections (which guarantee the higher oligomerity levels for Na, K-ATPase) are followed (or associated) by the changes in the enzyme-system allosterical properties for essential cations, especially for Na^+. As regards lipids, then in pathological states, besides other effects a considerable role can play their metabolism products (including the products of LP) which modify the lipid-protein contacts.

In conclusion, attention should be paid to an interesting aspect. The analysis of certain facts allows to assume, that there really exists a biological-logical relation between pathologies and alterations of the amount and cooperative properties of the Na, K-ATPase. For example, in the case of TBT cooperative Na^+ binding with Na, K-ATPase disappeared. At the same time it is known that the tumorous cells show higher Na^+ content and increased relationship of Na^+/K^+ which is connected with the

intensive transcription feature [10]. On the basis of the above-mentioned and literature facts we can put forward a hypothesis according to which the disturbance (disappearance) of the positive cooperative nature in Na^+ binding with Na, K-ATPase (as a guarantee for the effective transport Na^+ from cell), formed a basis for the Na^+ accumulation in the tumours cell that is connected with their intensive growth. Virtually, with this phenomena at Na, K-ATPase level, with simultaneos changes in enzyme amounts and with the excistence some endogenous inhibitors may, in our opinion, be explained the role of Na-pump in certain pathologies, characterized by the increased intracellular Na^+ for example, in hypertension genesis (see 7).

No doubt, the regulation of oligomeric transport and receptor proteins through their oligomerization level is one of the most universal and flexible regulatory principles, the disturbance or disappearance of which leads to certain pathological states.

References

1. Brown T.A., Horowitz B., Miller R.P., McDonough A.A., Farley R.A. Molecular cloning and sequence analysis of the $(Na^+ + K^+)$-ATPase β-subunit from dog kidney // Biochim. Biophys. Acta. - 1987. - Vol. 912. - P. 244-253.

2. Hexum T., Samson F.E., Himes R.H. Kinetic studies of membrane $(Na^+ - K^+ - Mg^{2+})$-ATPase // Biochim. Biophys. Acta. - 1974. - Vol. 212. - P. 322-331.

3. Kullisaar T., Zilmer M., Salum T., Tähepõld L. Cooperative interactions of essential cations with Na, K-ATPase in normal and tumours brain tissue // Abstr. of 14th Int. Congr. of Biochem. - Prague, 1988. - P. 111.

4. Schull G.E., Schwartz A., Lingrel J.B. Amino acid sequence of the catalytical subunit of the $(Na^+ - K^+)$-ATPase deducted from a complementary DNA // Nature. - 1985. - Vol. 316. - P. 691-695.

5. Stahl W.L. The Na, K-ATPase of nervous tissue // Neurochem. Internat. - 1988. - Vol. 8. - P. 449-476.

6. Vihalemm T., Salum T., Zilmer M., Laasik J. Protein-lipid intractions and the functioning of brain Na, K-ATPase // Abstr. of 14th Int. Congr. of Biochem. - Prague, 1988. - P. 111.

7. De Wardener H.E., Mac Gregor G.A. Le role possible d'un inhibitor circulant du transport de sodium dans l'hypertension essentielle // Nouv. presse Med. - 1980. - Vol. 9. - P. 1984-1988.

8. Болдырев А.А. Na^+, K^+-зависимая АТФаза как олигомерная система // Итоги науки и техники: сер. Биол. химия. - 1982. - Т. 17. - С. 75-146.

9. Болдырев А.А., Лопина О.Д., Прокопьева В.Д. Мембранные липиды как регуляторы межбелковых взаимодействий // Нейрохимия. - 1985. - Т. 4. - С. 80-95.

10. Казымин С.Д., Данко И.М. Энергетические особенности транспорта одновалентных катионов в клетках асцитных опухолей // Биохимия. - 1986. - Т. 51. - С. 95-102.

11. Капля А.А., Кравцов А.В., Кравцова В.В., Слуцкая Н.П., Горшняк В.П. Роль ионов магния в регуляции каталитической активности Na^+, K^+-АТФазы почек // Укр. биохим. ж. - 1988. - Т. 60. - С. 40-47.

12. Карелсон Э.И., Цильмер М.К., Тяхепыльд Л.Я. Простагландин E_2 как липофильный аллостерический модулятор Na-насоса // Вопр. мед. химии. - 1985. - Т. 31. - С. 84-87.

13. Паэсалу Э.И., Тарве У.С., Тяхепыльд Л.Я., Цильмер М.К. О Структурных и функциональных особенностях аллостерической регуляции активности Na^+, K^+-ФТФазы мозга // Вопр. нейрохимии: Сб. науч. трудов. - Л, 1977. - С. 61-70.

14. Салум Т.Т., Цильмер М.К., Куллисаар Т.Э., Вихалемм Т.Э., Тяхепыльд Л.Я. Особенности кооперативного связывания Na^+ и K^+ с Na^+, K^+-АТФазой мозга в зависимости от ее олигомерной структуры // Укр. биохим. ж. - 1988. - Т. 60. - С. 47-52.

15. Салум Т.Т., Цильмер М.К., Вихалемм Т.Э., Кулисаар Т.Э., Тяхепыльд Л.Я., Рандвере Т.О., Синисалу В.Н. Особенности температурной зависимости Na^+, K^+-АТФ-ферной реакции в нормальной и опухолевой ткани мозга // Укр. биохим. ж. - 1989. - Т. 61 (в печати).

16. Цильмер М.К., Карелсон Э.И., Салум Т.Т., Тяхепыльд Л.Я. Механизмы регуляции Na-насоса мозга природными и неприродными эффекторами // Учен. зап. Тартуск. ун-та: Вып. 766. - Тарту, 1987. - С. 151-168.

17. Цильмер М.К., Салум Т.Т., Вихалемм Т.Э. Действие цвитерионного детергента CHAPS (ХАПС) на Na^+, K^+-АТФазу и К-pNPPазу мозга // Успехи мед. науки. - Тарту, 1986. - С. 117-118.

ELECTROPHYSIOLOGICAL METHOD FOR STUDYING THE SYNAPTIC PHARMACOLOGY AT THE LEVEL OF A SINGLE PRESYNAPTIC FIBER

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Abstract

A direct undamaging micromethod for studying the synaptic pharmacology is described. The method is based on the registration of the so called quantum of EEG, i.e. the mass extracellular monosynaptic potential generated by the synapses of a single presynaptic fiber. Advantages of the method are illustrated by examples of experiments on the frog tectum. d-tubocurarine (0.1 mmol/l) differentially inhibits the quanta of EEG, $CdCl_2$ (10 - 200 μmol/l) inhibits all the quanta of EEG, and ethanol (0.2 - 0.5 mol/l) augments the presynaptic spike. When pair facilitation is also studied it is possible in some cases to differentiate between pre- and postsynaptic receptors.

Introduction

The main studies of synaptic pharmacology have been carried out with macromethods, e.g. registration of evoked potentials. Intracellular electrodes damaging the cell are also frequently used. Mass extracellular evoked responses and compound postsynaptic potentials might mislead us since relative refracterity and collective phenomena are possible when many nerve fibers are excited. Besides, as a rule, inhomogenous population of synapses is involved in these events. Synaptic pharmacology needs other approaches, stimulation of one nerve fiber and undamaging derivation of synaptic potentials are preferable.

Registration of the quantum of EEG meets these requirements. The quantum of EEG is a mass extracellular monosynaptic potential generated by a set of synapses of a single fiber when one presynaptic spike arrives in its terminal brush [5, 6]. The concept "the quantum of EEG" was introduced when

analysing theoretically the genesis of EEG, especially for understanding the high frequency EEG [5]. It has been stated that the quantum of EEG is an elementary impulse of mass potentials of the grey matter and that this impulse may be derived by the spike trigerred average of ECoG. This statement is based on the physics of bioelectricity and on the analysis of statistical structure of EEG.

Registration of the quantum of EEG is accomplished in various preparations by the neurophysiologists of the Kaunas Medical Institute [3, 4, 7, 9] and by a number of investigators in the USSR and abroad [2, 16, 17, 19]. Thus, there is a real new method in the physiology and pharmacology of central synapses. The method is equally good for proxymal and dendritic distal synapses, in all cases the population of synapses is probably homogeneous. When the presynaptic fiber or its cell is electrically stimulated we can derive the presynaptic spike of a single afferent and the extracellular postsynaptic potential, i.e. the quantum of EEG, evoked by the spike. This makes it possible to study the effects of pharmacological substances that have influence on the synaptic membrane and on the membrane of nerve fibers without complications due to pharmacological influence on the membrane potential of presynaptic cell, etc.

Some possibilities to differentiate between pre- and postsynaptic localization of pharmacological effects arise using the method of the quantum of EEG when marked frequency facilitation exists. Suppose, a blockator equally suppresses all the quanta of EEG of a train, it means the postsynaptic receptor is blocked independent of its state. Contrarily, when the first quanta of EEG are suppressed more in comparison with the later ones, the pharmacological effect is presynaptic: frequency facilitation counteracts the suppression of exocytose of the neurotransmitter [11, 13, 15]. There may be other possibilities, frequency depression may also be useful.

The choise of a neural object is very important for the success of the method. The following requirements should be observed: 1) the presynaptic cell or fiber must be accessible for electrostimulation; 2) the grey tissue where the quantum of EEG is derived should be of a clear and regular structure; 3) the presynaptic terminal brush should be compact and should have a large number of synapses; 4) the frequency facilitation should be significant.

The classical object of contemporary physiology of the CNS, the tectum opticum of the frog, meets all these requirements: 1) the presynaptic ganglion cells form a monocellular layer on the surface of the retina, among the cells there are very large ones that can be excited without irritating their neighbours; 2) cellular and molecular layers including sublayers of tangentially orientated afferent fibers are clearly marked, the apical dendites are strictly radial; 3) the terminal brush of optic afferents is of ellipsoidic shaps with dimensions 100 x 30 μm containing about a thousand synapses; 4) the pair facilitation reaches 2 [8, 10].

We suggest the registration of the quantum of EEG of the retino-tectal afferent in the frog with pair stimulation of the retina as an adequate and convenient micromethod for studying the pharmacological effects on the CNS synapses.

Methods

Surgical preparation of the animal. 0.1 - 0.2 mg of d-tubocurarino were injected intramuscularly. Ten minutes later when the frog was immobilized its oral cavity was ventilated with a moistened mixture of oxygen (95 %) and carbon dioxide (5 %). The ventilation lasted throughout the experiment. The immobilization was supported by an additional injection of 0.1 mg of d-tubocurarine every 20-30 min. 0.2 ml of 0.25 % novocaine was injected subcutaneously at the point aboive the tectum. 10-15 min later the skin was cut off with scissors, and the skull was trepanized. The dura mater was excised with special scissors, and the pia mater was eliminated with a tungsten wire sharpened to 1-2 μm, the latter operation was visually controled under a microscope. The opened surface of dorsal tectum was perfused with the Ringer solution (pH 7.2 - 7.3, 117 NaCl, 2.5 KCl, 1.8 $CaCl_2$, 1.2 $NaHCO_3$, 0.17 $NaH_2PO_4 \cdot 2H_2O$ - all the values in mmol/l). 0.02 ml of 0.25 % novocaine was introduced into each corner, besides a piece of cotton-wool moistened with novocaine was applied on the eye. Anasthesia completed, the upper eyelid, the nictating membrane and the sclera were excised. The lens, the hyaloid were sucked off. The naked retina was periodically moistened with the Ringer solution.

Electrical stimulation of the retina. According to Evert et al. [14] and our experience [9] the afferents from the nasoventral retina arrive to the dorsal tectum which is most convenient for microelectrode insertion. A block of 6 stimulating electrodes [1] was put on the nasoventral retina as much peripherally as possible because in the retinal periphery there is minimal density of ganglion cells. Every stimulating electrode is a glass-isolated 15 μm tungsten wire. One of the 30 variants of bipolar stimulation that are possible with the block of 6 electrodes may be chosen, thus, we can change the stimulated area without moving the electrodes along the retina. The distance between the centers of stimulating poles varied from 60 to 170 μm, the DC resistance was equal to 120-700 kΩ

The stimuli were one or two pulses of 1 - 5 V, 20-3 μs, the interval between the pulses in a pair was 5 - 50 ms, the frequency of stimulation was equal to 0.5 Hz. By changing the pulse duration we searched for the threshold of stimulation when the evoked response was of "all-or-nothing" type (Fig. 1). If needed, the location of the registrating microelectrode in the tectum was corrected, the stimulating bipole was changed, and sometimes even the stimulating block was moved along the retina.

Derivation. We used carbon microelectrodes [12], in recent experiments they were modified and a diminished electric noise in the low frequency range was achieved (the description of electrode modification will be published separately). According to the micrometer the microelectrodes were inserted into the depth of 0.2 - 0.3 mm where large retinal afferents terminate. The "all-or-nothing" responses consisted of a presynaptic wave, i.e. a spike of the terminal brush, and of a postsynaptic wave, i.e. the quantum of EEG, which arrived with a delay of 0.5 - 0.9 ms (Fig. 1a). Single or averaged responses were registered on the screen of an averager or a computer. The frequency of potential reading was 10.6 or 28.4 kHz, the overall frequency band was equal to 2 / 5000 Hz. When pair stimulation was applied the test quantum of EEG became larger. Pair facilitation is A_2/A_1, where A_2 and A_1 are the amplitude of the test and the conditioned quanta of EEG correspondingly. If the interval between the pulss in pair stimulation was small and the test

and the conditioned quanta of EEG overlapped, the A_2 was measured after automatic subtraction of the single response from the paired one (Fig. 1b).

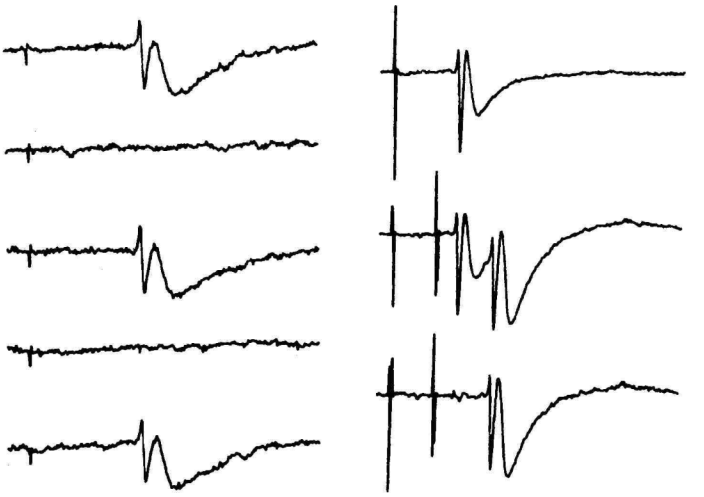

Fig. 1. - Examples of retino-tectal quanta of EEG. a) The threshold stimulus evokes "all-or-nothing" responses. At the beginning there is the stimulus artefact; the thin arrow marks the presynaptic spike, the thick one marks the quantum of EEG, average of 10 responses. 1. a response to a single stimulus, 2. a response to pair stimulation, 3. a test response obtained by subtraction of 1. from 2. Calibration is 100 μV and 5 ms, negativity is down.

Pharmacological substances were delivered to the tectum by application (d-tubocurarine solution of 0.1 mmol/l) or by perfusion with the Ringer solution at the rate of 2.5, 5 and 9 ml/h ($CdCl_2$ of 0.01 - 0.2 mmol/l, ethanol of 2.5 % or 5 %). As usual in experiments *in vivo*, we could not quantitatively determine the concentration of pharmacological substances in intercellular liquid. It is clear that this concentration is much lower in comparison with the applied or perfused solution. Due to mixing the solution with spino-cerebral liquid and due to the possible arrest of the pharmacological substance by the endogenous extracellular ligands.

Results

Some examples of the action of biologically active substances on the synaptic transmission in the CNS are given.

Specific N-cholinoblockator d-tubocurarine. Single quanta of EEG were diminished by d-tubocurarine 2-3 times 10-20 min after application (Fig. 2). The amplitude of the presynaptic spike was not sensitive to d-tubocurarine. 30 min rewashing of d-tubocurarine fully reestablished the quantum of EEG. When applied on paired quanta of EEG, d-tubocurarine acts in two different ways. In some cases it diminishes the conditioned quantum of EEG stronger than the test one, consequently, in these cases d-tubocurarine augments the pair facilitation by 0.5 - 0.9. In other cases the facilitation does not change, both quanta diminish proportionally. In the 8 former cases the control conditioned quantum of EEG was large (A_1 = 94 ± 5 μV, M ± S.E.M) but the facilitation was relatively small (f = 1.50 ± 0.02), in the 8 latter cases the opposite is true, A_1 = 69 ± 7 μV, f = 1.99 ± 0.02. We term these cases weak and strong facilitating quanta respectively. When the action of d-tubocurarine was maximal we applied Ca^{2+} (5 mmol/l) which eliminated the effect of d-tubocurarine in the group of weakly facilitating quanta; the Ca^{2+} only halfed the effects of d-tubocurarine on strongly facilitating quanta (Fig. 2b, 3).

The frequency facilitation of synaptic transmission is mainly caused by facilitated exocytosis of a neurotransmitter. When the control quantum of EEG, the control exocytosis, is dimished (enlarged) by raising extracellular Mg^{2+} (Ca^{2+}), the pair

facilitation becomes stronger (weaker) [10]. Thus, we can say that enlarged frequency facilitation by d-tubocurarine of weakly facilitating quanta of EEG

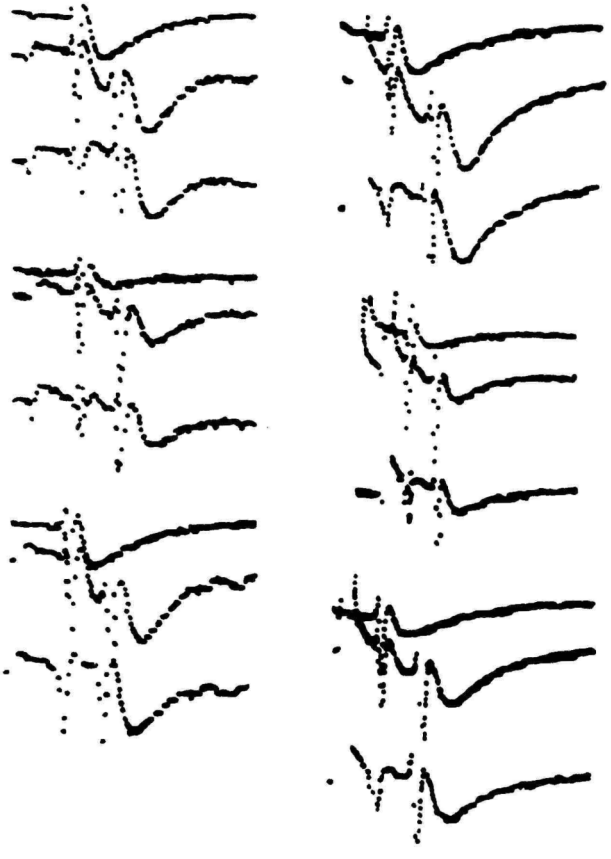

Fig. 2. - Effects of d-tubocurarine on the pairs of the quantum of EEG with weak (a) and strong (b) facilitation. 1. control, 2. 20 min after 0.1 mmol/l of d-tubocurarine, 3. 10 min after 5 mmol/l of Ca^{2+}. Facilitation equals to 1.5 ± 0.1 (1a); 2.2 ± 0.3 (2a); 1.5 ± 0.1 (3a); 1.9 ± 0.1 (1b); 1.8 ± 0.3 (2b); 1.8 ± 0.2 (3b). All the oscillograms are averages of 64 responses, calibration is 100 μV and 5 ms.

means that one of the causes of the diminishing of the amplitudes of these quanta by d-tubocurarine is its presynaptic action. This interpretation is supported by the fact of full elimination of du-tubocurrine blocade by high extracellular Ca^{2+}. We state that postsynaptic receptors in this case are not blocked by d-tubocurarine at all, the synapses are not N-cholinergic. When the quanta are strongly facilitating, when the diminishing of them by d-tubocurarine does not change the facilitation, the raised extrasellular Ca^{2+} cannot fully eliminate the d-tubocurarine effect which is ascribed to N-cholinergic nature of postsynaptic receptors.

It is clear that the method of registrating marked facilitating quanta of EEG enables us to demonstrate that d-tubocurarine may act presynaptically in the CNS, and that only a fraction of retino-tectal synapses in the frog is N-cholinergic contrarily to [18].

Neurotoxic Cd^{2+} ions. 18 quanta of EEG studied when the tectum was perfused with $CdCl_2$ solutions. The Cd^{2+} ions reversibly inhibited the quanta of EEG by 80 % on the average, while the presynaptic spikes were not appreciably changed. When the perfusion rate was raised up to 5 ml/h synaptic transmission was blocked by micromolar Cd^{2+} (Fig. 3). We noticed that synaptic delay increased by 0.15 ms when the quantum of EEG was not yet fully blocked, this effect was statistically significant. Thus, Cd^{2+} acts presynaptically primarily by blocking Ca^{2+} channels in the CNS synapses in full agreement with its action mechanism firmly established on other preparations. The cadmium in industrial waste may be toxic for human population because of its action on the CNS, too.

Ethanol. 14 quanta of EEG studied under perfusion of the Ringer solution with 0.22 or 0.54 mol/l of ethanol. The control presynaptic spikes were of 70 - 260 μV and the quanta of EEG were of 14 - 190 μV. The lower concentration enlarged the spikes by 6 - 10 % and the quanta of EEG by 7 - 22 % in 20 - 30 min, the larger concentration of ethanol enlarged the spikes by 11 - 38 % and the quanta of EEG by 14 - 34 %. In the latter case the velocity of spike propagation decreased by 3 - 10 % judging from the latency of the spikes. All the effects were reversible. The simplest explanation of the changes of the spike amplitude and velocity would be the

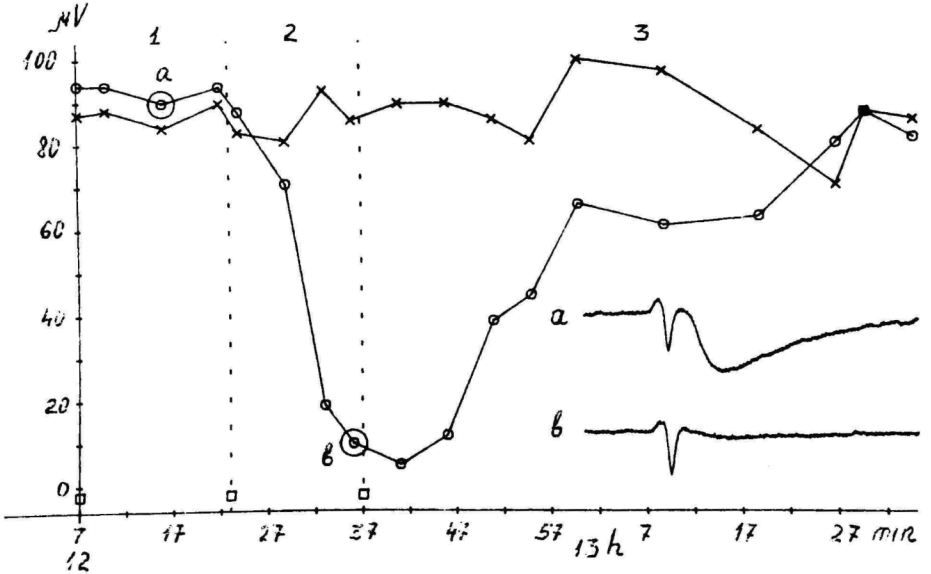

Fig. 3. - The effect of 10 μmmol/1 of $CdCl_2$ on the amplitude of the spike (crosses) and of the quantum of EEG (circles), perfusion rate is 5 ml/h. Everly point is measured on the average of 10 responses. Insert - examples of responses of points a and b respectively. 1 - control, 2 - perfusion, 3 - wash.

hyperpolarization of the membrane. The enlargement of synaptic potentials may be at least partly explained by the raised spike.

In conclusion, the micromethod of the quantum of EEG in conjunction with frequency facilitation and ionic manipulations is useful in pharmacological research of central synapses, it enables investigators to localize receptors of pharmacological substances in synaptic membranes, and to determine some features of the mechanism and specificity.

References

1. Блистрабас Р., Курас А. // Сб. науч. тр. вузов Лит. ССР. Медицина. - Вильнюс, 1988. - Т.- 28. - С. 145-148.
2. Воронин Л.Л., Кудряшов И.Е., Скребицкий В.Г. и др. // Нейрофизиология. - 1977. - Т. 9, № 2. - С. 124-134.
3. Груодис Ю., Гутман А., Курас А. и др. // Докл. АН СССР. - 1972. - Т. 204, № 5. - С. 1246-1249.
4. Груодис Ю.В., Курас А.В. // Журн. высш. нервн. деятельности им. И.П. Павлова. - Т. 29, № ,. - С. 1258-1261.
5. Гутман А.М. // Материалы VI Всесоюз. конф. по электрофизиологии центральной нервной системы. - Л.: Наука, 1971. - С. 91-92.
6. Гутман А.М. Биофизика внеклеточных токов мозга. - М., 1980. - 184 с.
7. Гутман А.М., Курас А.В. // Биофизика. - 1974. - Т. 19, № 5. - С. 894 - 898.
8. Гутман А.М., Курас А.В. // Нейрофизиология. - 1976. - Т. 8, № 4. - С. 434-437.
9. Курас А.В., Хусаиновене Н.П. // Нейрофизиология. - 1981. - Т. 13, № 6. - С. 643-646.
10. Курас А.В., Хусаиновене Н.П. // Нейрофизиология, - 1986. - Т. 18, № 1. - С. 45-55.
11. Alberts P. // Acta Physiol. Scand., Suppl. 506. - 1982. - P. 1-29.

12. Armstrong-James M., Millar J. // J. Neurosci. Meth. - 1979. - Vol. 1, N. 3. - P. 279-287.

13. Dunwiddie T.V., Haas H.L. // J. Physiol. - 1985. - Vol. 369, Dec. - P. 365-377.

14. Ewert J.-P., Hock F.J., Wiltersheim A. von // J. comp. Physiol. - 1974. - Vol. 92, N. 4. - P. 343-356.

15. Harris E.W., Cotman C.W. // Brain Res. - 1985. - Vol. 334, N. 2. - P. 348-353.

16. McIlwain J.T. // J. Neurophysiol. - 1978. - Vol. 41, N. 5. - P. 1343-1358.

17. Mendell L.M., Henneman E. // J. Neurophysiol. - 1971. - Vol. 34, N. 1. - P. 171-187.

18. Oswald R.E., Freeman J.A. // Life Sci. - 1980. - Vol. 27. - P. 527-533.

19. Witpaard J., Keurs H.E.D.J. ter // Vision Res. - 1975. - Vol. 15, N. 12. - P. 1333-1338.

THE SEASONAL VARIATIONS OF BLOOD PRESSURE, PULSE RATE, CEREBRAL BLOOD FLOW AND BRAIN MONOAMINE CONTENT IN RABBITS

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Abstract

In our previous studies a rather substantial difference between the initial values of the cerebral blood flow was found. On the other hand the brain monoamine content varies in different months of the year when studied. Comparative analysis of these parameters in rabbit brain was the aim of this paper. The content of noradrenaline (NA), dopamine (DA) and serotonine (5-HT) in cortical and subcortical structures and the cerebral blood flow (LCBF), the systemic arterial pressure (SAP) and pulse rate (PR) were learned. There were found seasonal variations in all parameters. A certain LCBF retardation in subcortical structures and cortex and the weakest effect of the stimulation was observed in May. There was a drop in SAP and some PR increase in the spring (April-May). Brain NA and 5-HT content showed seasonal changes with the lowest values near the winter months and reaching maximum in May as the PR does. Content of DA was low in May. So it seams possible that the LCBF in May can be explained by the decrease of brain DA at that time. The LCBF and the reactivity of cerebral microvessels depend on the monoamine content and show seasonal variability.

Introduction

Our previous research has proved the participation of the deep structures of the brain in the cerebral blood flow regulation. The locus in diencephalon has been found the stimulation of which causes microvessels' dilatation in the given region [17] and acceleration of local cerebral blood flow (LCBF) [18], while the systemic arterial pressure, the pulse rate and breathing do not change. Consequently, if the systemic factors regulating cerebral blood flow are stable, the regulation of cerebral blood flow will be carried out by means of

intracerebral mechanisms. As we know, the reactivity of brain has a mosaic character [12], hence the appearance of reacions having different directions and strengths in varied experiments. Alongside with that our attention was paid to a rather substantial difference between initial values. The question of the role of seasonal fluctuation arose. The present paper deals with some aspects of this problem.

Methods

Our work is based on the studies of one animal species - the rabbit, and they a lasted all the yar round, except summer. The rabbits involved were kept under similar vivarium conditions; they were given similar food. For this purpose a total of almost 300 rabbits (both male and female) in the age of less than one year were used. The research was carried out during 13 years in 3 stages.

Stage I: the systemic arterial pressure (SAP) and pulse rate (PR) were measured. For that purpose, in the animals with stereotaxically implanted electrodes, under urethan narcosis (1 g/kg i.m.) a cannula was introduced into the femoral artery. The cannula was connected to a hydrargyrum manometer via a polyvinyl tube. The SAP and PR were measured continuously during 5 min. The variations of the data of the reference group were by the month compared with those of the experimental group, whose brain structures had been electrically stimulaled (0.5 ms, 60 Hz, 1 V, 5 x 50 s).

Stage II: the local blood flow (LCBF) of an animal was measured by means of hydrogen clearance [16] during wakefulness without any premedication. Blood flow was measured simultaneously in the stimulated structure and in the precentral region of the cerebral cortex with previously implanted platinum electrodes. Stimulation (0.5 ms, 60 Hz, 2-4 V, 33 s) was conducted via a nichrome electrode. Before the experiment, the animals were subjected to a 10-day adaptation period. The experiments took place at noon under similar conditions. The LCBF initial values and those obtained after stimulation in different months were compared.

Stage III: the observations concerning the variations of brain monoamine content in rabbits under unique experimental conditions were carried out in 1972, 1973, 1981 and 1982. The content of

bioactive monoamines noradrenaline (NA), dopamine (DA) and serotonine (5-HT) in cortical and subcortical structures of the rabbit was learned.

The concentrations of NA, DA and its chief metabolite homovanillic acid (HVA) were assayed spectrofluorimetrically according to the methods described by M.K. Schellenberger and J.H. Gordon[8], and modified by P.F. Spano and N.H. Neff [9]. The serotonin (5-HT) and its metabolite 5-hydroxyindoleacetic acid (5-HIAA) concentrations were determined spectrofluorimetrically by the method of G. Curzon and A. Green [3].

For biochemical studies, the rabbits were decapitated and their brain structures were immediately frozen in liquid nitrogen. Minimally two weeks prior to each series of studies the animals were kept at a nearly constant temperature (20 °C). They were given food and water *ad libitum* and they were exposed to 12:12 hr regimen of alterating darkness and artificial light [14, 15]. The experiments were carried out in groups consisting of 2-6 animals.

Results and discussion

The initial values of SAP mostly varied within 90-120 mm Hg thus yielding the average value in a year 105 ± 1.4 mm Hg. The PR initial values for the same animals mostly 200-220 and the annual average - 210 ± 3.9 beats per minute.

When comparing the monthly initial values of the SAP and PR we found that spring data tended to differ those of autumn and winter and from the average year value. The comparison of the average data with those of the first half of the year (table 1) shows that there is a drop in blood pressure and some pulse rate increase in the spring.

Different nuclei of the thalamus, hypothalamus, septum and amygdala, as well as the ventral and dorsal hippocampus and the globus pallidus were electrically stimulated (III. 1).

The stimulation of the deep structures of the brain with the parameters given does not bring about any SAP and PR changes (table 2).

The initial values of LCBF varied in case of different rabbits. A certain variability was observed in one and the same rabbit in different experiments. The majority of the LCBF was situated in the range of 40-80 ml/100 g/min.

Table 1

The systemic arterial pressure (SAP) and the pulse rate (PR) changes from January to May (I - V)

The months	SAP (mm HG)	PR (beats per minute)
I	109.1	208.1
II	104.0	205.0
III	no data	no data
IV	97.9	212.8
V	91.5	220.9

As in the cases of SAP and PR, there are seasonal differences in the LCBF values. Thus, both in the cortex and in deep structures a certain LCBF retardation is observed in May (table 3).

Stimulation has resulted in the LCBF acceleration in deep structures and in both changes - increase or decrease of LCBF - in the cortex. The smallest effect of the stimulation is observed in May.

Consequently, in spring the SAP and LCBF of rabbits drops, while their pulse rate increases a little. At the same time, the reactivity of the brain vessels to a local irritation tends to decrease. The phenomen may have several reasons.

In order to establish the probable endogenic causes of the changes in cerebral blood flow in spring, we compared the results of our experiments with the dynamics of the monoamines' contents of the brain. The content on monoamine varies seasonally [1, 14, 15].

Some autors have shown that one of the purposes of seasonal variations of physiological parameters of experimental animals kept in constant temperature, light and food conditions are circa annual variations of geomagnetic fields. The geomagnetic storms occur usually during autumn and spring months [5]. The basic role of their structural-functional performances is influencing on neuroendocrinological regulation mechanisms.

The possible role of brain monoamines in the control of different aspects of the animal cardiovascular system has been extensively discussed in the literature [1, 6, 7].

Table 2

The systemic arterial pressure (SAP) and the pulse rate (PR) before and after the stimulation of the deep structurs of the brain

The stimulated structures	SAP (mm Hg)		PR (beats per minute)	
	Before stimulation	After stimulation	Before stimulation	After stimulation
Thalamus	107 ± 5.7	104 ± 4.7	213 ± 6.9	201 ± 4.0
Hypothalamus	103 ± 4.0	99 ± 2.2	209 ± 4.9	219 ± 7.1
Septum	109 ± 6.4	105 ± 6.1	192 ± 1.4	185 ± 5.6
Amygdala	104 ± 6.4	102 ± 6.5	216 ± 2.8	213 ± 3.9
Hippocampus	99 ± 2.0	95 ± 4.2	208 ± 6.6	198 ± 4.2
Globus pallidus	104 ± 1.7	101 ± 1.4	211 ± 7.2	209 ± 9.6
All together (n = 96)	405 ± 1.4	101 ± 1.4	210 ± 3.9	209 ± 5.9

Table 3

The local cerebral blood flow (LCBF) changes in different months (I - V = the months from January to May)

The months	LCBF of the deep structures		LCBF of the cortex (ml/100 g/min)	
	Before stimulation	After stimulation	Before stimulation	After stimulation
I	67.6	74.2	87.8	101.3
II	79.7	113.4	103.6	124.6
III	85.6	120.1	no dates	
IV	87.3	101.6	91.1	77.3
V	67.1	70.4	46.9	46.3

Note! The deep structure in these experiments are thalamus hypothalamus and septum.

From a methodological point of view, the most convenient approach to this problem seems obviously to be the search for a clearcut causal correspondence between a given pattern of cardiovascular processes and its specific biochemical correlate. Brain NA showed marked seasonal changes with the lowest values near the winter months (Table 4). From this time it began to increase, reaching its maximum in May. Thereafter a progressive and significant lowering was noticed. NA has got a calorigenic effect. The seasonal variations of muscular energetics by seasonal variation of pulse rate were shown [4].

The results of our experiments (Table 4) show that the content of 5-HT in the rabbits brain cortex has peak values in January (during the first weeks) and in May. The dynamics of the content of indolamines is connected with the variability of the functions of CNS [1]. Reciprocally to the seasonal variations of indolamine melantonine is varying the content of gonadotropic hormones (whose peak value is reached in March-April) [6]. The content of 5-HT in our experiments during these months was also low.

The variation of heart rate, blood pressure, minute volume etc. has a rather regular character with maximal values in winter [13]. In our experiments the SAP in January was higher than in May. The activation of heart rate in connection with O_2 consumption in response to exercise is higher during winter months [4].

As it is well known, the basic physiological patterns of mammals are controlled by two opposite somato-vegetative coordinating structures of the CNS. Those behavioural states characterized by a marked expenditude of energy are postulated to be modulated by the so called "ergotropic system", adrenergic in nature [7, 11]. They usually involve a high degree of responsiveness to external stimulation, sympathetic predominance and increased motor activity. On the other hand, physiological states such as sleep and winter rest, which are common traits in energy storage, could be regulated by another "trophotropic system".

The functional activation of sympathico-adrenal system is increasing in autumn and winter months. This is concerned with noradrenaline concentration change and with the increase of heart rate. During winter, when animal behaviour and life-processes

Table 4

Variability of the mean concentrations of serotonin (5-HT), noradrenaline (NA), dopamine (DA) and dopamines metabolite HVA in μg/g of wet tissue of the rabbits brain cortex during the first half of the year

Months	5-HT	NA	DA	HVA
I	0.60 ± 0.06	0.16 ± 0.01	0.38 ± 0.07	0.33 ± 0.04
II	0.47 ± 0.04	0.17 ± 0.01	0.41 ± 0.05	0.28 ± 0.02
III	0.33 ± 0.02	0.17 ± 0.02	0.72 ± 0.09	0.43 ± 0.05
IV	0.37 ± 0.05	0.19 ± 0.03	0.55 ± 0.06	0.49 ± 0.05
V	0.66 ± 0.08	0.31 ± 0.04	0.44 ± 0.04	0.64 ± 0.07
VI	0.46 ± 0.06	0.24 ± 0.02	0.51 ± 0.05	0.48 ± 0.03

clearly demonstrate the predominance of energy-saving mechanisms, with lower motor activity an important decrease in the O_2 consumption and in general metabolic rate associated with an increased storage of carbohydrate, fat and proteins in different organs is noted. In this period arterial hypotension and a lowered vasomotor responsiveness are observed [7]. All these features suggest *prima facie* a functional preponderance of parasympathic mechanism.

Conversely, the O_2 consumption and the general endocrine - metabolic rate, as well as circulatory responses are greatly enhanced during mating and estivation. These facts, in the phase with the significant and opposite displacement in the DA and 5-HT brain content again make consider a possible causal relationship between monoamines and cardio-vascular patterns in this period.

As it is known, the vasodilatation has more a DA-ergic nature and vasoconstriction takes place as a result of NA-ergic activation. So it seems possible that the low LCBF in May can be explained by the increase of brain NA and decrease of brain DA at that time. Decreasing SAP in spring may be in connection with the character of the anaesthesia, which is correlated with the increased concentrations of NA and 5-HT in the brain [11, 13]. The practically constant SAP and PR during the stimulation are due do the weak stimulus in these experimente (1 V) and to the lowered reactivity of the brain in narcosis.

It is concluded that the LCBF and the reactivity of cerebral microvessels depend on the monoamine content of the brain and show seasonal variability.

References

1. Arendt J., Wirz-Justice A., Bratke J. Circadian, diurnal and circaannual rhythms of serum melantonin (MT) and platelet serotonin (5-HT) in man // Chrinologia. - 1977. - Vol. 4, N. 2. - P. 96-97.

2. Chatfield P.O., Lyman C.P., Purpura D.P. The effects of temperature on the spontaneous and induced electrical activity in the cerebral cortex of the golden hamster // Electroenceph. clin. neurophysiol. - 1951. - Vol. 3. - P. 225-230.

3. Curzon G., Green A. Rapid method for the determination of 5-hydropytryptamine and 5-hydroxyndoleactic acid in a small regions of rat brain // Brit. J. Pharmacol. - 1970. - Vol. 39. - P. 653-655.

4. Erikssen J., Rodahl K. Seasonal variation in work performance and heart rate response to exercise // Eur. J. Appl. Physiol. - 1979. - Vol. 42, N. 2. - P. 133-140.

5. Heroux O. Circadian and circaannual variations in growth and cold resisltance of warm acclimated rats and their relationship with the geomagnetic activity // In. J. Biometeorol. - 1979. - Vol. 23, N. 1. - P. 51-62.

6. Reunberg A. Advances in clinical chronopharmacology // Trends Pharmacol. Sci. - 1979. - Vol. 1. - P. 81-83.

7. Segura E.T., D'Agostino S.A. Seasonal variations of blood pressure, vasomotor reactivity and plasmatic catecholamines in the toad // Acta Physiol. Latinoam. - 1964. - Vol. 14. - P. 231-237.

8. Shellenberger M.K., Gordon J.H. A rapid, simplified procedure for simultaneous assay of norepinephrine, dopamine and 5-hydroxytryptamine from discrete areas of brain. // Anal. Biochem. - 1971. - Vol. 8. - P. 123-130.

9. Spano P.F., Neff N.H. Procedure for the simultaneous determination of dopamine, 3-methoxy-4-hydroxyphenylacetic acid and 3,4-dihydroxyphenylacetic acid in brain // Anal. Biochem. - 1971. - Vol. 42. - P. 113-118.

10. Буреш Я., Петрань М., Зазар И. Электрофизиологические методы исследования. М.: ИЛ, 1962. - С. 384-426.

11. Деряпа Н.Р., Мошкин М.П., Посный В.С. Проблемы медицинской биоритмологий. - М: Медицина, 1985.

12. Москаленко Ю.Е., Демченко И.Т., Вайнштейн Г.Б., Зеликсон Б.В. Некоторые аспекты проблемы регуляции внутричерепного кровообращения // Первый Всесоюз. съезд нейрохирург. - М., 1972. - Т. 5. - С. 27-29.

13. Оранский И.Е. Биологические ритмы и бальнотерапия. - М.: Медицина, 1977. - 120 с.

14. Оттер М. Сезонные колебания содержания катехопатинов в мозге белых крыс. - Бюлл. эксп. биол. мед. - 1980. - № 2. - С. 215-217.

15. Оттер М.Я. Сезонные и суточные изменения содержания и скорости метаболизма серотонина в мозге мышей. - Бюлл. эксп. биол. мед. - 1982. - № 7. - С. 84-86.

16. Унификация исследований мозгового кровообращения / Под ред. Ю.Е. Москаленко. - Л.: Наука, 1986. - 35 с.

17. Шоттер А.В., Роосаар П.О. Изучение роли глубинных мозговых структур в регуляции интрацеребральной микроциркуояции. - Физиол. журн. СССР. - 1982. - Т. LXVIII, № 9. - С. 1196-1205.

18. Шоттер А.В., Шелякин А.М., Каасик А.-Э.А. Изучение роли диэнцефальных структур в регуляции мозгового кровообращения. - Физиол. журн. СССР. - 1988. - Т. IXXIV, № 3. - С. 359-366.

THE PROGRESS IN TEACHING PHARMACOLOGY IN TARTU UNIVERSITY DURING PERIOD 1944-1989

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Abstract

A short survey of the development of teaching in pharmacology in Tartu University during the period 1944-89 is given. During this period the amount of pharmacological information increased nearly two-fold but the number of hours for its presentation remained practically unchanged (150-170 hrs.). Beside the publication of text-books and many teaching aids it was necessary to revise all system of lectures, practical training and testing of students knowledge. At last a renewed teaching system was established.

Introduction

During the Second World War most of the apparatus and glassware of the chair of pharmacology had got lost. Therefore the teaching process began almost from zero. In 1944/45 there were one professor (Georg Kingisepp) and two assisting students from the 5th course.

The lectures were read to the students of medical, stomatological, pharmaceutical and veterinary faculties. There were no heating, gas or water, no laboratory animals or reactives. The lack of them produced difficulties in practical laboratory work. The vivarium was to be reconstructed, 150 frogs were caught and white mice and guinea pigs for reproduction were borrewed. The drug collection was reorganized on the basis of Soviet Pharmakopoea. During summer one of the assistants had to collect medical plants for demonstration preparations. It took 2-3 years to normalize the process of training in the department.

Until 1948 the teaching program was compiled in the department. In 1948 and in 1951 the all-union programs in pharmacology were issued. In these programs a special stress was laid on the priority and achievements of Soviet and Russian medical

science. The equipment of the department did not allow to carry out practical trainings on a contemporary level. Only the simpliest experiments, which did not need any laboratory animals could be carried out by students. In 1952, the vivarium was reconstructed and became possible to use laboratory animals. In 1952 the teaching staff became more or less stable. In the following years the equipment and materials were obtained. New apparatus for research and training was acquired. In 1965 an additional room of the department was reconstructed for experimental laboratory work, in which all tables were equipped with water, gas, compressed air and electricity. Three textbooks of pharmacology (1948, 1963 and 1982), also some teaching aids and syllabuses were published. After the formation of the Estonian Agricultural Academy in the 50-s the teaching of pharmacology to veterinary students was finished. As a new subject the clinical pharmacology was introduced. In 1964/65 and 1965/66 only a few chapters were read to 5th year students as an optional discipline. Beginning from 1971 this course became a regular one. Lectures on several topics were divided between the teachers from pharmacology department and clinical disciplines. Clinical pharmacology was read to 6th year students of medicine during 66-76 hrs. In 1981 by a decree of the USSR Ministry of Health Tartu University was determined as an experimental basis for teaching clinical pharmacology. To the lectures seminars were added. Refresher courses in pharmacology for postgraduate pharmacists were started in the 60s. From 1976, on the basis of department of pharmacology refresher courses in clinical pharmacology and pharmacotherapy for postgraduate physicians were organized.

Lectures

The course of pharmacology has traditionally consisted of lectures and laboratory training. During the period from 1944 to 1989 considerable changes in the contents and form of teaching process have taken place. In first two decades it was possible to discuss the more important preparations individually, to draw attention on their dosage and combinations used, also to present the most interesting experimental data. Later, with the growth of a number of drugs, and appearance of principally new information about mechanisms of action, it became

impossible to present such detailed information. Beginning of 1972 professor Lembit Allikmets was elected the head of department. It became necessary to change the way of presentation of the growing material. In these years a new system of presentation was worked out. It was put into proper form and several teaching materials were issued by the University Press (1972, 1977, 1981, 1987). The classification of drugs in latin also gave the students a full survey of the whole pharmacological system. In lectures a more detailed analysis of pharmacological properties of a whole drug group could be present instead of description of single preparations. A more or less certain system for presentation of the material was proposed: a short historical survey, general characteristics of the whole drug group, data about chemical structure, pharmacokinetics, mechanism of action, therapeutic and side effects, therapeutic principles and toxicity. In such a scheme it was possible only to compare the main properties of single preparations. The publication of a textbook (1982) in Estonian and textbooks in Russian enabled not to read the whole course but to concentrate on the most topical problems. The students had to work through the themes left out on their own. This part of the course has been increasing from year to year and in 1988/89 it was about 30-35 % of the whole material. As the role of student's individual work has increased, the necessity for effective system of testing their knowledge arose. During the period considered the use of visual aids in lectures has undergone considerable modifications. In the 40s and 50s the lectures were illustrated mainly by tables, especially chemical formulas drawn by the teaching staff. In the lectures drugs and pharmaceutical preparations were also demonstrated. Demonstration of experiments on animals was also of importance, although the auditorium of the Old Anatomical Theatre was not suitable for it. The lecture demonstrations were carried out either during the lecture time or concentrated on the spring term in hours for consultations. The number of lecture demonstrations was maximal in 1955-1957, then in decreased and lecture demonstrations were abandoned in the 60s. Obtaining of contemporary projecting equipment in 70s permitted us to use slides for lecture illustration. A wide collection of slides was established. Since then the demonstration of slides and transparents has become an inseparable part of lectures.

The film-projecting apparatus allowed to demonstrate films. The increased importance of projection material in lectures have supersede tables.

Practical (laboratory) training

The role of laboratory training in teaching of pharmacology has been used in order to introduce the methodological aspects of pharmacology and to work out the prescribing skill. In order to solve these assignements several different methods were used, and at last the teaching concept was changed. The traditional laboratory training in pharmacology until the mid 70s was as follows. The students worked in groups of 5-6, later 3-4. The laboratory class began with an introduction in which beside theoretical aspects the methodology of definite experiments was introduced by the teacher. After that the students carried out experiments on laboratory animals or on themselves. The results were protocolled. The training was finished with a conclusion, made by the teacher. This scheme was improved with the issue of methodological instructions, which were completed as a teaching aid in 1975. It helped to spare time. The conclusions became more detailed and were combined with the frontal questioning of the students. The role of testing students knowledge in theoretical topics was increased. In the first after-war years there was no control of theoretical knowledge, but later more and more attention has been concentrated on it. Inspite of its importance it began to limit the time for practical experimental work. Regardless of the improvements made, the classical scheme of practical training did not give satisfactory results. The preparation of experiments and their practical realizing took too much time. The students' attention was pointed principally on the technical aspects of the experiments, not so much on the results achieved. The results of the experiments, which were rather primitive from contemporary scientific stand-points, did not give any expected results in 100% of cases. Not seldom the artefacts deformed the correct results. All this made it more difficult for the teacher to form a correct conclusion and often in was not in accordance with his pedagogical principles. All this diminished the value of practical training. Taking it all into account the classical scheme of practical training was modified. Beside experiments seminars were introduced and by the end of the 70s the role of

ezperimental work practically lost its importance. In seminars papers written by the students were discussed. The discussion was with frontal questioning. Later on a new scheme of practical training still developed. The concept of training was also changed. It became clear that it was practically impossible to teach the students the contemporary pharmacological experimental methodology, moreover, for a student and especially for a practising physician the knowledge of only pharmacological methodology is not sufficient. It is important to teach and develop the pharmacological mode of thinking. A thesis was formulated: a student has to acquire the skill of choosing a proper drug considering all conditions of the assignement, also the pharmacokinetic, pharmacodynamic and toxicological properties of the drug; the student has to know how to prescribe the drug chosen. In order to develop thinking and analizing skills several methods were tried out, starting with the analysis of problematic slides, as it was done in the First Moscow Medical Institute. This system did not suit the teachers of the department and it was soon abandoned. The training of prescribing skills was carried out parallel to practical training.

In the first decades until the publication of a special teaching aid (1975), the prescribing of drugs was lectured during practical training hours. Till 60s these "minilectures" took place trough the whole years, later on they were concentrated at the beginning of the autumn term. During the laboratory training the students had to prepare the simplest prescribing forms (powders, pills, ointments, solutions ets.). Special practical trainings of prescribing skills were carried out several times in both terms. After publication of the teaching manual (1975) there was no need for those lectures any more and the time spared could be used for practical writing of the prescriptions. From the 1961/62 academic years in the autumn and spring termas special prescribing tests were introduced. The student had to write 5 prescriptions without any mistakes on condition that the spelling of the drug name and the dosage were given. As the students could usually manage it only after 4 or 5, seldom 10 or more attempts, the prescribing art considerably improved. In order to help the students prepare for these tests, a collection of prescribing tests was published (1976). This collection included not only test assignements but also the assignements basing

on indications, which were later included into spring term tests. The assignements basing on indications of principal drug effect were also analized and solved in practical training time, their importance gradually increased and the analizing tactics improved. The test collection was republished (1982, 1986). To the beginning of the 80s a standard scheme for solving such situational assignements was finally worked out. From this time the solving of situational tasks became a main part in practical training beside written papers and discussion. In the last years the solving of situational tasks has to be done as follows:

- the student words the exact effect needed;
- describes all mechanisms enabling to reach it;
- names all drug groups and drugs having the effect needed;
- while choosing the appropriate drug the student compares the pharmacokinetics, main and side effects of each drug in question;
- looks up the prescribing form, dosage, dosing schedule of the drug chosen;
- writes the prescription

Such analysis can be made either individually or in groups, also teaching aids or text-books can be used. The experience of last years shows that this scheme stimulates the students' thinking and mobilizes their passive knowledge.

The testing of students' knowledge

The final level of students' knowledge is checked on examinations in the spring term. This is traditionally an oral examination which is based on question slips, in addition to that the student has to write 2 prescriptions. This scheme has existed practically without any principal changes through all the period in question. Considerable changes have taken place in the current testing system. Until the last 2 years rather substantial testing took place each term. These tests were carried out in different years either orally or in written form. Up to the beginning the 1970s colloquiums and prescribing tests took place each term. During the last 2 years (1987/88 and 1988/89) at students' request the total number of colloquium has been increased to 7 and they have been shortened. The teaching process was planned so that the lectures and practical trainings were concentrated on certain

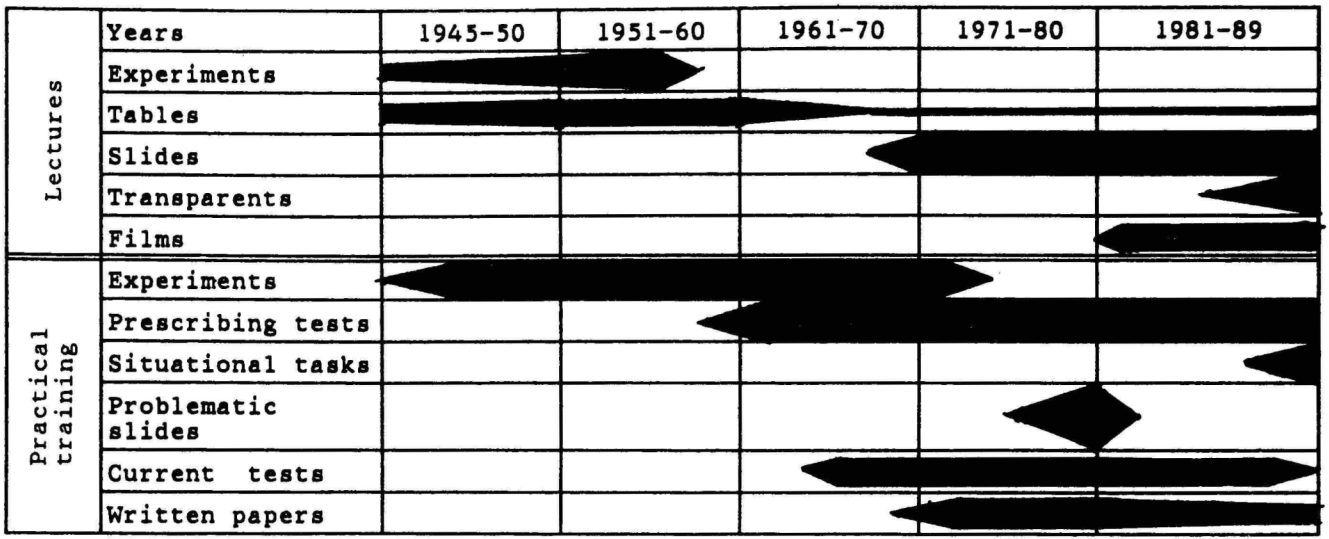

Fig. 1. Methods used in teaching pharmacology in Tartu University during period 1944-1989.

topics, each such cycle finished with consultations and with a colloquium. Thr colloquium included also problems worked trough by the students themselves.

In the middle of 60s in practical training regular tests were introduced. The form of these tests has undergone certain changes. At one time programmed tests were used, at the other either oral questioning or written tests applied. Written tests proved to be the most effective ones, permitting to assess both theoretical knowledge as well as the prescribing skills of the students. The questionnaires for the tests have been issued regularly (1979, 1982, 1988). The tests were timed at begin of training in order to check the previous level of knowledge or after training to fix the obtained information. Each version has both positive and negative aspects. The fact that the valuable time of teaching must be wasted on tests is the most negative side of their both. Testing with accompanying analysis takes at least 15–20 minutes which compared to the 90 minutes of practical training time in the spring term is apparently too much. In the last 2 years both at students' request and considering the increase of the number of colloquia these regular tests have not been used any more. The knowledge control was limited to current questioning during trainig time.

As it is shown the teaching process in the chair of pharmacology of Tartu University has continually developed. Finally the concept of lectures and practical trainings, and their contents and forms have become more contemporary and, we hope, also more effective.

Literature

Textbooks, published by the department members

1. Allikmets L. Antibiootikumid. - Tartu, 1989.
2. Allikmets L., Nurmand L. Farmakoloogia. - Tallinn: Valgus, 1982. - 1. kd.
3. Kingisepp G. Farmakoloogia. - Tartu: RK Tead. Kirj., 1948.
4. Kingisepp G. Farmakoloogia. - Tallinn: ERK, 1963.
5. Nurmand L. Üldfarmakoloogia. - Tartu, 1976; idem. - Tartu, 1977. - In Russian.

ИЗМЕНЕНИЕ ПЕРИФЕРИЧЕСКИХ БЕНЗОДИАЗЕПИНОВЫХ РЕЦЕПТОРОВ (ω_3) КРОВЯНЫХ КЛЕТОК КРЫС ПРИ РАЗЛИЧНЫХ МОДЕЛЯХ СТРЕССА: ВЛИЯНИЕ ТРАНКВИЛИЗАТОРОВ НЕБЕНЗОДИАЗЕПИНОВОГО РЯДА

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Р е з ю м е

В настоящей работе изучалось изменение периферических (ω_3) бензодиазепиновых рецепторов (БДР) при некоторых моделях стресса.

Как показали опыты, электроболевое раздражение значительно понижало связывание ^{3}H-диазепама с тромбоцитами. Плавательный стресс и процедура хронической инъекции повышали количество мест связывания ^{3}H-Ро 5-4864 как на тромбоцитах, так и на лимфоцитах.

Транквилизаторы небензодиазепинового ряда, фенибут и буспирон, в опытах *in vivo* по-разному влияли на БДР кровяных клеток. Так, фенибут 100 мг/кг значительно повышал аффинность связывания с ^{3}H-Ро 5-4864 и проявлял тенденцию к понижению числа мест связывания радиолиганда на тромбоцитах. Буспирон (5 мг/кг) не изменял параметров связывания ^{3}H-Ро 5-4864. Предварительное введение фенибута (100 мг/кг) предупреждало эффект плавательного стресса на ω_3 БДР как на тромбоцитах, так и на лимфоцитах. Буспирон (5 мг/кг) не влиял на связывание ^{3}H-Ро 5-4864 БДР тромбоцитов при плавательном стрессе, однако в этих условиях он понижал связывание радиолиганда на лимфоцитах крови крыс.

В работе обсуждаются возможные механизмы изменения ω_3 БДР кровяных клеток при разных моделях стресса. Предполагается возможность использования БДР кровяных клеток как чувствительный маркер определения эмоционального статуса.

ПЕРИФЕРИЧЕСКИЕ МЕСТА СВЯЗЫВАНИЯ БЕНЗОДИАЗЕПИНОВ НА ТРОМБОЦИТАХ И ЛИМФОЦИТАХ КРЫСЫ: ВОЗДЕЙСТВИЕ ГАМК-ЕРГИЧЕСКИХ ВЕЩЕСТВ

А.Ю. Адояан, Л.К. Ряго

Р е з ю м е

По современным представлениям агонисты ГАМК рецепторов не изменяют параметров связывния лигандов периферических бензодиазепиновых рецепторов *in vitro*.

В настоящей работе нами изучалось влияние мусцимола, дипропилацетата и фенибута на взаимодействие бензодиазепиновых радиолигандов с периферическими местами связывания (ПМС) бензодиазепинов (БД) *in vivo*. Исследование проводились на интактных тромбоцитах и лимфоцитах мечеными агонистами БР [^{3}H]Ро 5-4864 или [^{3}H] диазепамом. Как показали результаты опытов, не только агонист ГАМК рецепторов мусцимол, а также дипропилацетат и фенибут повышали аффинность периферических БД мест связывания как на тромбоцитах, так и на лимфоцитах.

Число БД мест связывания на клетках крови не изменяется под воздействием ГАМК-ергических веществ *in vivo*. В работе обсуждается модулирующая роль ГАМК-ергических веществ на ПМС БД кровяных клеток.

РОЛЬ ПОТЕНЦИАЛ-ЗАВИСИМЫХ КАНАЛОВ КАЛЬЦИЯ В РАЗВИТИИ АБСТИНЕНЦИИ ПОСЛЕ ОТМЕНЫ ХРОНИЧЕСКОГО ВВЕДЕНИЯ ДИАЗЕПАМА

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Р е з ю м е

В экспериментах на крысах самцах хроническое введение диазепама (10 мг/кг/день, в/бр., 30 дней) вызывало развитие спонтанного абстинентного синдрома. Введение этим животным антагониста бензодиазепиновых рецепторов CGS 8216 (5 мг/кг, в/бр.) вызывало дополнительное усиление признаков

абстиненции. Антагонисты каналов кальция верапамил (10 мг/кг) и нифедипин (5 и 10 мг/кг), вводимые за 30 мин до CGS 8216, ослабляли признаки абстиненции. После отмены хронического введения диазепама у животных наблюдалось снижение связывания [^{3}H]нитрендипина и одновременно увеличение захвата $^{45}Ca^{2+}$ синаптосомами в условиях деполяризации. Повышенный захват $^{45}Ca^{2+}$ после отмены диазепама угнетался добавлением диазепама (1 мкМ) или верапамила (1 мкМ) в среду инкубации. В этих концентрациях вещества не влияли на захват $^{45}Ca^{2+}$ синаптосомами контрольных животных. Предполагается, что повышение захвата кальция через активированные потенциал-зависимые каналы может вызывать развитие признаков абстиненции.

ЗЕВАНИЯ У КРЫС, ВЫЗВАННЫЕ АНТАГОНИСТАМИ КАЛЬЦИЯ

А.М. Жарковский, К.Э. Черешка, Г.Э. Цеберс

Р е з ю м е

При системом введении антагонисты кальция вызывают зевания у крыс. По силе действия вещества располагаются следующим образом: нифедипин = флунаризин > верапамил = дильтиазем. Поскольку флунаризин и верапамил вызывают зевания при их введении в желудочки мозга, следовательно, можно предполагать, что зевания, вызванные антагонистами кальция, центрального происхождения.

Совместное введение дофаминомиметиков апоморфина и квинпирола или агониста М-холинорецепторов пилокарпина с антагонистами кальция вызывает повышение этого поведенческого эффекта. Нейролептики сульпирид, галоперидол и SCH-23 390, а также антагонист М-холинорецепторов скополамин дозозависимо угнетают зевания, вызванные нифедипином. Есть основание предполагать, что в механизме возникновения зеваний задействованы потенциал-зависимые каналы кальция.

ОСОБЕННОСТИ МЕХАНИЗМА ГИПОТЕНЗИВНОГО ДЕЙСТВИЯ ЦИКЛИЧЕСКИХ АНАЛОГОВ АНГИОТЕНЗИНА II И БРАДИКИНИНА

В.В. Голубева, М.П. Раткевича, Е.А. Порункевич, В.Е. Клуша

Р е з ю м е

Нами показано участие гистаминового и серотонинового компонентов в механизме гипотензивного действия модифицированных циклических аналогов ангиотензина II и брадикинина. Блокада H_1-рецепторов дифенгидрамином и ингибирование синтеза серотонина парахлорофенилаланином достоверно уменьшает гипотензивный эффект, вызванный циклопептидами.

Найденная нами способность циклического ангиотензина и циклического брадикинина высвобождать гистамин из перитонеальных тучных клеток крыс в экспериментах *in vitro*, являясь еще одним доказательством в пользу участия гистамина в гипотензивном действии циклопептидов, дает возможность предполагать, что именно тучные клетки могут быть клетками-мишенями для циклопептидов.

Стабилизация мембран тучных клеток инталом полностью предотвращает развитие гипотенозного эффекта малых доз циклических ангиотензина и брадикинина (2 и 10 мкг/кг соответственно) и уменьшает продолжительность гипотензивного действия больших доз циклического ангиотензина и брадикинина (5 и 50 мкг/кг соответственно). Добавление интала в концентрации 0,1 мг/мл в инкубационную среду на 50 % снимает высвобождение гистамина циклическим брадикинином в дозе 10^{-6}М из перитонеальных тучных клеток крыс в экспериментах *in vitro*.

Таким образом совокупность полученных данных дает возможность рассматривать циклические производные ангиотензина и брадикинина как соединения со своеобразным механизмом действия - способностью высвобождать гистамин и серотонин из тучных клеток крыс.

Обсуждается возможность высвободить другие вазоактивные субстанции из тучных клеток крыс циклическими производными вазоактивных пептидов.

ВЛИЯНИЕ СИСТЕМНОГО ВВЕДЕНИЯ АГОНИСТОВ ХЦК-8 НА ПАРАМЕТРЫ СВЯЗЫВАНИЯ РЕЦЕПТОРОВ В ЦНС

Я. Харро, Р.-А. Кийвет, М. Пыльд, А. Ланг, Э. Васар

Изучалось влияние системного введения церулеина (0,1 - 10 мкг/кг) и пентагастрина (0,5 - 50 мкг/кг) на бензодиазепиновые, опиатные в ГАМК-рецепторы в мозге крыс. Показано, что ХЦК-8 агонисты могут вызывать изменения в параметрах связывания меченых лигандов этих рецепторов. Введение крысам церулеина и пентагастрина в низких дозах понижало число бензодиазепиновых рецепторов в фронтальной коре и повышало это число в гиппокампе. Связывание ^{3}H-ГАМК в мембранах гиппокампа тоже повышалось. Введение пентагастрина в более высоких (седативных) дозах изменяло параметры связывания с опиатными рецепторами. Влияние нейропептидов на параметры центральных рецепторов медиаторов при системном введении может быть основой поведенческих эффектов этих веществ, ограниченно проникающих через гематоэнцефалический барьер.

NMDA-РЕЦЕПТОР: ОСНОВНЫЕ ИТОГИ ИЗУЧЕНИЯ И ПЕРСПЕКТИВЫ ИСПОЛЬЗОВАНИЯ АНТАГОНИСТОВ В КЛИНИКЕ

С.А. Гутников

Р е з ю м е

Аминокислоты опосредуют около половины всей синаптической передачи в мозге млекопитающих. Из рецепторов возбуждающих аминокислот наиболее изучен N-метил-d-аспартатный (NMDA) рецептор. В обзоре рассматривается структура комплекса NMDA-рецептор/катионный канал, агонисты и антагонисты, их эффекты у животных. Обосновывается возможность использования антагонистов для лечения эпилепсии, гипоксии (в т.ч. перинатальной), инсульта, нейродегенеративных заболеваний (болезнь Альцгеймера, хорея Геттингтона, боковой амиотрофический склероз). Высказывается предположение об участии NMDA-рецепторов в патогенезе психозов и лекарственной зависимости.

УРОВЕНЬ ОЛИГОМЕРИЗАЦИИ - РЕГУЛЯТОРНЫЙ ФАКТОР ФУНКЦИОНИРОВАНИЯ ТРАНСПОРТНЫХ (И РЕЦЕПТОРНЫХ) БЕЛКОВ

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Р е з ю м е

Показано, что кооперативность Na^+ и K^+ Na, K-АТФазы мозга зависит от степени олигомеризации фермента и что лиганд-белковые взаимодействия устанавливают уровень олигомерной структуры фермента. Предполагается, что один из биологических механизмов генеза патологических процессов с повышенным внутриклеточным содержанием ионов Na (опухолевые болезни и гипертония) связан с нарушением на высшем уровне олигомеризации (аллостерически свойств) Na, K-АТФазы, которая приводит к уменьшению положительной кооперативности связывания ионов Na. Нарушение уровня олигомеризации транспортных и рецепторных белков связано с развитием патологических процессов.

ЭЛЕКТРОФИЗИОЛОГИЧЕСКИЙ МЕТОД ДЛЯ ИЗУЧЕНИЯ ФАРМАКОЛОГИИ СИНАПСОВ НА УРОВНЕ ОТДЕЛЬНОГО ПРЕСИНАПТИЧЕСКОГО ВОЛОКНА

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Р е з ю м е

В работе дана характеристика прямого неповреждающего метода для изучения фармакологии синапса. Метод основан на регистрации квантов ЭЭГ, т.е. потоков моносинаптических потенциалов, генерируемых отдельными пресинаптическими волокнами. Метод представлен на примере эксперимента проведенного на лягушках. d-Тубокурарин (0,1 Ммоль/л) избирательно угнетает кванты ЭЭГ, $CdCl_2$ (10 - 200 Мкмоль/л) полностью угнетает кванты ЭЭГ. В отличие от последних этанол (0.2 - 0.5 моль/л) повышает пресинаптические пики. Параллельно в работе изучались возможности разграничения между пре- и постсинаптическими рецепторами.

СЕЗОННЫЕ ИЗМЕНЕНИЯ СИСТЕМНОГО АРТЕРИАЛЬНОГО ДАВЛЕНИЯ, ЧАСТОТЫ ПУЛЬСА, ВНУТРИМОЗГОВОГО КРОВОТОКА И СОДЕРЖАНИЯ МОНОАМИНОВ В МОЗГЕ КРОЛИКОВ

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Р е з ю м е

В долголетних опытах на кроликах, проводимых ежемесячно, было выяснено, что системное артериальное давление (САД), частота пульса (ЧП), внутримозговой кровоток (ВМК) и содержание моноаминов - норадреналина (НА), дофамина (ДА), и серотонина (5-ОТ) - имеют сезонные варианты. Весной САД и ВКТ у кроликов понижаются, частота пульса повышается. Наряду с этим весной, особенно в мае месяце, наблюдается и максимальное содержание НА и 5-ОТ в мозге кролика, содержание ДА наоборот, понижено. Причиной понижения САД может служить и понижение содержания ДА, т.к. ДА играет роль вазоконструкции. Понижение САД весной может быть связано с характером анестезии у кроликов в этот сезон. Глубина анестезии, в свою очередь, коррелируется повышением содержания НА и 5-ОТ.

РАЗВИТИЕ ПРЕПОДАВАНИЯ ФАРМАКОЛОГИИ В ТАРТУСКОМ УНИВЕРСИТЕТЕ ЗА ПЕРИОД 1944-1989 гг.

Л.Б. Нурманд

Р е з ю м е

В статье приводятся краткие сведения о состоянии кафедры фармакологии в послевоенные годы и о ее развитии до настоящего времени. За этот период объем курса фармакологии расширился вдвое, в то время как количество часов осталось практически неизмененным. Кроме издания учебников (3) и учебно-методических пособий, надо было ввести существенные изменения в лекционный курс, лабораторные занятия и систему контроля. В частности, подробное изложение отдельных препаратов на лекциях ввиду ограниченности времени и обширности материала было заменено ознакомлением студентов с общими свойствами отдельных групп лекарственных веществ, экспериментальные работы - семинарскими занятиями и решением ситуационных задач, были введены коллоквиумы по основным главам фармакологии.

Contents

Содержание

Ученые записки Тартуского университета.
Выпуск 866.
МОЛЕКУЛЯРНАЯ ФАРМАКОЛОГИЯ РЕЦЕПТОРОВ III.
Труды по медицине.
На английском языке.
Резюме на русском языке.
Тартуский университет.
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