ISSN 0494-7304 0207-4419

TARTU ÜLIKOOLI

TOIMETISED

УЧЕНЫЕ ЗАПИСКИ ТАРТУСКОГО УНИВЕРСИТЕТА

ACTA ET COMMENTATIONES UNIVERSITATIS TARTUENSIS

929

MOLECULAR PHARMACOLOGY OF RECEPTORS IV

TARTU 1991

TARTU ÜLIKOOLI TOIMETISED
УЧЕНЫЕ ЗАПИСКИ ТАРТУСКОГО УНИВЕРСИТЕТА
ACTA ET COMMENTATIONES UNIVERSITATIS TARTUENSIS
Alustatud 1893.a. VIHIK 929 ВЫПУСК Основаны в 1893.г.

MOLECULAR PHARMACOLOGY OF RECEPTORS IV

Editor Lembit H. Allikmets

TARTU 1991

This Edition continues the series of earlier regular publications of the Department of Pharmacology, University of Tartu. First, it deals with the pharmacological studies of sigma, phencychdine and cholecystokinin receptors in the mechanism of action of antipsychotic, anxiolytic and cholinergic drugs. The other topic concerns the changes in central and peripheral benzodiazepine receptors during long term treatment with their ligands, and also the changes in calcium channels in alcohol and benzodiazepine abstinence.

Editor

CONTENTS

THE INVOLVEMENT OF SIGMA AND PHENCYCLIDINE RECEPTORS IN THE ACTION OF ANTIPSYCHOTIC DRUGS

Aavo Lang, Eero Vasar,
Jaanus Harro & Andres Soosaar
Psychopharmacology Lab, Tartu University,
202 400 Tartu, Estonia

The effects of acute and long-term treatment with typical neuroleptic haloperidol, atypical antipsychotic drug clozapine and selective sigma antagonist BMY 14802 were studied in behavioural and radioligand binding experiments. It was shown that haloperidol was the most potent drug in all provided acute behavioural studies, reflecting "antipsychotic activity". Clozapine and BMY 14802 were less potent drugs to inhibit apomorphine-, amphetamine- and MK-801-induced behaviours in the acute experiments, depending on their lower affinity for dopamine$_2$ and sigma receptors compared with haloperidol. Nevertheless, clozapine was a comparatively selective antagonist of apomorphine-induced yawning and MK-801-induced motor excitation. Chronic treatment (for 15 days) with clozapine, differently from haloperidol and BMY 14802, caused the significant increase of phencyclidine (PCP) receptor density in the rat forebrain and the increased sensitivity of rats to motor stimulating effect of PCP agonist MK-801, indicating the probable involvement of PCP receptors in the action of clozapine.

KEY WORDS: Haloperidol; Clozapine; BMY 14802; Behavioural effects; Radioligand binding; Dopamine receptors; Sigma receptors; PCP receptors

INTRODUCTION

An original classification of opioid receptor, introduced by Martin et al. [13], identifies sigma receptors as the sites accounting for the "mania" in spinal dogs induced by N-allylnormetazocine (SKF 10,047) and related benzomorphans. The psychotomimetic action of benzomorphans have since been attributed to nonopioid sites that are not sensitive to naloxone and etorphine [16]. In the radioligand binding studies the important differentiation has been made between 2 distinct binding sites for SKF 10,047: 1) the phencyclidine (PCP)

site, with low affinity for SKF 10,047, is known to be related to the N-methyl-D-aspartate (NMDA) subtype of glutamate receptor; 2) a site with high affinity for SKF 10,047, which is now known as the sigma receptor [10; 17]. This sigma receptor exhibits high affinity for some neuroleptic drugs (i.e. haloperidol, chlorpromazine) [10; 11; 19]. Several studies suggest a significant functional connection of PCP and sigma receptors with dopaminergic neurons. According to the studies of Deutch et al. [6] PCP increases dopamine release in mesolimbic/cortical region and decreases it in nigrostriatal structures. The prototypical sigma receptor agonist (+)SKF 10,047 has been shown to stimulate the activity of dopamine neurons in ventral tegmental area (A_{10}) of the rat brain [7]. Rimcazole, a selective sigma antagonist, effectively blocks the (+)SKF 10,047-induced excitation of dopamine neurons in ventral tegmental area, while having no effect on spontaneous firing of A_{10} neurons [2]. Wachtel and White [20] have demonstrated that the chronic administration of BMY 14802, a selective sigma antagonist, reduces the number of spontaneously active A_{10} dopamine cells without affecting the activity of dopamine cells in the substantia nigra (A_9).

The observation that benzomorphans with high affinity for the sigma receptor are psychotomimetics in humans has prompted the suggestion that the selective antagonists at the sigma receptor may represent a class of novel antipsychotic compounds without the extrapyramidal side effects [18]. Although, Janowsky and Berger [8] have found, that clozapine, an atypical neuroleptic, moderately active compound at dopamine$_2$ receptors is rather potent at PCP sites. Byrd et al. [1] have demonstrated that long-term treatment with haloperidol significantly increases the number of PCP binding sites in the rat brain. Thus, for clarifying the role of dopamine$_2$, PCP and sigma receptors in the action of antipsychotic drugs we studied the effects of acute and chronic treatment with the classical neuroleptic haloperidol, the atypical antipsychotic compound clozapine and the selective antagonist at sigma receptors BMY 14802 in the behavioural and radioligand binding experiments.

MATERIALS AND METHODS

Animals. Male albino rats, weighing 250–300 g, and male albino mice, weighing 25–30 g, were used in experiments. Animals were housed under standard laboratory conditions (temperature 20±3 °C), with free access to food and water.

Acute behavioural studies.

Apomorphine-induced yawning in rats. Test was performed as described by Morelli et al. [15]. Haloperidol, clozapine and BMY 14802 were injected intraperitoneally 30 min before the administra-

tion of apomorphine. Number of yawns was counted during 1h after the treatment with apomorphine (0.1 mg/kg, s.c.). The commercial solution of haloperidol (Gedeon Richter, Hungary) was diluted in the saline, BMY 14802 (Bristol-Myers, USA) was dissolved in the saline and clozapine (Sandoz, Switzerland) was made soluble in the saline with the help of 1–2 drops of Tween-85 (Ferak, Germany).

Apomorphine-induced climbing in mice was studied according to the method of Moore and Axton [14]: apomorphine (3 mg/kg, s.c.) and test compounds were injected respectively 5 min and 30 min prior to the placement of animals into the individual wire net cages, where the climbing activity was registered during 30 min.

Amphetamine- and MK-801-induced motor excitation in mice was measured in individual cylindrical cages, (Ø40 cm) with 2 photocells located in the wall. Locomotor activity was counted between 15 and 45 min after administration of amphetamine (7.5 mg/kg, s.c.) or MK-801 ((+)-5-methyl-10,11-dihydro-5-H-dibenzo[a,d] cycloheptan-5,10-imine maleate) (0.25 mg/kg, i.p.). The test compounds were injected 30 min before the measurement of motor activity.

The ED_50 values for all drugs were calculated from the dose-response curves.

Apomorphine-induced stereotyped behaviour in rats. Apomorphine (0.5 mg/kg, s.c.) was injected 30 min and the test drugs 60 min prior to the registration of stereotyped behaviour according to the scale of Costall and Naylor [5]. The stereotyped behaviour was measured simultaneously with aggressive behaviour.

Apomorphine-induced aggressiveness in rats was studied in the grouped animals (8 rats in the test cage). The animals were sensitized previously to apomorphine aggressiveness by 3-weeks chronic treatment with apomorphine (1 mg/kg daily, s.c.). The number of rats showing apomorphine (0.5 mg/kg, s.c.) induced aggressive behaviour was registered. Haloperidol, clozapine and BMY 14802 were administered 30 min before the treatment with apomorphine. In the case of apomorphine-induced stereotyped behaviour and aggressiveness the dose of drugs, inducing complete antagonism with the behavioural effects of apomorphine, was registered.

Behavioural studies after chronic treatment. Haloperidol (0.5 mg/kg daily, i.p.), clozapine (10.0 mg/kg daily, i.p.) and BMY 14802 (10.0 mg/kg daily, i.p.) were administered for 15 days. 48 h after the last injection of the test drug the MK-801-induced behaviour was investigated. MK-801 (0.2 mg/kg) was administered s.c. 30 min prior to the estimating of stereotyped behaviour according to the scale of Costall and Naylor [5]. After that the animals were placed into the open field (1 x 1 x 0.4 m). The number of crossed lines and rearings during 5 min was counted. The intensity of ataxia was measured according to the method of Contreras et al.

[4]. Apomorphine-induced behaviour was investigated also 48 h after the last injection of haloperidol, clozapine and BMY 14802. Apomorphine (0.15 mg/kg) was injected s.c. 15 min prior to the experiment. The stereotypy, number of crossed lines, rearings and head-dippings were estimated.

Binding studies. For binding studies the animals were killed by decapitation 48 h after the last injection of the drugs. The brains were rapidly removed from the skull and the brain structures were dissected on the ice. The brain structures were stored at −20°C until the following procedures. Brain tissues were thawed on the day of experiment. Pooled tissues from 4 animals were used in all radioligand experiments. Tissues were homogenized with a Potter-S homogenizer in 20 vol ice-cold 50 mM Tris-HCl buffer (pH 7.4 or 7.7 in the case of [^{3}H]spiperone, [^{3}H]haloperidol or [^{3}H]TCP, respectively). Membranes were washed twice by centrifugation at 48000 x g for 15 min. After the last centrifugation the tissues were suspended in the incubation buffer for the appropriate binding assay. The radioligand binding studies were repeated at least three times.

[^{3}H]spiperone (109 Ci/mmol, Amersham, final concentrations 0.06-2 nM) was incubated 30 min at 37°C with the membrane preparation (1 mg wet weight/tube) in the 0.5 ml of incubation buffer consisting of Tris-HCl 50 mM, NaCl 120 mM, KCl 5 mM, $CaCl_2$ 2 mM, $MgCl_2$ 1 mM (pH 7.4). The nonspecific binding was determined in the presence of 500 nM raclopride. The reaction was stopped by the rapid centrifugation at 11000 x g for 4 min.

[^{3}H]haloperidol binding. Homogenates (12 mg wet weight/tube) were incubated with increasing concentrations (2.5–80 nM) of [^{3}H]haloperidol (8.9 Ci/mmol, NEN) in the absence and presence of 10 μM haloperidol to define specific binding. Raclopride (500 nM) was added to each tube to block [^{3}H]haloperidol binding to dopamine$_2$ receptors. Incubation was carried out at room temperature in the total volume of 1 ml 50 mM Tris-HCl buffer (pH 7.7). After a 90-min incubation at room temperature membrane-bound [^{3}H]haloperidol was separated from free radioligand by rapid filtration through Whatman GF/B glass fibre filters which were presoaked with 0.05% polyethyleneimine. After filtration , the filters were washed twice (4.5 ml each) with the incubation buffer.

PCP receptors were detected on the membranes using 7.5 nM [^{3}H]TCP (60 Ci/mmol, NEN) in the presence of 2–100 nM of MK-801. The incubation of brain membranes (12 mg wet weight/tube) was carried out in the total volume of 0.5 ml 5 mM Tris-HCl buffer (pH 8.1 at 20° C) for 45 min at room temperature. The incubation was terminated by rapid filtration as described above.

The mean apparent equilibrium dissociation constants (K_d) and maximum number of binding sites (B_{max}) were calculated from

binding studies performed 48 hours after the last injection of the test drugs using nonlinear iterative computer curve-fitting program (Enzfitter) of Leatherbarrow [12].

The IC_{50} values for haloperidol, clozapine and BMY 14802 were detected using methods described above. The concentrations of [^{3}H]spiperone, [^{3}H]haloperidol and [^{3}H]TCP used in displacement experiments were 0.1 nM, 1.7 nM and 2 nM respectively. 10–12 concentrations of test drugs were used to inhibit [^{3}H]ligand binding. The experiments were repeated at least 4 times. The IC_{50} values were determined by log-plot analysis.

RESULTS

As shown in table 1, the studied drugs inhibited drug-induced behaviour in the following order of the potency: haloperidol > clozapine > BMY 14802. Clozapine and BMY 14802, differently from haloperidol, were unable to inhibit apomorphine-induced stereotypies in rats. BMY 14802 did not block also the apomorphine-induced aggressiveness in the rat. The ratios calculated between the effective doses of clozapine and BMY 14802 versus haloperidol indicated the relatively higher potency of clozapine and BMY 14802 in the inhibition of MK-801-induced motor excitation in mice and apomorphine-induced yawning behaviour in rats.

In the radioligand binding studies haloperidol was the most potent inhibitor of [^{3}H]spiperone binding at dopamine$_2$ sites, clozapine was a moderately potent compound and BMY 14802 had only weak affinity at dopamine$_2$ receptors. In [^{3}H]haloperidol binding studies at sigma sites haloperidol shared significantly higher affinity if compared to BMY 14802, whereas clozapine was ineffective to inhibit [^{3}H]haloperidol binding. All the studied compounds were ineffective to inhibit [^{3}H]TCP binding (data are not shown). It is noteworthy that the ratio between IC_{50} values of clozapine and haloperidol against [^{3}H]spiperone in the rat striatum was very similar to the ratio between ED_{50} values of clozapine and haloperidol against apomorphine-induced climbing, amphetamine-induced motor excitation and apomorphine-induced aggressiveness (table 1). In the case of comparison of BMY 14802 and haloperidol very similar relation was found between their IC_{50} values against [^{3}H]haloperidol binding in the rat cerebellum and their ED50 values against apomorphine-induced yawning, amphetamine-induced motor excitation and MK-801-induced motor excitation (table 1). MK-801 (0.2 mg/kg) caused the stereotyped behaviour and ataxia in the rat, but also increased the motor activity of the animals. Repeated treatment (for 15 days) with clozapine (10 mg/kg daily) significantly potentiated the effect of

Table 1

The antagonism of haloperidol, clozapine and BMY 14802 with the behavioural effects of apomorphine, amphetamine and MK-801 in rodents and inhibition of *in vitro* radioligand binding by haloperidol, clozapine and BMY 14802 in the rat brain

DRUG-INDUCED BEHAVIOUR	HALO-PERI-DOL	CLOZA-PINE	RATIO CLZ vs. HAL	BMY 14802	RATIO BMY vs. HAL
Apomorphine-induced yawning (rat)	0.13	3.4	26	7.5	58
Apomorphine-induced climbing (mouse)	0.35	24.0	69	45.0	129
Amphetamine-induced motor excitation (mouse)	0.37	17.0	46	30.0	81
MK-801-induced motor excitation (mouse)	0.43	6.4	15	27.0	63
Apomorphine-induced stereotypy (rat)	0.67	>31	>46	>115	>172
Apomorphine-induced aggressiveness (rat)	0.67	31	46	>115	>172
RADIOLIGAND BINDING					
[^{3}H]spiperone binding in striatum	5.5	300	55	5100	927
[^{3}H]haloperidol binding in cerebellum	1.2	>10000	>8333	83	69

ED_{50} (μmol/kg) values are presented in the case of behavioural studies, only in the case of stereotyped behaviour and aggressiveness the doses of drugs which completely block the behaviour are represented. Radioligand binding studies results are IC_{50} values in nM.

MK-801 on motor activity of the rat (table 2). Long-term treatment with haloperidol and BMY 14802 had a tendency to increase the motor excitation induced by MK-801, but this potentiation did not reach the statistical level.

Apomorphine (0.15 mg/kg) induced in the rat stereotyped behaviour and significantly decreased the motor activity in the saline treated group (table 3). The chronic pretreatment with clozapine and BMY 14802 evidently reversed the motor depressant effect of low dose of apomorphine. Long-term treatment with clozapine, differently from haloperidol and BMY 14802, also reduced the intensity of stereotyped behaviour in the rat. The chronic treatment with haloperidol also antagonized the motor depressant effect of apomorphine, however, this antagonistic effect of haloperidol was not statistically evident (table 3).

Table 2

The effect of chronic administration of haloperidol, clozapine and BMY 14802 on behavioural effects of apomorphine in the rat

TREATMENT	INTENSITY OF STEREO-TYPED BE-HAVIOUR	No OF CROSSED LINES	No OF BEARINGS	No OF HEAD-DIPS
SALINE+ SALINE	–	27.3±4.4	13.1±2.8	3.0±0.8
SALINE+ APOMORPHINE	3.0±0.1	14.3±2.5[a]	4.1±2.0[a]	1.3±0.4[a]
HALOPERIDOL+ APOMORPHINE	2.8±0.3	21.6±3.0	4.8±3.5[a]	4.4±0.6[b]
CLOZAPINE+ APOMORPHINE	2.3±0.3[b]	25.0±4.7[b]	8.0±2.6[b]	2.8±0.9
BMY 14802+ APOMORPHINE	2.8±0.4	29.0±4.5[b]	8.8±3.4[b]	4.3±1.3[b]

[a] –p<0.05 (if compared to saline+saline treated animals)

[b] –p<0.05 (if compared to saline+apomorphine treated animals) Mann-Whitney U-test

Table 3

The effect of chronic administration of haloperidol, clozapine and BMY 14802 on behavioural effects of MK-801 in the rat

TREATMENT	INTENSITY OF STEREO-TYPED BE-HAVIOUR	INTENSITY OF ATAXIA	No OF CROSSED LINES	No OF REARINGS
SALINE+ SALINE	–	–	48±4	8.4±1.4
SALINE+ MK-801	1.3±0.3	1.3±.03	97±14[a]	1.4±0.3[a]
HALOPERIDOL+ MK-801	1.6±0.3	1.8±0.3	118±12	2.3±0.8
CLOZAPINE+ MK-801	1.3±0.3	1.8±0.3	139±19[b]	6.0±2.7[b]
BMY 14802+ MK-801	1.3±0.2	1.1±0.2	120±23	2.0±0.9

[a] –p<0.05 (if compared to saline+saline treated animals)

[b] –p<0.05 (if compared to saline+MK-801 treated animals) Mann-Whitney U-test

Long-term treatment with haloperidol significantly increased the apparent number and reduced the affinity of [^{3}H]spiperone binding sites in the striatum and mesolimbic structures (figure 1). The other studied compounds clozapine and BMY 14802 seemed to affect preferentially [^{3}H]spiperone binding in the mesolimbic structures, however these changes were not statistically evident.

Only the long-term treatment with clozapine induced the increase of [^{3}H]TCP binding in the rat brain, whereas BMY 14802 and haloperidol were completely ineffective. In the frontal cortex the increase of density of PCP receptors induced by repeated administration of clozapine was statistically evident (figure 2).

The long-term treatment with BMY 14802, the selective sigma antagonist, caused the increase of density and the decrease of affinity of sigma receptors in the frontal cortex, but not in the cerebellum (figure 3). On the contrary, the chronic treatment with haloperidol decreased the density of sigma sites in the cerebellum (figure 3), but not in the frontal cortex. Long-term treatment with clozapine did not cause any statistically evident changes in [^{3}H]haloperidol binding at sigma receptors.

DISCUSSION

According to the present study, haloperidol is the most potent drug among studied compounds in the behavioural and radioligand binding studies at dopamine$_2$ and sigma receptors. Therefore, it is possible that both dopaminergic and "sigmaergic" mechanisms are involved in the action of haloperidol. The significance of sigma receptors in the modulation of amphetamine-, apomorphine- and MK-801-induced behavioural effects is obvious, because the selective sigma antagonist BMY 14802 is rather effective in most behavioural studies, except apomorphine-induced stereotypy and aggressiveness in the rat. The ratio of ED_{50} values of BMY 14802 and haloperidol against apomorphine-induced yawning, amphetamine- and MK-801-induced motor excitation is quite similar to the ratio of their IC_{50} values at sigma receptors in the rat cerebellum. The results of long-term treatment with BMY 14802 and haloperidol seem to support the idea about functional interaction between dopamine neurons and sigma receptors. Repeated treatment with haloperidol and BMY 14802 caused different changes in the binding of [^{3}H]spiperone and [^{3}H]haloperidol in the rat brain, but similar changes at the behavioural level. After long-term treatment with haloperidol and BMY 14802 apomorphine (0.15 mg/kg) is not able to suppress the motor activity of the rat and the motor stimulant effect of MK-801 is also somewhat increased.

Clozapine, differently from haloperidol and BMY 14802, is rel-

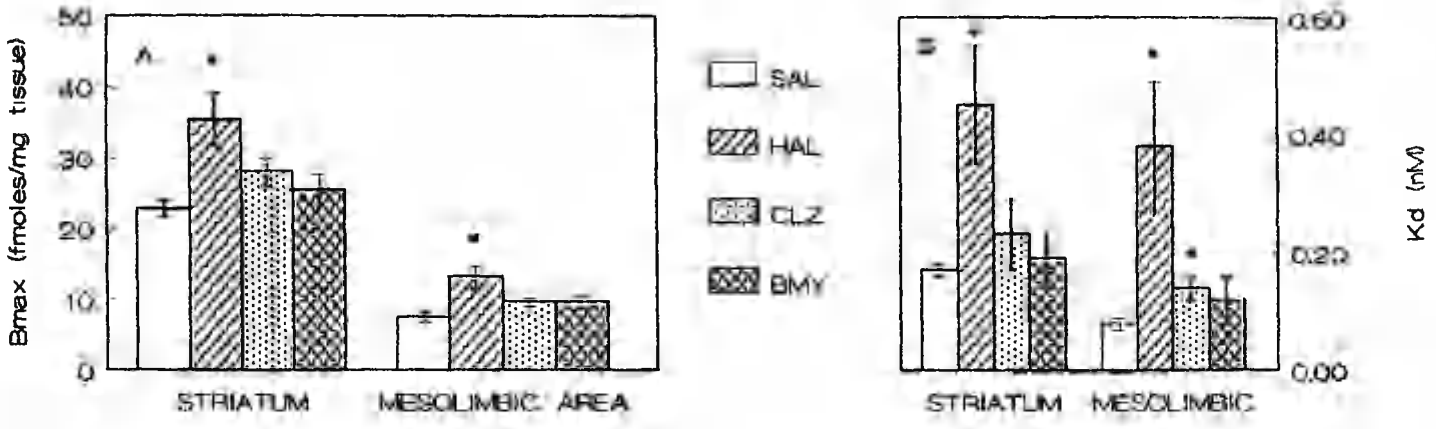

FIGURE 1. The effect of long-term treatment (for 15 days) with haloperidol, clozapine and BMY 14802 on [^{3}H]spiperone binding in the rat striatum and mesolimbic region. Part A shows density and part B shows affinity of dopamine$_2$ receptors. [B_{max} – density of binding sites; K_d – dissociation constant. * – $P<0.05$ compared to saline treated animals (Student's t-test). SAL=saline, HAL=haloperidol, CLZ=clozapine, BMY=BMY 14802]

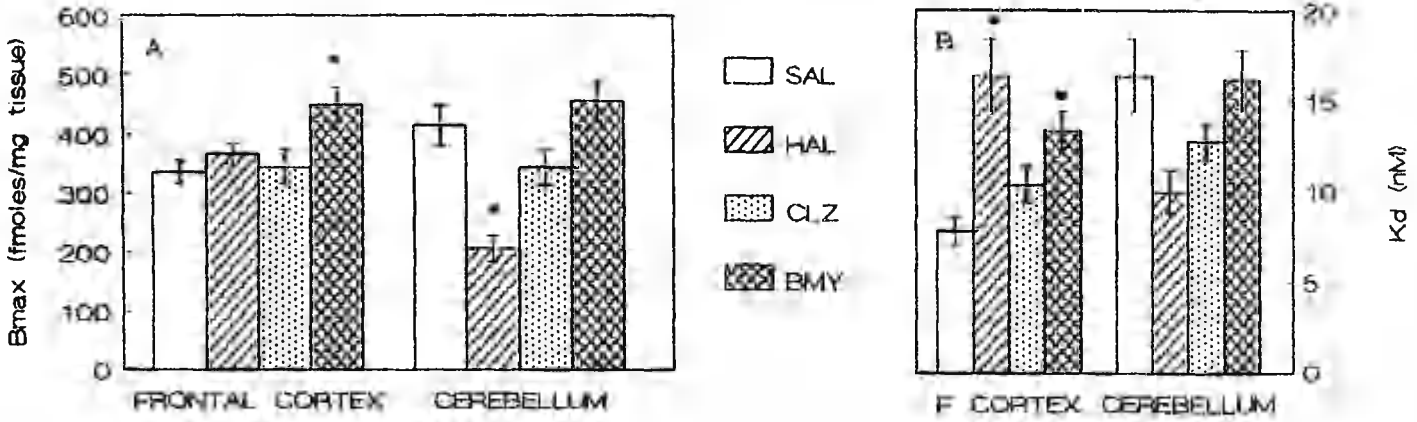

FIGURE 2. The effect of long-term treatment (for 15 days) with haloperidol, clozapine and BMY 14802 on [^{3}H]haloperidol binding in the rat frontal cortex and cerebellum (see also the explanation to fig. 1).

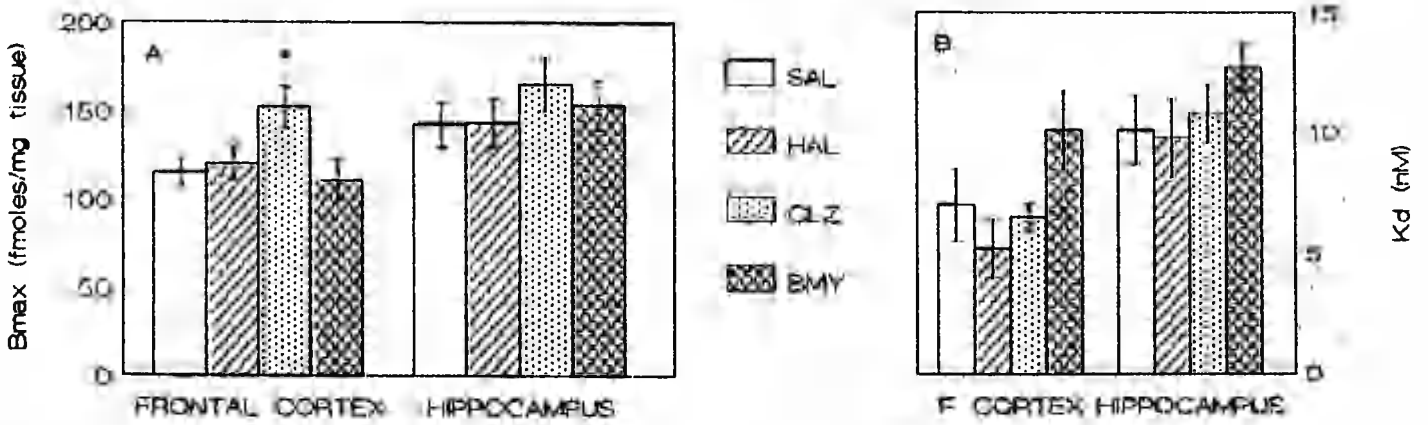

FIGURE 3. The effect of long-term treatment (for 15 days) with haloperidol, clozapine and BMY 14502 on [^{3}H]TCP binding in the rat frontal cortex and hippocampus (see also the explanation to fig. 1).

atively potent antagonist of MK-801, PCP agonist, induced motor excitation. Taking into consideration the finding of Janowsky and Berger [8] that clozapine is an effective inhibitor of [^{3}H]MK-801 binding at PCP-receptors, it is possible that this anti-MK-801 effect is explainable by direct interaction of clozapine with PCP-sites. But our experiments do not confirm this finding, clozapine even in concentrations up to 100 μM is not able to inhibit [^{3}H]TCP binding. However, there may exist the possibility that MK-801 and TCP interact with different sites at NMDA-ion channel. Long-term treatment with clozapine, differently from the action of haloperidol and BMY 14802, potentiates MK-801-induced motor excitation. This phenomenon is parallel with the increased number of PCP-sites in the rat frontal cortex after chronic administration of clozapine, i.e. a repeated treatment with clozapine induces the hypersensitivityed PCP receptors. A long-term treatment with clozapine does not significantly change the density of dopamine$_2$ receptors, but the involvement of dopamine$_2$ receptors in the action of clozapine is obvious. There is a good correlation between the ratio of IC_{50} values of clozapine and haloperidol at dopamine$_2$ receptors and their ED_{50} values in the behavioural experiments (apomorphine-induced climbing and aggressiveness, amphetamine-induced motor excitation). Clozapine is a relatively potent and selective antagonist of apomorphine-induced yawning and aggressiveness. The apomorphine-induced yawning and aggressiveness are evoked by the stimulation of dopamine$_2$ receptors [21; our unpublished data]. According to the existing data clozapine, differently from the typical neuroleptic drugs (haloperidol, chlorpromazine), selectively decreases the dopaminergic activity in the mesolimbic structures, without affecting the activity of nigrostriatal system [3]. The different interaction of clozapine with the mesolimbic and nigrostriatal dopaminergic system seems to be the reason why clozapine antagonizes apomorphine aggressiveness, but not stereotyped behaviour.

In conclusion, it is probable that not only dopamine$_2$ receptors, but also sigma and PCP receptors are involved in the action of neuroleptic drugs. The selective sigma antagonists indirectly decrease the activity of dopamine neurons, but they do not seem to be strong antipsychotic drugs, because they do not antagonize apomorphine-induced aggressiveness. Kane et al. [9] have shown that clozapine is an effective drug in the medication of schizophrenic patients resistant to conventional neuroleptic treatment. Thus, one could speculate that the interaction with PCP receptors has the significance in the beneficial clinical action of clozapine.

REFERENCES

1. **Byrd J.C., Bykov V., Rothman R.** Chronic haloperidol treatment up-regulates rat brain PCP receptors // Eur. J. Pharmacol. 1987. Vol. 140. P. 121–122.
2. **Ceci A., Smith M., French E.D.** Activation of the A_{10} mesolimbic system by the σ-receptor agonist (+)SKF 10,047 can be blocked by rimcazole, a novel putative antipsychotic // Eur. J. Pharmacol. 1988. Vol. 154. P. 53–57.
3. **Chiodo L.A., Bunney B.S.** Typical and atypical neuroleptics: differential effects of chronic administration on the activity of A_9 midbrain dopaminergic neurons // J. Neurosci. 1983. Vol. 3. P. 1607–1619.
4. **Contreras P.C., Rice K.C., Jacobson A.E., O'Donohue T.L.** Stereotyped behavior correlates better than ataxia with phencyclidine-receptor interactions // Eur. J. Pharmacol. 1986. Vol. 121. P. 9–18.
5. **Costall B., Naylor R.J.** Stereotyped and circling behaviour induced by dopaminergic agonists after lesions of the midbrain raphe nuclei // Eur. J. Pharmacol. 1974. Vol. 29. P. 206–222.
6. **Deutch A.Y., Tam S.-E., Freeman A.S., Bowers M.B., Roth Jr., R.H.** Mesolimbic and mesocortical dopamine activation induced by phencyclidine: contrasting pattern to striatal response // Eur. J. Pharmacol. 1987. Vol. 134. P. 257–264.
7. **Freeman A.S., Bunney B.S.** The effects of phencyclidine and N-allylnormetazocine on midbrain dopamine neuronal activity // Eur. J. Pharmacol. 1984. Vol. 104. P. 287–293.
8. **Janowsky A., Berger S.P.** Clozapine inhibits [^{3}H]MK-801 binding to the glutamate receptor-ion channel complex // Schizophrenia Res. 1989. Vol. 2. P. 189.
9. **Kane J., Honigfeld, G., Singer J., Meltzer H.** Clozapine for the treatment-resistant schizophrenic // Arch. Gen. Psychiatry. 1988. Vol. 45. P. 789–796.
10. **Largent B.L., Gundlach A.L., Snyder S.H.** Pharmacological and autoradiographic discrimination of sigma and phencyclidine receptor binding sites in brain with (+)- [^{3}H]SKF 10,047, (+)-[^{3}H]-3-[3-hydroxyphenyl]-N-(1- propyl)piperidine and [^{3}H]-1-[1(2-thienyl)cyclohexyl] piperidine // J. Pharmacol. Exp. Ther. 1986. Vol. 238. P. 739–748.
11. **Largent B.L., Wikstrōm H., Snowman A.M., Snyder S.H.** Novel antipsychotic drugs share high affinity for σ receptors // Eur. J. Pharmacol. 1988. Vol. 155. P. 345–347.
12. **Leatherbarrow R.J.** Enzfitter, a non-linear regression data analysis program for the IBM PC. Amsterdam, The Netherlands: Elsevier Science Publisher, 1987.
13. **Martin W.R., Eades C.G., Thompson J.A., Huppler R.E., Gilbert P.E.** The effects of morphine- and nalorphine-like drugs in the nondependent and morphine-dependent chronic spinal dog // J. Pharmacol. Exp. Ther. 1976. Vol. 197. P. 517–532.
14. **Moore N.A., Axton M.S.** Production of climbing behaviour in mice requires both D1 and D2 receptor activation // Psychopharmacol. 1988. Vol. 94. P. 263–266.

15. Morelli M., Longoni R., Spina L., Di Chiara G. Antagonism of apomorphine-induced yawning by SCH 23390: evidence against the autoreceptor hypothesis // Psychopharmacol. 1986. Vol. 89. P. 259–260.
16. Su T.P. Evidence for sigma opioid receptors: binding of [^{3}H]SKF-10,047 to etorphine-inaccessible sites in guinea- pig brain // J. Pharmacol. Exp. Ther. 1982. Vol. 223. P. 284–290.
17. Tam S.W. (+)-[^{3}H]SKF 10,047, (+)-[^{3}H]ethyl- ketocyclazocine, mu, kappa, sigma and phencyclidine binding sites in guinea pig brain membranes // Eur. J. Pharmacol. 1985. Vol. 109. P. 33–41.
18. Tam S.W., Steinfels G.F. Cook L. Biochemical behavioral aspects of sigma and phencyclidine receptors: similarities and differences // Sigma and phencyclidine-like compounds as molecular probes in biology / Ed. by E.F. Domino, J.M. Kamenka NPP Books, 1988. P. 383–396.
19. Taylor D.P., Dekleva J. Potential antipsychotic BMY 14802 selectively binds to sigma sites // Drug. Develop. Res. 1987. Vol. 11. P. 65–70.
20. Wachtel S.R., White F.J. Electrophysiological effects of BMY 14802, a new potential antipsychotic drug, on midbrain dopamine neurons in the rat: acute and chronic studies // J. Pharmacol. Exp. Ther. 1988. Vol. 244. P. 410–416.
21. Yamada K., Tanaka M., Shibata K., Furukawa T. Involvement of septal and striatal dopamine D-2 receptors in yawning behavior in rats // Psychopharmacol. 1986. Vol. 902. P. 9–13.

THE INTERACTION OF CCK ANTAGONISTS WITH THE LOCOMOTOR ACTIVITY IN THE ALBINO MOUSE

Eero Vasar, Jaanus Harro, Aavo Lang,
Anu Põld and Andres Soosaar
Psychopharmacology Lab, Institute of General and Molecular Pathology, Tartu University

The influence of CCK-A antagonist devazepide and CCK-B/gastrin antagonist L-365,260 on the locomotor activity of mice were studied in different experiments. Devazepide (1-methyl-3-(2indoloyl)amino-5-phenyl-3H-1,4-benzodiazepin-2-one) and L-365,260 (3R(+)-N-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1H-1,4-benzodiazepin-3-yl)-N'-(3-methyl-phenyl)urea) had the opposite effect on the spontaneous locomotor activity, caerulein- and apomorphine-induced hypomotility in the mouse. Devazepide in high doses (0.1–1 mg/kg IP) reduced the spontaneous motor activity, whereas L-365,260 in a high dose (1 mg/kg IP) increased the activity of mice. Devazepide (0.1–10 μg/kg) moderately antagonized the sedative effect of apomorphine (0.1 mg/kg SC) and caerulein (25 μg/kg SC), whereas L-365,260 (1–10 μg/kg) significantly potentiated the actions of dopamine and CCK agonists. Concomitant administration of caerulein (15 μg/kg SC) and apomorphine (0.1 mg/kg SC) caused nearly a complete loss of locomotor activity in the mouse. CCK-B/gastrin agonist pentagastrin (250 μg/kg SC) injected together with apomorphine (0.1 mg/kg) also caused a very signifcant reduction of motor activity. Devazepide and L-365,260 (0.1–10 μg/kg) were completely ineffective against caerulein-induced potentiation of apomorphine's hypomotility. Devazepide in high doses (0.1–1 mg/kg), reducing the spontaneous motor activity of mice, counteracted to the motor excitation induced by d-amphetamine (5 mg/kg IP). CCK agonist caerulein (100 μg/kg SC) had a similar antiamphetamine effect. Devazepide (1–100 μg/kg) and L-365,260 (1 μg/kg) completely reversed the antiamphetamine effect of caerulein.

The results of the present study are reflecting the distinct role of CCK-A and CCK-B receptors in the regulation of motor activity. The opposite effect of devazepide and L-365,260 on caerulein- and apomorphine-induced hypolocomotion is probably related to the antagonistic role of CCK-A and CCK-B receptor subtypes in the regulation of mesencephalic dopaminergic neurons. The

antiamphetamine effect of caerulein is possibly linked to the stimulation of CCK-A receptors in the mouse brain, whereas the blockade of both subtypes of the CCK-8 receptor is involved in the antiamphetamine effect of devazepide.
Key words: Caerulein; CCK-A receptors; CCK-B receptors; Devazepide; L-365,260; Locomotor activity; Apomorphine; Amphetamine; Pentagastrin

INTRODUCTION

Dopamine coexists with cholecystokinin octapeptide (CCK-8) in some mesencephalic neurons, innervating mesolimbic and cortical regions [19]. Mesolimbic dopamine is known to have a significant role in the regulation of motor activity in rodents [3,5]. The systemic treatment with CCK agonists (CCK-8 and caerulein) in low doses significantly suppresses locomotor activity in rodents [33] and in higher doses the compounds are able to block stereotyped behaviour and hyperlocomotion induced by dopamine agonists [24, 31, 33]. It is suggested that several behavioural effects of CCK-8 and caerulein are generated through the peripheral mechanisms [26]. It is thought that the motor depressant effect of CCK-8 and the suppression of dopaminergic activity by large doses of CCK agonists are of peripheral origin and could be abolished by abdominal vagotomy in rats [7, 16]. Devazepide, the highly selective antagonist at peripheral CCK (CCK-A subtype) receptors completely reversed the motor depression induced by CCK-8 in mice [22] and in rats [29]. Nevertheless, not all authors have been able to reproduce the finding that vagotomy can reverse the behavioural effects of CCK agonists in rodents. Moroji and Hagino [27] have demonstrated that bilateral subdiaphragmatic vagotomy does not prevent the behavioural effects of systemically administered caerulein in mice. The suppression of electrical self-stimulation by caerulein is completely insensitive to vagotomy in rats [10]. Altar and Boyar [1] have shown that peripherally injected CCK-8 interacts through CCK-B receptors (brain or central subtype) with the central dopaminergic mechanisms. Recently two different subtypes of the CCK receptor (CCK-A and CCK-B) have been shown to exist in the brain of rodents [11, 25]. The CCK-B subtype is ubiquitous in the brain, whereas CCK-A receptors were shown to be localized in certain discrete regions of brain, including the area postrema, nucleus of the solitary tract and the interpeduncular nucleus [18, 25]. However, the recent behavioural, electrophysiological and homogenate radioligand binding studies [2, 8, 14, 28] show CCK-A receptors to have a more widespread distribution in the brain of mammals than suggested by the above CCK autoradiographic studies.

The aim of the present study is to analyze the role of CCK-A and CCK-B receptors in the regulation of the motor activity of mice. Therefore, two highly selective CCK antagonists devazepide (CCK-A antagonist) [4] and L-365,260 (CCK-B/gastrin antagonist) [23] were used to reveal the role of CCK receptor subtypes in the regulation of motor activity and in the action of peripherally injected caerulein, an agonist at CCK receptors. The action of devazepide and L-365,260 was studied on the spontaneous motor activity, apomorphine-induced hypolocomotion and amphetamine-induced hyperlocomotion, and on the behavioural effects of caerulein (caerulein-induced hypolocomotion, potentiation of apomorphine-induced hypomotility by caerulein, antiamphetamine effect of caerulein) in mice.

METHODS

Animals. Male albino mice , weighing 20–25 g, were used throughout the study. Mice were maintained at 20±3° C with food and water ad lib. All the experiments were performed between 3 and 9 p.m.

Procedure. Spontaneous locomotor activity and hypolocomotion induced by apomorphine and caerulein were studied in an open-field. Animals were placed singly into the centre of the open-field area (30x30x18 cm, divided by lines into 16 equal squares) and observed during 3 min. The number of crossed lines, rearings and hole-dippings was counted. Apomorphine (a dopamine agonist, 0.1 mg/kg) and caerulein (a potent CCK-8 agonist, 15 and 25 μg/kg) were given subcutaneously 15 min before the experiment. CCK-B/gastrin agonist pentagastrin (0.1 and 0.25 mg/kg SC) was given, in one experiment, both alone and together with apomorphine. CCK antagonists (devazepide and L-365,260) were administered intraperitoneally 30 min prior to open-field test.

Amphetamine-induced hyperlocomotion and antiamphetamine effect of caerulein were measured in the individual cages. The cage for registration of motor activity was a cylinder with an inner diameter 40 cm and 2 photocells (located in walls) for detection of motor activity. Motor activity was counted between 15 and 45 min after intraperitoneal administration of d-amphetamine (an indirect dopamine agonist, 5 mg/kg). CCK antagonists were given intraperitoneally 15 min before the injection of d-amphetamine. Caerulein (100 μg/kg) was given subcutaneously 5 min after the administration of amphetamine.

Drugs. The following drugs were used in the present study: caerulein (Bachem), d-amphetamine (Sigma), pentagastrin (Sanitas), apomorphine (Sigma), devazepide and L-365,260 (Merck Sharp

& Dohme). Caerulein, d-amphetamine and apomorphine were prepared in saline. Some drops of 0.001 N HCl were added for stabilizing the injection solution of apomorphine. Devazepide (1-methyl-3-(2indoloyl)amino-5-phenyl-3H-1,4-benzodiazepin-2-one) and L-365,260 (3R(+)-N-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1H-1,4-benzodiazepin -3-yl)-N'-(3methyl-phenyl)urea) were made soluble in saline by adding 1–2 drops of Tween-85. The same vehicle, 1–2 drops of Tween-85 in saline, was the control injection for CCK antagonists. Each treatment was given in a volume of 0.1 ml/10 g body weight.

Statistical analysis. Results are expressed in the tables and figures as means ± S.E.M. The behavioural data were analyzed using one-way analysis of variance (ANOVA). Post hoc comparisons between individual groups were made by using Newman-Keuls test.

RESULTS

In the behavioural studies CCK antagonists (devazepide and L-365,260) in low doses failed to affect the locomotor activity of mice in an open-field (figure 1), only in high doses they were able to change the behaviour of animals. Devazepide (0.1–1 mg/kg) appeared to decrease the number of crossed lines in an open-field [$F(5,54)= 2.88$, $p< 0.05$] (figure 1), whereas L-365,260 (1 mg/kg) had the opposite effect [$F(5,54)= 2.52$, $p< 0.05$]. The systemic administration of caerulein in moderate dose (25 μg/kg) reduced the number of crossed lines [$F(1,18)=4.3$, $p< 0.05$] and head-dippings [$F(1,18)=4.1$, $p< 0.05$] in the open-field test (table 1). The pretreatment of animals with devazepide (0.1–10 μg/kg) only moderately antagonized the effect of CCK agonist. However, in high dose (100 μg/kg), devazepide even potentiated the effect of caerulein [$F(5,54)= 2.62$, $p< 0.05$ for crossings; $F(5,54)= 4.08$, $p< 0.005$] for head-dippings. L-365,260 (1–100 μg/kg) potentiated the sedative effect of caerulein [$F(5,54)= 3.64$, $p< 0.01$ for crossings; $F(5,54)= 3.49$, $p< 0.01$ for rearings; $F(5,54)= 6.53$, $p<0.0001$ for head-dippings] (table 1). Dopamine agonist apomorphine in low dose (0.1 mg/kg) reduced the motor activity of mice [$F(1,18)= 4.82$, $p< 0.05$ for crossings] (table 2). L-365,260 (1–10 μg/kg) significantly potentiated the sedative effect of apomorphine in the mouse [$F(5,54)=3.94$, $p< 0.005$ for crossed lines; $F(5,54)=2.52$, $p<0.05$ for rearings; $F(5,54)=8.04$, $p< 0.00001$ for head-dippings]. Devazepide (1–10 μg/kg) only moderately reduced the effect of apomorphine, whereas in high doses (100 and 1000 μg/kg) it potentiated,to some extent, the effect of dopamine agonist [$F(5,54)=4.68$, $p<0.001$ for crossed lines; $F(5,54)=2.83$, $p<0.05$ for rearings) (table 2). Pretreatment with caerulein (15 μg/kg) very significantly potentiated apomorphine-induced hypolocomotion in

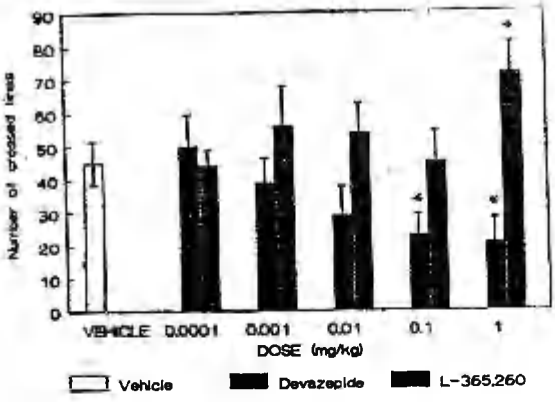

Figure 1. THE EFFECT OF CCK-8 ANTAGONISTS ON THE SPONTANEOUS MOTOR ACTIVITY OF MICE IN AN OPEN-FIELD. L-365,260 (0.0001-1 mg/kg, i.p.) and devazepide (0.0001-1 mg/kg, i.p.) were administered 30 min before the experiment. The number of crossed lines during 3 min is presented in the figure. Each bar represents the mean ± S.E.M. for 10 animals. Data subjected to one-way analysis of variance and Newman-Keuls test. * – $p < 0.05$ (significantly different from vehicle treated animals).

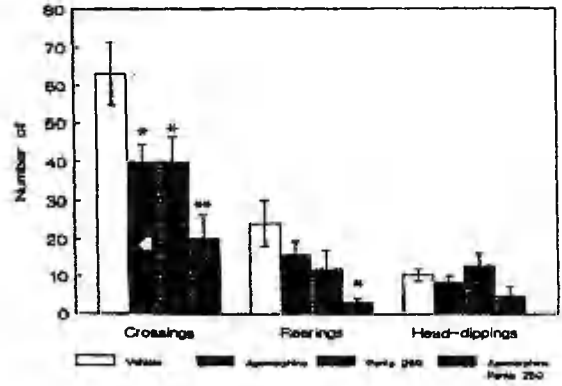

Figure 2. THE EFFECT OF PENTAGASTRIN ON APOMORPHINE-INDUCED HYPOLOCOMOTION. Apomorphine (0.1 mg/kg, s.c.) was given 15 min and pentagastrin (0.25 mg/kg, i.p.) 10 min prior to the open-field test. The number of crossings, rearings and head-dippings during 3 min is presented here. Each bar represents the mean ± S.E.M. for 10 animals. Data were subjected to one-way analysis of variance and followed by Newman-Keuls test. * – $p < 0.05$; ** – $p < 0.01$ (statistically evident difference from vehicle treated mice)

FIGURE 3

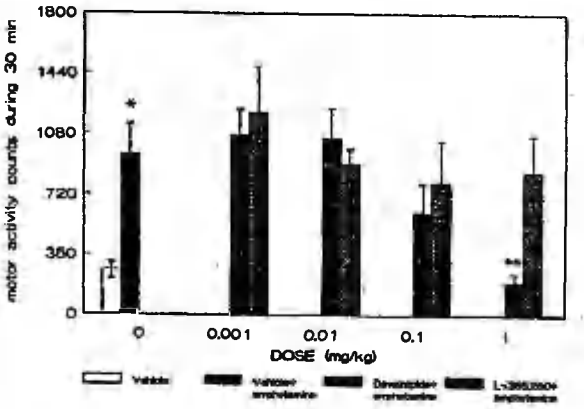

Figure 3. THE INTERACTION OF CCK ANTAGONISTS WITH AMPHETAMINE-INDUCED HYPERLOCOMOTION. CCK antagonists (0.001–1 mg/kg, i.p.) were given 15 min before d-amphetamine (5 mg/kg, i.p.), whereas d-amphetamine was injected 15 min prior to the experiment. The locomotor activity of mice was measured in the individual cages. The number of counts was registered during 30 min. Each bar represents the mean ± S.E.M. for 10 animals. Data were subjected to one-way analysis of variance and Newman-Keuls test. * – $p < 0.05$ (significantly different from vehicle treated group); ** – $p < 0.05$; *** – $p < 0.01$ (if compared to vehicle+d-amphetamine).

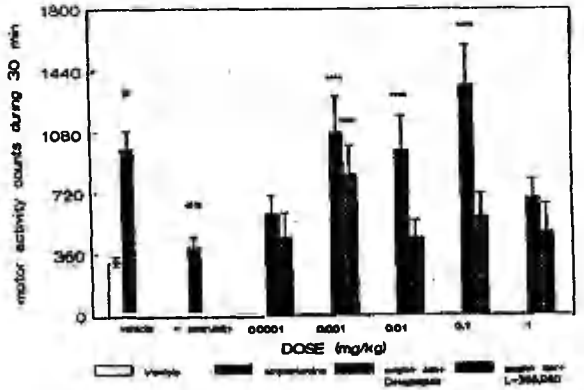

Figure 4. THE INTERACTION OF CCK ANTAGONISTS WITH ANTIAMPHETAMINE EFFECT OF CAERULEIN. CCK antagonists (0.0001–1 mg/kg, i.p.) were injected 30 min, d-amphetamine (5 mg/kg, s.c.) 15 min and caerulein (0.1 mg/kg, s.c.) 10 min before the experiment. The number of motor activity counts was registered in the individual cages during 30 min. Each bar represents the mean ± S.E.M. for 10 animals. Data were subjected to one-way analysis of variance and followed by Newman-Keuls test. * – $p < 0.05$ (significantly different from vehicle+saline); ** – $p < 0.05$ (if compared to vehicle+d-amphetamine); *** – $p < 0.05$; *** – $p < 0.01$ (if compared to d-amphetamine+caerulein).

Table 1

The interaction of L-365,260 and devazepide with hypolocomotion in the mouse

Drug/dose	Crossed lines	Rearings (during 3 min) Mean values ± S.E.M.	Head-dippings
Vehicle	77±6.2	29±6.3	22±2.9
Vehicle + caerulein 25 μg/kg	56±5.6*	16±4.3	9±1.4*
Devazepide 0.1 μg/kg + caerulein 25 μg/kg	54±5.4*	13±4.8	16±3.3**
Devazepide 1 μg/kg + caerulein 25 μg/kg	58±12.4	16±5.7	18±3.4**
Devazepide 10 μg/kg + caerulein 25 μg/kg	66±7.5	19±3.2	14±1.5**
Devazepide 100 μg/kg + caerulein 25 μg/kg	38±8.0*	13±5.7	10±1.8*
L-365,260 1 μg/kg + caerulein 25 μg/kg	35±6.8*,**	5±1.8*,**	10±1.8*
L-365,260 10 μg/kg + caerulein 25 μg/kg	41±8.5*	8±4.6*	13±2.5*
L-365,260 100 μg/kg + caerulein 25 μg/kg	40±10.5*	10±5.2*	8±1.6*
L-365,260 1000 μg/kg + caerulein 25 μg/kg	56±9.4	14±4.8	14±1.4**

CCK antagonists were administered 30 min and caerulein 15 min before the experiment. * – $p < 0.05$ (Newman-Keuls test after significant one-way ANOVA, if compared to vehicle treated mice). ** – $p < 0.05$ (Newman-Keuls test, in comparison to vehicle + caerulein treatment).

Table 2

The effect of devazepide and L-365,260 on apomorphine-induced hypolocomotion in mice

Drug/dose	Crossed lines	Rearings (during 3 min) Mean values ± S.E.M.	Head-dippings
Vehicle	60±8.8	14.2±3.0	7.4±0.9
Vehicle + apomorphine 0.1 mg/kg	40±3.3*	10.3±3.0	7.5±1.2
L-365,260 1 μg/kg + apomorphine 0.1 mg/kg	29±8.5*	5.4±1.6*	2.6±0.7*,**
L-365,260 10 μg/kg + apomorphine 0.1 mg/kg	23±5.8*,**	5.7±1.5*	2.3±0.7*,**
L-365,260 100 μg/kg + apomorphine 0.1 mg/kg	32±5.7*	7.5±2.4	2.8±0.8*,**
L-365,260 1000 μg/kg + apomorphine 0.1 mg/kg	36±5.6*	9.6±2.5	4.0±0.6*
Vehicle	79±10.2	22±4.8	7.9±2.6
Vehicle + apomorphine 0.1 mg/kg	45±5.6*	11±2.9	5.5±1.8
Devazepide 1 μg/kg + apomorphine 0.1 mg/kg	55±6.6	13±2.6	5.1±1.6
Devazepide 10 μg/kg + apomorphine 0.1 mg/kg	54±5.8	12±2.6	6.1±2.0
Devazepide 100 μg/kg + apomorphine 0.1 mg/kg	33±10.0*	6±2.7*	2.8±0.9
Devazepide 1000 μg/kg+ apomorphine 0.1 mg/kg	34±7.5*	8±2.3*	3.0±1.0

CCK antagonists were given 15 min prior to apomorphine, whereas apomorphine was injected 15 min before the experiment. * – $p < 0.05$ (Newman-Keuls test, following significant one-way ANOVA, in comparison to vehicle treated mice); ** – $p < 0.05$ (Newman-Keuls test, if compared to vehicle+apomorphine).

Table 3

The effect of devazepide and L-365,260 on caerulein-induced potentiation of motor depressant effect of apomorphine

Drug/dose	Crossed lines	Rearings (during 3 min) Mean values ± S.E.M.	Head-dippings
Vehicle	85±8	35±5.8	9.8±2.8
Vehicle + caerulein 15 μg/kg	62±12	24±3.2	7.2±1.3
Vehicle + apomorphine 0.1 mg/kg	54±3*	21±2.6*	8.5±1.6
Caerulein 15 μg/kg + apomorphine 0.1 mg/kg	15±5**	1.3±0.8**	1.9±0.6**
L-365,260 0.1 μg/kg + caerulein 15 μg/kg + apomorphine 0.1 mg/kg	20±6**	5.9±3.0**	0.8±0.3**
L-365,260 1 μg/kg + caerulein 15 μg/kg + apomorphine 0.1 mg/kg	13±5**	1.6±1.4**	2.6±1.1**
L-365,260 10 μg/kg + caerulein 15 μg/kg + apomorphine 0.1 mg/kg	10±4**	1.8±0.8**	1.8±0.7**
Devazepide 0.1 μg/kg + caerulein 15 μg/kg + apomorphine 0.1 mg/kg	13±5**	1.4±1.2**	1.6±0.7**
Devazepide 1 μg/kg + caerulein 15 μg/kg + apomorphine 0.1 mg/kg	10±5**	1.2±0.8**	1.0±0.5**
Devazepide 10 μg/kg + caerulein 15 μg/kg + apomorphine 0.1 mg/kg	21±10**	3.9±2.7**	3.0±1.3**

CCK antagonists were injected 30 min, apomorphine 15 min and caerulein 10 min before the experiment. * – $p<0.05$ (Newman-Keuls test after significant one-way ANOVA, if compared to vehicle treated mice); ** – $p< 0.01$ (Newman-Keuls test in comparison to vehicle+apomorphine treatment).

the mouse [$F(3,36)=38.4$, $p<0.000001$ for crossed lines, $F(3,36)=20.7$, $p<0.00001$ for rearings, $F(3,36)=5.01$, $p<0.01$ for head-dippings) (table 3). The coadministration of apomorphine and caerulein caused nearly a complete loss of motor activity in mice. Several animals lay motioneless in the center of open-field area. Neither devazepide, nor L-365,260 could antagonize the effect of concomitant treatment with apomorphine and caerulein (table 3). Pentagastrin (100 μg/kg) neither changed the motor activity of mice nor affected the sedative effect of apomorphine (0.1 mg/kg) (data not shown). 250 μg/kg pentagastrin statistically reduced the number of crossings (figure 2). The coadministration of pentagastrin (250 μg/kg) and apomorphine significantly suppressed the motor activity in mice, however the potentiation appeared less evident than in the case of caerulein [$F(3,36)=6.98$, $p<0.001$ for crossed lines; $F(3,36)=4.12$, $p<0.01$ for rearings].

An indirect dopamine agonist d-amphetamine (5 mg/kg) increased the number of motor activity counts nearly three times (figure 3). L-365,260 in low dose increased the effect of d-amphetamine to some extent, whereas devazepide in high dose (1 mg/kg), suppressing the spontaneous motor activity, completely antagonized the motor stimulation induced by d-amphetamine [$F(9,86)=3.1$, $p<0.005$ for 30 min period]. Caerulein (100 μg/kg) also very potently reversed the motor excitation induced by dopamine agonist (figure 4). The pretreatment of mice with devazepide in wide dose range (1–100 μg/kg) completely blocked the antiamphetamine effect of caerulein [$F(7,104)=9.56$, $p<0.000001$ for 30 min period]. The administration of L-365,260 only in low dose (1 μg/kg) also counteracted the antiamphetamine effect of CCK agonist [$F(7,104)=4.48$, $p<0.0001$ for 30 min period].

DISCUSSION

In the behavioural studies CCK-B/gastrin antagonist L-365,260 and CCK-A antagonist devazepide have the opposite effect on the spontaneous locomotor activity and on the apomorphine- and caerulein-induced hypolocomotion in mice. The spontaneous motor activity is affected only by high doses of CCK antagonists, but the hypolocomotion induced by CCK and dopamine agonists is changed by low doses of devazepide and L-365,260. Devazepide (0.1–1 mg/kg) reduces the spontaneous motor activity of mice, whereas L-365,260 (1 mg/kg) increases this behaviour. It is very puzzling that CCK antagonists affect apomorphine- and caerulein-induced hypolocomotion a similar way. Devazepide antagonizes moderately the sedative effect of low dose of apomorphine and caerulein, whereas L-365,260 significantly potentiates the action of CCK and dopami-

ne agonists. According to the existing data the motor suppressant effect of apomorphine and caerulein is thought to be related to the decreased activity of dopaminergic cells in the mesencephalon [30, 33]. The behavioural effects of CCK antagonists probably reflect the distinct role of CCK-A and CCK-B receptors in the regulation of presynaptic dopaminergic activity in the mouse brain. The stimulation of CCK-B receptors is increasing the dopaminergic activity, whereas the interaction of CCK agonist with CCK-A receptors suppresses it in the mouse brain. CCK-A receptors located in the nucleus of solitary tract seem to be involved in the motor depressant effect of CCK-8 [6, 20]. Nevertheless, it is worthy to stress that in the present study devazepide, differently from the investigation of Khosla and Crawley [22], only moderately antagonizes the motor depressant effect of apomorphine and caerulein. Hamilton et al. [17] have shown that devazepide only partially antagonizes the suppression of self-stimulation induced by caerulein in the rat. All these experiments support the idea that not only the CCK-A receptor subtype is mediating the effect of caerulein. The concomitant treatment with low dose of apomorphine and CCK agonist causes nearly a complete loss of motor activity in the mice. The animals are lying motionless in the middle of the open field. Devazepide and L-365,260 in low doses, not affecting per se locomotor activity of animals, are completely ineffective against the motor depression induced by the simultaneous administration of caerulein and apomorphine. Nevertheless, according to the studies of Hommer et al. [21] and Crawley [9] CCK receptors potentiating dopamine-induced hypolocomotion and suppression of the electrical activity of dopamine neurons in the rat mesencephalon are belonging to the CCK-B subtype. Altar and Boyar [1] have found that the antagonistic effect of CCK-8 agonists (CCK-8, desulfated CCK-8 and CCK-4) on amphetamine evoked dopamine release in the mouse striatum is also related to the CCK-B receptor subtype. The similar potentiation of apomorphine's effect by pentagastrin and caerulein seems to support the above mentioned idea that CCK-B receptors are involved in the suppression of dopaminergic activity in the mesencephalon. Thus, one could speculate that the subtype of CCK-B receptors insensitive to L-365,260 is existing on the mesencephalic dopaminergic neurons. It is quite possible that these CCK-B receptors and CCK-A receptors belonging to the nucleus of the solitary tract are related to the motor depressant effect of caerulein in mice.

The interaction of CCK antagonists with antiamphetamine effect of caerulein and amphetamine-induced hyperlocomotion is somewhat different from their action on CCK and dopamine agonists elicited hypolocomotion. It is suggested that the different pharmacology of CCK-8 against dopamine-induced hypolocomotion and

hyperlocomotion is related to the involvement of distinct brain regions in the development of two opposite behavioural effects of dopamine in the rat [8, 9]. The potentiation of dopamine-induced hypolocomotion is linked to the interaction of CCK-8 with dopamine "autoreceptors" in the ventral tegmental area, whereas the potentiation of dopamine-induced hyperlocomotion is related to the posteromedial part of the nucleus accumbens [9]. CCK-B/gastrin antagonist L-365,260 does not change significantly amphetamine-induced hyperlocomotion, but paradoxically it reverses in the low dose the antiamphetamine effect of caerulein. The effect of devazepide is dependent on the dose of CCK-A antagonist used. In low doses, interacting with CCK-A receptors, it completely antagonizes the antiamphetamine effect of caerulein, but in high doses, interacting also with CCK-B receptors (see 12, 13), devazepide per se reverses the effect of d-amphetamine. The antiamphetamine effect of devazepide is in agreement with our previous studies where the other CCK antagonist proglumide (5–15 mg/kg) also blocks the effect of d-amphetamine (our unpublished data). According to the studies of Moroji and Hagino [27] antiamphetamine effect of caerulein in mice is completely resistant to the vagotomy. It is worthy to note that nearly 10 times higher doses of caerulein are required for blocking the amphetamine-induced hyperlocomotion as compared to the sedative effect of caerulein. Accordingly, it seems very probable that CCK-A receptors involved in the antiamphetamine effect of caerulein are distinct from the CCK-A receptors related to caerulein and apomorphine-induced hypolocomotion. The above mentioned study [27] is raising the possibility that these CCK-A receptors are located in the mouse brain. The study by Hagino et al. [15] also supports the idea that it is the intracerebroventricular administration of CCK-8 and caerulein, but not desulfated CCK-8 and CCK-4, that antagonizes amphetamine caused motor excitation in the mouse. The recent behavioural, electrophysiological and radioligand binding studies [9, 28, 32] have established even wider distribution of CCK-A receptors in the rat brain than it has been stated previously [18, 25]. The possible mediation of antiamphetamine effect of caerulein through the CCK-A receptors in the mouse brain is reflecting the substantial difference between CCK-A receptors in the mouse and rat brain. Crawley et al. [8, 9] have shown that CCK-8 by interacting with CCK-A receptors is facilitating dopamine-induced hyperlocomotion in posteromedial part of the nucleus accumbens of the rat. The different pharmacology of CCK-A receptors in the mouse and the rat brain seems to account for the interspecies differences in the behavioural effects of caerulein in the mouse and the rat [31].

In conclusion, CCK-A and CCK-B receptor subtypes seem to have a distinct role in the regulation of motor activity. The opposite

effect of devazepide and L-365,260 on caerulein- and apomorphine-induced hypolocomotion is probably reflecting the antagonistic role of CCK-A and CCK-B receptor subtypes in the regulation of mesencephalic dopaminergic cells. The stimulation of CCK-A receptors seems to suppress their activity, whereas the stimulation of the CCK-B receptor subtype has the opposite effect. Nevertheless, another CCK-B receptor subtype, completely insensitive to L-365,260, is possibly involved in the CCK agonist-induced potentiation of apomorphine's hypomotility. The antiamphetamine effect of caerulein seems to be linked to the stimulation of CCK-A receptors in the mouse brain, whereas the blockade of both subtypes of the CCK-8 receptor is probably involved in the antiamphetamine effect of devazepide.

Acknowledgement. The authors wish to thank Merck Sharp & Dohme for the generous gift of devazepide and L-365,260.

REFERENCES

1. Altar C.A.0, Boyar W.Cc Brain CCK-B receptors mediate the suppression of dopamine release by cholecystokinin // Brain Res. 1989. Vol. 483. P. 321–326.
2. Barrett R.W., Steffey M.E., Wolfram C.A.W. Type-A CCK sites in cow brain: characterization using (–)-[^{3}H]-L-364,718 membrane binding assays // Mol.Pharmacol. 1989. Vol. 36. P. 285–290.
3. Bradbury A.J., Costall B., Naylor R.J., Nenmeyer J.L. Motor inhibition induced by apomorphine derivatives in the mouse. // J.Pharm.Pharmacol. 1983. Vol. 35 P. 494–499.
4. Chang R.S.L., Lotti V.J. Behavioral and pharmacological characterization of an extremely potent and selective nonpeptide cholecystokinin antagonist // Proc.Natn.Acad.Sci. USA 1986. Vol. 83. P. 4923–4926.
5. Costall B., Domeney A.M., Naylor R.J. The continuity of dopamine receptors antagonism can dictate the long-term behavioural consequences of a mesolimbic infusion of dopamine. Neuropharmacol. 1985. Vol. 24. P. 193–197
6. Crawley J.N., Schwaber J.S. Abolition of the behavioral effects of cholecystokinin following bilateral radiofrequency lesions of parvocellular subdivision of the nucleus tractus solitarius // Brain Res. 1984. Vol. 295. P. 289–299.
7. Crawley J.N., Kiss J.Z. Tracing the sensory pathway from the gut to brain regions mediating the actions of cholecystokinin on feeding and exploration // Ann.N.Y.Acad.Sci. 1985. Vol. 448. P. 586–588
8. Crawley J.N., Stivers J.A., Blumstein L.K., Paul S.M. Cholecystokinin potentiates dopamine-mediated behaviors evidence for modulation specific to a site of coexistence // J.Neurosci. 1985. Vol. 5. P. 1972–1983.
9. Crawley J.N. Microinjection of cholecystokinin into the rat ventral tegmental area potentiates dopamine-induced hypolocomotion // Synapse. 1989. Vol. 3. P. 346–355.

10. De Witte P., Goldman S., Gewiss M., Poels J.F., Van Boxez P., Van Der Veken E., Vanderhaeghen J.J. Similar effect of caerulein on intracranial self-stimulation in vagotomized and non-vagotomized rats // Neurochem.Int. 1986. Vol. 8. P. 339–343.
11. Dourish C.T., Hill D.R. Classification and function of CCK receptors // Trends Pharmacol.Sci. 1987. Vol. 8. P. 207–208.
12. Dourish C.T., Rycroft N., Iversen S.D. Postponement of satiety by blockade of brain cholecystokinin (CCK-B) receptors // Science. 1989. Vol. 245. P. 1509–1511.
13. Dourish C.T., O'Neill M.F., Coughlan J., Kitchener S.J., Hawley D., Iversen S.D. The selective CCK-B antagonist L-365,260 enhances morphine analgesia and prevents morphine tolerance in the rat: evidence for mediation of CCK/opioid interactions by CCK-B receptor // Eur.J.Pharmacol. 1990. Vol. 176. P. 35–44.
14. Gerhardt G.A., Friedmann M., Brodie M.S., Vickroy T.W., Gratton A.P., Hoffer B.J., Rose G.M. The effect of cholecystokinin (CCK-8) on dopamine-containing nerve terminals in the caudate nucleus and nucleus accumbens of the anesthetized rat: an in vivo electrochemical studies // Brain Res. 1989. Vol. 499. P. 157–163
15. Hagino Y., Moroji T., Iizuka R. A behavioural pharmacological study on intracerebroventricularly administered CCK-8 related peptides in mice // Neuropeptides 1989. Vol. 13. P. 107–113.
16. Hamamura T., Kazahaya Y., Otsuki S. Ceruletide suppresses endogenous dopamine release via vagal afferent system studied by in vivo intracerebral dialysis // Brain Res. 1989. Vol. 483. P. 78–83.
17. Hamilton M.H., Rose LC., Herberg L.J., De Belleroche J.S. Effect of intracerebroventricular and systemic injections of caerulein, a CCK analogue, on electrical self-stimulation and its interaction with the CCK-A receptor antagonist, L-364,718 (MK-329) // Psychopharmacol. 1990. Vol. 101. P. 384–389.
18. Hill D.R., Campbell N.J., Shaw T.M., Woodruff G.N. Autoradiographic localization and characterization of peripheral type CCK receptors in rat CNS using highly selective nonpeptide CCK antagonists // J.Neurosci. 1987. Vol. 7. P. 2967–2976.
19. Hökfelt T., Rehfeld J.F., Skirboll L., Ivemark B., Goldstein M., Markey K. Evidence for coexistence of dopamine and CCK in mesolimbic neurones // Nature (London) 1980. Vol. 285. P. 476–478.
20. Hommer D.W., Palkovits M., Crawley J.N., Paul S.M., Skirboll L.R. Cholecystokini-induced excitation in the substantia nigra: evidence for peripheral and central components // J.Neurosci. 1985. Vol. 5. P. 1387–1397.
21. Hommer D.W., Stoner G., Crawley J.N., Paul S.M., Skirboll L.R. Cholecystokinin-dopamine coexistence: electrophysiological actions corresponding to cholecystokinin receptor subtype // J.Neurosci. 1986. Vol. 6. P. 3039–3043.
22. Khosla S., Crawley J.N. Potency of L-364,718 as an antagonist of the behavioural effects of peripherally administered cholecystokinin // Life Sci. 1988. Vol. 42. P. 153–159.

23. Lotti V J., Chang R.S.L. A new potent and selective non-peptide gastrin antagonist and brain cholecystokinin receptor (CCK-B) ligand: L-365,260 // Eur.J.Pharmacol. 1989. Vol. 162. P. 273–280.
24. Matsubara K., Matsushita A. β-endorphin involvement in the antidopaminergic effect of caerulein // Jap.J.Pharmacol. 1986. Vol. 40. P. 417–422.
25. Moran T.H., Robinson P.H., Goldrich M., Mc Hugh P.R. Two brain CCK receptors: implications for behavioral actions // Brain Res. 1986. Vol. 362. P. 175–179.
26. Morley J.E. Behavioral effects of administered cholecystokinin // ISI Atlas Pharmacol. 1987. Vol. 1. P. 49–51.
27. Moroji T., Hagino Y. Bilateral subdiaphragmatic vagotomy does not prevent the behavioural effects of systematically administered ceruletide in mice // Neuropeptides. 1987. Vol. 9. P. 217–224.
28. Rovati L. New pentanoic acid derivatives with potent CCK antagonistic properties: different activity on periphery vs. central nervous system // Cholecystokinin antagonists / Eds. R.Y. Wang, R. Schoenfeld. New York. Alan R. Liss, Inc., 1988. P. 1–11.
29. Soar J., Hewson G., Leighton G.E., Hill R.G., Hughes J. L-364,718 antagonizes the cholecystokinin-induced suppression of locomotor activity // Pharmacol.Biochem.Behav. 1989. Vol. 33. P. 637–640.
30. Strômbom U. Antagonism by haloperidol of locomotor depression induced by small doses of apomorphine // J.Neural Transm. 1977. Vol. 40. P. 191–194.
31. Vasar E., Allikmets L., Ryzhov L, Prakhye L, Soosaar A., Mirzayev S. Intraspecies differences in the behavioural effects of caerulein, an agonist of CCK-8 receptors, in mice and rats // Bull.Exp.Biol.Med. 1988. Vol. 105. P. 168–171.
32. Vickroy T.W., Bianchi B.R. Pharmacological and mechanistic studies of cholecystokinin-faciliatated [^{3}H]-dopamine efflux from rat nucleus accumbens // Neuropeptides. 1989. Vol. 13. P. 43–50.
33. Zetler G, Neuropharmacological profile of cholecystokinin-like peptides // Ann.N.Y.Acad.Sci. 1985. Vol. 448. P. 448–469.

PILOCARPINE-INDUCED LIMBIC SEIZURES — AN INVOLVEMENT OF CCK RECEPTORS

E. Vasar, J. Harro, A. Lang, A. Soosaar
Psychopharmacology Lab, Tartu University

A muscarinic agonist pilocarpine (380 mg/kg) induced in all injected male mice the fatal seizures. The pretreatment of mice with CCK-8 (25–200 μg/kg) antagonized significantly the effect of pilocarpine, whereas the CCK-B/gastrin agonist pentagastrin (CCK-5, 2500 μg/kg) only moderately inhibited the action of muscarinic agonist. Devazepide (10–1000 μg/kg), a selective antagonist at CCK-A receptors, and L-365,260 (10–1000 μg/kg), a selective antagonist at CCK-B receptors, antagonized the anticonvulsant effect of CCK-8. However, only a high dose (1 mg/kg) of devazepide and L-365,260 reversed significantly the action of CCK-8.

In rats the administration of pilocarpine (380 mg/kg) decreased significantly the number of [^{3}H]-pCCK-8 binding sites in the several forebrain structures (the frontoparietal cortex, striatum and hippocampus). The comparison of [^{3}H]-pCCK-8 binding in the brain structures of rats with and without seizures revealed evidently higher decrease of CCK-8 receptors' density in animals experiencing seizures. In the hippocampus the difference between the values of responders and non-responders was statistically evident. The significant reduction of [^{3}H]-pCCK-8 binding density in the rat brain during pilocarpine-induced seizures probably reflects the involvement of CCK-B receptors. However, the weak reversal of pilocarpine-induced seizures by CCK-5, and nearly similar action of L-365,260 and devazepide against the anticonvulsant effect of CCK-8 in the mouse seems to support the involvement of both subtypes of the CCK-8 (CCK-A and CCK-B) receptor in the modulation of pilocarpine-induced limbic seizures in rodents.
KEY WORDS: LIMBIC SEIZURES; CCK-8 RECEPTORS; PILOCARPINE; DEVAZEPIDE; L-365,260; CCK-8 ; MOUSE; RAT.

INTRODUCTION

An involvement of cholecystokinin octapeptide (CCK-8) in the regulation of seizure activity has been suggested by numerous phar-

macological studies. Thus, systemic or intracerebral administration of CCK-8 and its analogue caerulein inhibits seizures with different genesis [6, 20, 21]. On the other hand, the unspecific CCK-8 antagonist proglumide reverses the anticonvulsant effect of caerulein against picrotoxin and quinolinate-induced seizures, and potentiates seizures induced by quinolinate, an agonist at N-methyl-D-aspartate receptors [19, 20]. The highest levels of CCK-8 immunoreactivity and receptors are found in the different limbic and cortical structures (piriform cortex, amygdala, hippocampus etc.) [5, 12, 17], which are known to be involved in the regulation of seizure activity [4, 14]. Limbic seizures with varied genesis have been demonstrated to cause nearly complete loss of CCK-8 immunoreactivity from hippocampal mossy fiber system [3]. The potent convulsant picrotoxin is shown to reduce CCK-8 immunoreactivity in the several limbic regions [7]. The systemic treatment with muscarinic agonist pilocarpine is shown to cause very typical limbic seizures in rodents [18]. Magnani et al. [10, 11] have shown that the systemic treatment with CCK-8 and caerulein significantly affects the release of acetylcholine from the cerebral cortex of the rat "in vivo". Therefore, the aim of present work was to establish the role of CCK-8 receptors in the regulation of limbic seizures induced by pilocarpine in mice and rats. CCK-8, CCK-B/gastrin agonist pentagastrin (CCK-5) and two selective antagonists at CCK-8 receptors L-365,260 (antagonist of "brain" or CCK-B receptors) and devazepide (antagonist of "visceral" or CCK-A receptors) [1, 9] were used for clarifying this problem. Simultaneously with the behavioural experiments, the effect of pilocarpine-induced seizures was studied on the parameters of CCK-8 receptors in the different brain structures of the rat.

MATERIALS AND METHODS

Male albino mice (25–30 g) and male Wistar rats (250–300 g) were used throughout the experiment. The mice were placed into individual observation boxes 15 min before the start of experiment. After this habituation period CCK antagonists — L-365,260 (3R(+)-N-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1H-1,4-benzodiazepin-3-yl)-N'-(3-methyl-phenyl)urea, CCK-B antagonist, 0.01-1 mg/kg) and devazepide (formerly MK-329, CCK-A antagonist, 0.01–1 mg/kg) — were injected 15 niin, and CCK-8 (25–200 μg/kg) and pentagastrin (CCK-5, 2.5 mg/kg) 10 min prior to muscarinic agonist pilocarpine (380 mg/kg). Mice were observed for 60 min and the latencies of onset of tremor, and tonic seizures and death were registered. In the radioligand binding experiments with [propionyl-^{3}H]propionylated CCK-8 (^{3}[H]-pCCK-8) scopolamine methylnitrate (an antagonist at peripheral muscarinic

receptors) was injected 30 min prior to saline or pilocarpine (380 mg/kg). Two subgroups of rats — responders and non-responders to pilocarpine-induced limbic seizures — were selected for radioligand binding studies. Animals, respectively with and without seizures, were killed by decapitation 60 min after the administration of pilocarpine. The brains were removed rapidly from the skulls and the frontoparietal cortex, mesolimbic structures (nucleus accumbens, tuberculum olfactorium), piriform cortex, striata and hippocampus were dissected [15]. The method of Praissman et al. [16] in our slight modification was used for ^{3}[H]-pCCK-8 binding studies. Saturation curves were analyzed using ENZFITTER program for IBM microcomputers [8].

RESULTS

Systemic treatment with muscarinic agonist pilocarpine (380 mg/kg) evoked in all injected male mice (n=39) the fatal seizures. The pretreatment of mice with CCK-8 (25–200 μg/kg) significantly antagonized the effect of 380 mg/kg pilocarpine (figure 1). 50 μg/kg CCK-8 obviously reversed the effect of muscarinic agonist, the further increase of CCK-8 dose did not enhance the effect of neuropeptide. 13 mice from 39 tested survived pilocarpine-induced seizures after administration of 200 μg/kg CCK-8. CCK-8 antagonist devazepide in the high dose (1 mg/kg) evidently antagonized the anticonvulsant effect of CCK-8 (figure 2). CCK-B antagonist L-365,260 also after the administration of high dose (1 mg/kg) reversed the anticonvulsant action of CCK-8 (figure 3). However, L-365,260 (10–1000 μg/kg), differently from devazepide, completely blocked the antagonism of CCK-8 against the pilocarpine-induced lethality.

Pilocarpine up to 1 mM did not interact with ^{3}[H]-pCCK-8 binding in the radioligand studies "in vitro". The administration of high dose of pilocarpine (380 mg/kg) changed the parameters of ^{3}H-pCCK-8 binding sites in the several forebrain structures (table). Pilocarpine reduced significantly the number of ^{3}[H]-pCCK-8 binding sites in the striatum, frontoparietal cortex and hippocampus (table). In the hippocampus affinity of ^{3}H-pCCK-8 binding sites was also increased after administration of pilocarpine. The comparison of [^{3}H]-pCCK-8 binding parameters in the animals, responding and non-responding to pilocarpine-induced seizures, revealed more significant changes in the brain structures of rats, experiencing seizures (table). In the hippocampus the difference between the values of [^{3}H]-pCCK-8 binding in responders and non-responders was statistically evident (table).

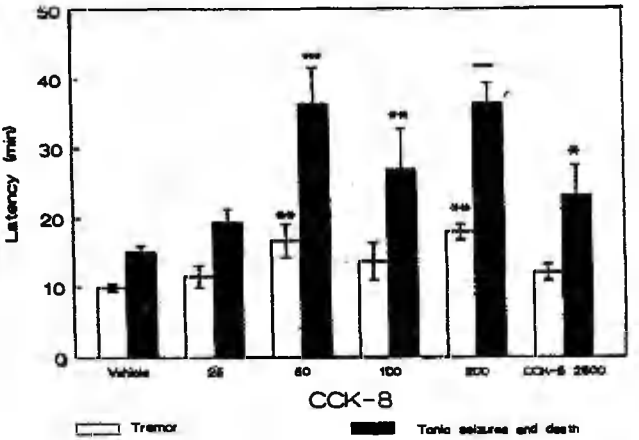

Figure 1. The effect of CCK-8 (25–200 μg/kg) and pentagastrin (CCK-5, 2500 μg/kg) on pilocarpine-induced seizures in mice. CCK-8 and CCK-5 were given 10 min prior to pilocarpine (380 mg/kg). The animals were observed for 60 min after the administration of pilocarpine. Significant differences between vehicle/pilocarpine and CCK-8 or CCK-5/pilocarpine treated groups were determined by Newman-Keuls test after significant ANOVA. $F_{5,116}= 8.71$, $p<0.0001$ (for tremor); $F_{5,116}= 10.46$, $p<0.000001$ (for tonic seizures and death). * – $p< 0.05$; ** – $p< 0.01$; *** – $p<0.005$ (if compared to pilocarpine treated mice).

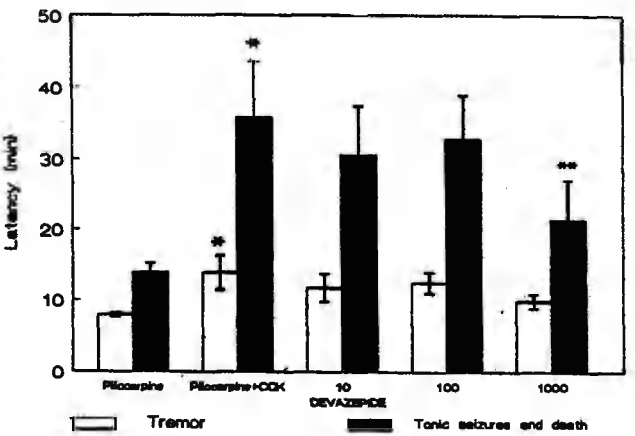

Figure 2. The effect of devazepide (10–1000 μg/kg) on the anticonvulsant action of CCK-8 (200 μg/kg) against pilocarpine-induced seizures in mice. Devazepide was injected 15 min and CCK-8 10 min prior to pilocarpine. Significant differences between pilocarpine, CCK-8/pilocarpine and devazepide/CCK-8/pilocarpine treated groups were determined by Newman-Keuls test after significant ANOVA. $F_{4,77}=2.4$, $p<0.05$ (for tremor), $F_{4,77}=2.5$ (for tonic seizures and death). * – $p<0.05$ (if compared to pilocarpine treated animals); ** – $p<0.05$ (if compared to CCK-8/pilocarpine treated mice).

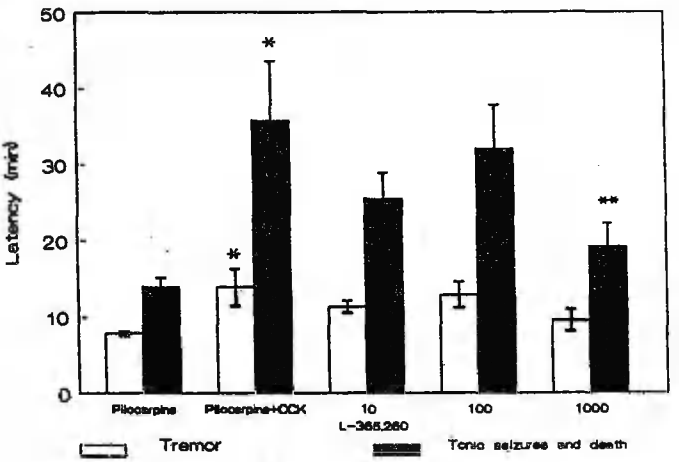

Figure 3. The effect of L-365,260 (10–1000 μg/kg) on the anticonvulsant effect of CCK-8 (200 μg/kg) against pilocarpine-induced seizures in mice. L-365,260 was injected 15 min and CCK-8 10 min prior to pilocarpine. Significant differences between pilocarpine, CCK-8/pilocarpine and L-365,260/CCK-8/pilocarpine treated groups were determined by Newman-Keuls test after significant ANOVA. $F_{4,77}= 2.86$, $p<0.05$ (for tremor); $F_{4,77}= 3,69$, $p<0.01$ (for tonic seizures and death). * – $p<0.05$ (if compared to pilocarpine treated moce); ** – $p<0.05$ (if compared to CCK-8/pilocarpine treated animals).

DISCUSSION

The results of present study are reflecting a significant role of CCK-8 receptors in the modulation of epileptogenic effect of a muscarinic agonist pilocarpine. CCK-8 potently antagonizes the seizures induced by the lethal dose of pilocarpine. One third of mice survive pilocarpine-induced seizures after pretreatment with 200 μg/kg CCK-8. CCK-b/gastrin agonist pentagastrin only moderately reduces the convulsant action of pilocarpine. Accordingly, it seems probable that the peripherally injected CCK-8 affects the cholinergic neurotransmission in the brain. It is suggested that several behavioural effects of CCK-8 and caerulein are generated through primarily peripheral mechanisms [13]. It is thought that the sedative effect of large doses of CCK-8 is of peripheral origin and could be abolished by abdominal vagotomy [2]. Magnani et al. [10, 11] have shown that CCK-8, in the doses 10 μg/kg and higher, potently inhibits the release of acetylcholine from the rat cerebral cortex. This effect of CCK-8 is not affected by bilateral vagotomy or by the lesion of dopaminergic cells in the substantia nigra. The selective CCK antagonists devazepide and L-365,260 reverse the anticonvulsant effect of CCK-8. However, it happens only after the administration of

Table

The binding parameters of [^{3}H]-pCCK-8 in the brain structures of responding and non-responding rats to pilocarpine (380 mg/kg) seizures

Brain structures		Saline	Non-responders	Responders
Mesolimbic area	K_d	0.42±0.02	0.40±0.03	0.50±0.15
	B_{max}	4.51±0.32	4.13±0.22	3.55±0.53
Piriform cortex	K_d	0.43±0.02	0.63±0.06	0.68±0.08
	B_{max}	6.30±0.20	5.22±0.29	5.58±0.75
Frontoparietal	K_d	0.33±0.02	0.41±0.03	0.26±0.02
cortex	B_{max}	5.15±0.30	3.76±0.76	2.71±0.23a
Striatum	K_d	0.27±0.01	0.45±0.07	0.36±0.04
	B_{max}	5.23±0.28	4.25±0.19a	3.74±0.24b
Dorsal hippo-	K_d	0.63±0.04	0.37±0.05a	0.15±0.02b,c
campus	B_{max}	1.87±0.17	1.18±0.28a	0.56±0.05b,c

The brain structures of 4–5 rats have been pooled. The mean values ± S.E.M. of 4 independent experiments are presented in table. K_d – dissociation constant in nM; B_{max} – apparent number of binding sites in pmoles per gram original tissue wet weight a – $p < 0.05$; b – $p < 0.01$ (compared to saline treated rats, Student's t-test); c – $p < 0.05$ (compared to non-responders, Student's t-test).

very high dose (1 mg/kg) of CCK antagonists. It is noteworthy that the effect of L-365,260 is somewhat stronger.

L-365,260, in wide dose range (10–1000 μg/kg), antagonize also the effect of CCK-8 on pilocarpine-induced lethality. Nevertheless, the both subtypes of CCK-8 (CCK-A and CCK-B) seem to be involved in the anticonvulsant effect of CCK-8. According to the radioligand binding studies "in vitro", pilocarpine (up to 1 mM) does not interact directly with CCK-8 receptors in the brain. However, the systemic administration of very high dose of pilocarpine (380 mg/kg) is reducing the density of CCK-B receptors in the frontoparietal cortex, striatum and hippocampus of the rat brain. The comparison of ^{3}[H]-pCCK-8 binding parameters in rats, responding and non-responding to the administration of pilocarpine with seizures, reveals markedly higher reduction of CCK-B receptors in animals with seizures. It supports the idea that CCK-B receptors are involved in the modulation of seizures induced by muscarinic agonist.

In conclusion, it is very likely that the both subtypes of CCK-B receptor are involved in the modulation of limbic seizures induced by the muscarinic agonist pilocarpine. This idea is supported by the findings that CCK-B/gastrin agonist pentagastrin only moderately antagonized the effect of pilocarpine, the selective CCK-8 antagonists devazepide and L-365,260 have nearly similar effect on the anticonvulsant effect of CCK-8, and during pilocarpine-induced

seizures the density of CCK-8 receptors is significantly reduced in the several brain regions.

Acknowledgment. The authors are grateful to Merck, Sharp and Dohme Ltd. for the generous gift of devazepide and L-365,260.

REFERENCES

1. Chang R.S.L., Lotti V.J. Biochemical and pharmacological characterization of an extremely potent and selective nonpeptide cholecystokinin antagonist. Proc.Natn.Acad.Sci.USA. 1983. Vol. 83. P. 4923–4926.
2. Crawley J.N., Kiss J.Z. Tracing the sensory pathway from gut to brain regions mediating the actions of cholecystokinin on feeding and exploration // Ann.N.Y.Acad.Sci. 1985. Vol. 448. P. 586–588.
3. Gall C. Seizure induce dramatic and distinctly different changes in enkephalin, dynorphin and CCK immunoreactivities in mouse hippocampal mossy fibers // J.Neurosci. 1988. Vol. 8. P. 1852–1862.
4. Handforth A., Ackermann R.F. Electrically induced limbic status and kindled seizures // Anatomy of epileptogenesis / Eds. B.S. Meldrum, J.A. Ferrendelli and H.G. Wieser. London: John Libbey and Co Ltd., 1988. P. 71–88.
5. Harris Q.L.G., Lewis S.J., Shulkes A., Vajda F.J.E. Regional concentrations of cholecystokinin in the rat: the effects of kindled and non-kindled seizures // Neuropharmacol. 1988. Vol. 27. P. 547–549.
6. Kadar T., Pesti A., Penke B., Telegdy G. Inhibition of seizures induced by picrotoxin and electroshock by cholecystokinin octapeptides and their fragments in rats after intracerebroventricular administration // Neuropharmacol. 1984. Vol. 23. P. 955–961.
7. Kato T., Emi Y., Takita M., Kushima Y. Time dependent changes in the concentrations of immunoreactive cholecystokinin octapeptide sulfate in rat brain regions after systemic injection of picrotoxin // Neurochem.Int. 1988. Vol. 12. P. 493–497.
8. Leatherbarrow R.J. Enzfitter, a non-linear regression data analysis program for the IBM PC. Amsterdam: Elsevier Science Publisher, 1987.
9. Lotti V.J., Chang R.S.L. A new potent and selective non-peptide gastrin antagonist and brain cholecystokinin receptor (CCK-B) ligand: L-365,260 // Eur.J.Pharmacol. 1989. Vol. 162. P. 273–280.
10. Magnani M., Mantovani P., Pepeu G. Effect of cholecystokini octapeptide and ceruletide on release of acetylcholine from cerebral cortex of the rat in vivo // Neuropharmacol. 1984. Vol. 23. P. 1305–1309.
11. Magnani M., Florian A., Casamenti F., Pepeu G. An analysis of cholecystokinin-induced increase in acetylcholine output from cerbral cortex of the rat // Neuropharmacol. 1987. Vol. 26. P. 1207–1210.
12. Marley P.D., Rehfeld J.F., Emson P.C. Distribution and chromatographic characterisation of gastrin and cholecystokinin in the rat central nervous system // J.Neurochem. 1984. Vol. 42. P. 1523–1530.
13. Morley J.E. Behavioural effects of administered cholecystokinin // ISI Atlas Pharmacol. 1987. Vol. 1. P. 49–51.

14. Patel S. Chemically induced limbic seizures in rodents // Anatomy of epileptogenesis / Eds. B.S. Meldrum, J.A. Ferrendelli, G.H. Wieser London: John Libbey and Co. Ltd., 1988. P. 89–106.
15. Paxinos G., Watson C. The rat brain in stereotaxic coordinates. North Ryde: Academic Press Australia, 1982.
16. Praissman M., Martinez P.A., Saldino C.F., Berkowitz Steggles A.W., Finkelstein J.A. Characterization of cholecystokinin binding sites in rat cerebral cortex using a [125I]-CCK-8 probe resistant to degradation // J.Neurochem. 1983. Vol. 40. P. 1406–1413.
17. Saito A., Sankaran K., Goldfine D., Williams J.A. Cholecystokinin receptors in the rat brain: characterization and distribution. 1980. Vol. 208. P. 1155–1156.
18. Turski W.A., Cavalheiro E.A., Schwarz M., Czuczwar S.J., Kleinrok Z., Turski L. Limbic seizures produced by pilocarpine in rats: behavioural, electroencephalic and neuropathological study // Behav.Brain Res. 1983. Vol. 9. P. 315–322.
19. Vasar E., Rägo L., Soosaar A., Nurk A., Maimets M. Modulatory effect of caerulein on benzodiazepine receptors. Bull.Exp.Biol.Med. 1985. Vol. 102. P. 711–713.
20. Vasar E., Allikmets L., Ryzhov L, Prakhye L, Soosaar A., Mirzayev S. Intraspecies differences in the behavioral effects of caerulein, an agonist of CCK-8 receptors, in mice and rats // Bull.Exp.Biol.Med. 1988. Vol. 105. P. 168–170.
21. Zetler G. Caerulein and its analogues: neuropharmacological properties // Peptides 1985. Vol. 6. P. 33–46.

EFFECT OF Ro 15-4513 ON THE BEHAVIOUR AND POTASSIUM-EVOKED CALCIUM UPTAKE FOLLOWING ETHANOL WITHDRAWAL IN RATS

A. Zharkovsky and A. Shavrin

Department of Pharmacology
and Molecular Pharmacology
& Pharmacokinetics Research Unit,
Tartu University, 202400 Tartu, Estonia

In the present investigation the effect of benzodiazepine receptor inverse agonist Ro 15-4513 on potassium(55 mM)-evoked calcium accumulation in ethanol-intoxicated (EI) and ethanol-withdrawn (EW) rats has been studied. Ethanol was administered in increasing concentrations from 2% to 10% with drinking water during two months. Daily intake of ethanol at the end of chronic administration was 15 g/kg. Ro 15-4513 (4 mg/kg, i.p.) did not change the calcium influx in synaptoneurosomes of control rats. No changes in uptake were found 2 hrs after the last ethanol administration (EI rats). At 24 hrs of ethanol withdrawal calcium accumulation was significantly increased ($P>0.05$). Nifedipine (5 mg/kg, i.p.) completely reversed an increase of calcium influx in EW rats. Ro 15-4513 (4 mg/kg, i.p.) did not affect calcium accumulation in EW-rats but significantly enhanced influx in EI animals. Simultaneously Ro 15-4513 induced the signs of withdrawal in EI rats.

These data suggest that Ro 15-4513 may induce the signs of withdrawal when given during ethanol intoxication and therefore may be potentially dangerous in patients suffering from the chronic ethanol abuse.

KEY WORDS: CHRONIC ETHANOL, Ro 15-4513, CALCIUM INFLUX, SYNAPTONEUROSOMES

INTRODUCTION

Ro 15-4513 (ethyl 8-azido-5, 6-dihydro-5-methyl-6-oxo-4H-imidazo-[1,5-a][1,4] benzodiazepine-3-carboxylate) has been reported to possess behavioural effects of benzodiazepine receptor inverse agonists [2, 3, 12]. Recent studies have shown that Ro 15-4513 is capable of antagonizing some of the behavioural effects of ethanol

[2, 11, 12, 17]]. There were speculations concerning a possible clinical application of this compound as a spetcific ethanol antagonist. However, the usefulness of this compound as ethanol spetcific antagonist during chronic ethanol intoxication has been recently questioned since this compound might exacerbate the signs of withdrawal. In fact, the administration of Ro 15-4513 increased the incidence of seizures in mice or rats during 5–8 hours of ethanol withdrawal [1, 13]. There are some questions whether the administration of Ro 15-4513 is able to precipitate the symptoms of withdrawal during chronic ethanol administration or this compound is able only to exacerbate the existing symptoms of spontaneous withdrawal. Previous studies have shown that ethanol intoxication and the development of the withdrawal signs were associated with the changes in dihydropyridine sensitive voltage-dependent calcium channels. Dolin et al. [7] demonstrated that calcium channel antagonist nimodipine protected against ethanol-withdrawal seizures and the density of dihydropyridine binding sites is increased during ethanol withdrawal. Previous reports suggested that ethanol-induced intoxication and sedation correlate closely with the blockade of voltage dependent calcium entry into presynaptic nerve terminals [10] and ethanol is able to inhibit the fast phase of potassium-evoked calcium uptake. Recent results have also shown that potassium-evoked calcium entry intio cortical synaptosomes of animals withdrawn from chronic ethanol is also increased [16]. These data suggest that alteration in the calcium channel functions produced by the acute and chronic ethanol may be involved in the behavioural signs of ethanol intoxication and withdrawal reactions developed upon chronic ethanol administration.

Our experiments were designed to study the effect of Ro 15-4513 on behaviour and potassium-evoked calcium uptake by the cortical synaptosomes isolated from the rat brain at various times following chronic ethanol administration.

MATERIALS AND METHODS

Animals. The male albino rats with initial weight 200–230 g were housed in groups of 8–10 animals. Animals were maintained on food and water ad libitum.

Ethanol treatment. The groups of animals were given ethanol in the drinking water in increasing concentrations from 2% to 10% during two months. Daily intake of ethanol at the end of chronic administration was 15±1 g/kg. The food intake of the control group was restricted to maintain a weight gain comparable to that of ethanol-treated animals. At the end of ethanol administration the bottles containing ethanol solution were replaced by water.

All control and ethanol administered rats were divided into two groups. The first group was used in experiment I, the second droup – in experiment II.

Experiment I. Animals for this experiment were taken 1.5 hours after ethanol withdrawal when a considerably high ethanol concentrations were present in their blood and were referred to as ethanol intoxicated animals. Animals from control and ethanol group were injected either vehicle or Ro 15-4513 (4 mg.kg, i.p.) and were placed into single observation boxes. After 15 min of habituation period animals were observed for the incidence of myoclonic jerks as an indication of withdrawal for 30 min. After performing of behavioural observations animals were rapidly sacrificed by the cervical dislocation and their brains were taken for the biochemical assay.

Experiment II. Animals for this experiment were taken 24 hours after ethanol withdrawal and were referred to as ethanol withdrawn animals. These animals were also injected with either saline or Ro 15-4513 and 45 min later were directly taken for the biochemical assay without behavioural testing.

$^{45}Ca^{2+}$ uptake assay

Membrane vesicles (microsacs) were prepared according to the method of Harris and Allan [9]. The tissue was homogenized by hand (10 strokes) in 4.5 ml of ice-cold assay buffer (millimolar): NaCl, 145; KCl, 5; $MgCl_2$, 1; glucose, 10; $CaCl_2$, 1; and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 10, adjusted to pH=7.5 with TRIS base using a glass Teflon homogenizer. The homogenate was centrifuged at 900 g for 15 min. The supernatant was decanted, and the pellet was suspended in 8 ml of assay buffer and centrifuged once again. The final pellet was suspended in assay buffer to yield a preparation containing 5–6 mg of protein per 1 ml of suspension. Protein content was determined by the method of Lowry et al., 1951). Aliquots of microsacs (1–2 mg protein/ml) were preincubated in volume 900 ul in glass tubes at °C for 10 min, and at 37°C for 10 min. The uptake was initiated by adding about 100 ul of solution containing 1 uCi of $^{45}Ca^{2+}$ and 50 mM NaCl (resting uptake) or 50 mM KCl (depolarization-induzed uptake). The incubation was continued for the further 5 sec and was terminated by a rapid filtration through GF/B filters followed by four 4 ml washings with ice-cold buffer containing 145 mM KCl. All experiments were performed in triplicates. Filters were placed into scintillation vials containing dioxane based scintillation cocktail and, after shaking, were counted in Beckman scintillation counter LS-6800.

STATISTICS

Student's t-test and analysis of variance was performed for the evaluation of the data.

RESULTS

Two hours after withdrawal from chronic ethanol treatment when ethanol was still present in high concentrations in the blood there were no observable signs of spontaneous withdrawal. Administration of Ro 15-4513 in ethanol-indoxicated animals induced myoclonic jerks (Fig.1). In contrast, administration of Ro 15-4513 in control animals did not induce any behavioural changes (data not shown).

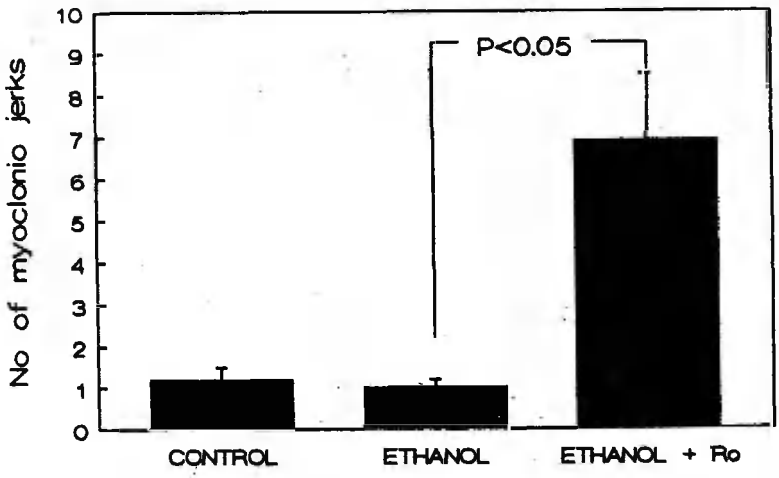

Figure 1. Ro 15-4513 — evoked myoclonic jerks in ethanol-indoxicated rats. Ro 15-4513 (4 mg/kg) was injected 2 hours after ethanol withdrawal. In control animals Ro 15-4513 did not induce myoclonic jerks (Data not shown).

In ethanol-intoxicated animals no changes in potassiumfevoked $^{45}Ca^{2+}$ uptake were observed in comparison to control (Fif. 2). Administration of Ro 15-4513 (4 mg/kg) in ethanol-intoxicated rats resulted in the significant increase of $^{45}Ca^{2+}$ uptake (Fig. 2). In contrast, Ro 15-4513 did not induce any changes in control animals. Similarly, *in vitro* addition of 10 um Ro 15-4513 to the incubation medium did not induce any changes in calcium flux (data not shown). 24 Hours after ethanol withdrawal a significant increase

in potassium-evoked $^{45}Ca^{2+}$ uptake was observed and this increase was not further enhanced by the pretreatment with Ro 15-4513 (Fig 3).

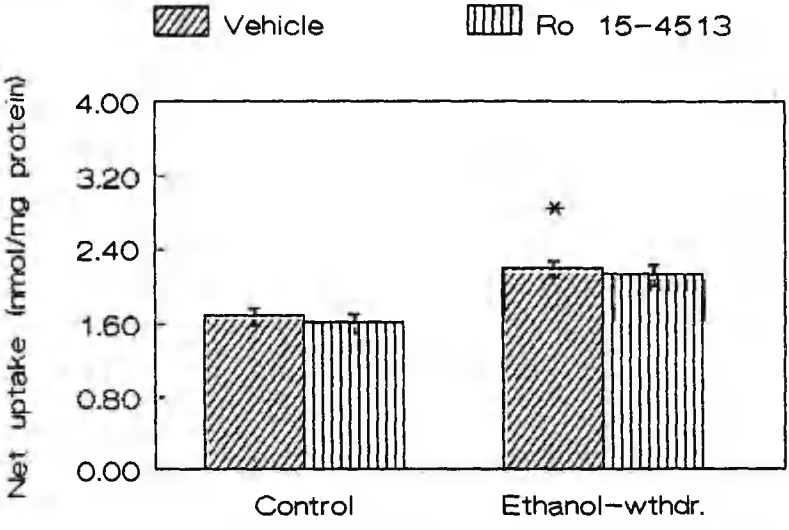

Figure 2. Effect of *in vivo* Ro 15-4513 (4 mg/kg) administration on potassium-evoked $^{45}Ca^{2+}$ uptake by the cortical synaptoneurosomes of ethanol-indoxicated rats.

DISCUSSION

The results of the present study demonstrated that administration of Ro 15-4513 during chronic ethanol intoxication results in the appearance of behavioural signs resembling those seen in rats with spontaneous withdrawal. The appearance of these signs suggests that administration of Ro 15-4513 is capable of precipitating the withdrawal signs in these animals. These data contradict previous observations where no precipitated withdrawal was found upon the administration of Ro 15-4513 in ethanol-intoxicated mice [1]. The species differences and route of ethanol administration, however, might account for this discrepancy. Appearance of the withdrawal signs was accompained by an increase in the potassium-evoked $^{45}Ca^{2+}$ uptake by synaptoneurosomes upon Ro 15-4513 administration in ethanol-intoxicated rats. Although the mechanism of Ro 15-4513-induced increase in the calcium channel function in ethanol-intoxicated rats is not known, one may propose that these changes might reflect the alteration of neuronal membranes induced by chronic ethanol administration. Since primary site of action of Ro 15-4513

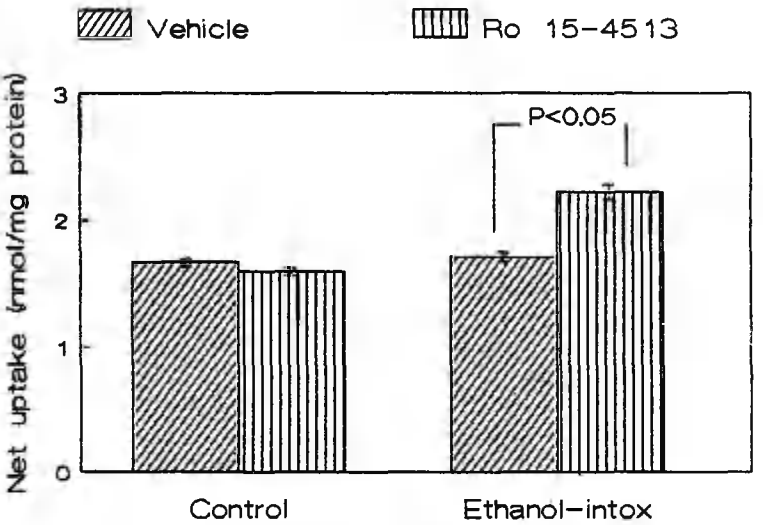

Figure 3. Effect of ***in vivo*** Ro 15-4513 (4 mg/kg) administration on potassium-evoked $^{45}Ca^{2+}$ uptake by the cortical synaptoneurosomes of ethanol-indoxicated rats. * – $P<0.05$ (Students t-test).

is GABA-benzodiazepine-barbiturate complex linked to the chloride ionophore [2] and ***in vitro*** addition of Ro 15-4513 did not affect calcium channel function one can propose that changes in $^{45}Ca^{2+}$ flux induced by ***in vivo*** administration of Ro 15-4513 are secondary and probably develop in response to the alteration of chloride channel function. Recent studies have shown that ***in vitro*** addition of ethanol in concentrations 25–200 mM inhibited $^{45}Ca^{2+}$ flux through voltage-sensitive calcium channels, which might be a consequence of elevation of intraneuronal calcium [5, 6, 9]. The lack of any changes in calcium flux in chronically ethanol-intoxicated rats found in this study may suggest a development of tolerance to the acute effects of ethanol. The data obtained from experiment II demonstrated an increase in potassium evoked $^{45}Ca^{2+}$ flux 24 hours after termination of chronic ethanol administration. Although behavioural data found by others demonstrated that administration of Ro 15-4513 might exacerbate the behavioural signs of ethanol withdrawal (Becker and Anton, 1989), the administration of Ro 15-4513 in our experiments did not produce any further increase in calcium flux. it is not excluded that 24 hours after withdrawal a maximum increase in calcium flux was achieved and an additional administration of RO 15-45123 was not able to induce any further increase.

In conclusion, the data found in the present stydy suggest that Ro 15-4513 may induce the signs of withdrawal when given during

ethanol intoxication and therefore may be potentially dangerous in patients suffering from chronic ethanol abuse.

REFERENCES

1. Becker H.C., Anton R.F. The benzodiazepine receptor inverse agonist Ro 15-4513 exacerbates, but does not precipitate, ethanol withdrawal in mice // Pharmacol. Biochem. Behav. 1989. Vol. 229. P. 163–167.
2. Bonetti E.P., Burkard W., Gabl M., Möhler H. The partial benzodiazepine agonist Ro 15-4513 antagonizes acute ethanol effects in mice and rats // Br. J. Pharmacol. 1985. Vol. 86. P. 463.
3. Gorda M.G., Giorgi O., Longoni B., Biggio G. Ro 15-4513, as a partial inverse agonist for benzodiazepine recognition sites, has a proconflict and proconvulsant effects in the rat // Eur. J. Pharmacol. 1989. Vol. 159. P. 233–239.
4. Daniel L.C., Brass E.P., Harris R.A. Effect of ethanol on intracellular ionized calcium concentrations in synaptosomes and hepatocytes // J. Pharmacol. Exp. Ther. 1987. Vol. 32. P. 831–837.
5. Daniell L., Brass E.P., Harris R.A. Effect of ethanol on intracellular ionized calcium concentrations in synaptosomes and hepatocytes // Mol. Pharmacol. 1988. Vol. 32. P. 831–837.
6. Davidson M., Wilse P., Shanley B. Ethanol increases synaptosomal free calcium concentration // Neurosci. Lett. 1988. Vol. 89. P. 165–169.
7. Dolin S., Little H., Hudspith M., Pagonis C., Littleton J. Increased dihydropyridine-sensitive calcium channels in rat brain may underlie ethanol physical dependence // Neuropharmacol. 1987. Vol. 26. P. 275–279.
8. Harris R.A., Allan A.M. Functional coupling of GABA receptors to chloride channels in brain membranes // Science. 1985. Vol. 228. P. 1108–1110.
9. Harris R.A., Bruno. P. Membrane disordering by anaesthetic drugs: relationship to synaptosomal sodium and calcium fluxes // J. Neurochem. 1985. Vol. 44. P. 1274–1281.
10. Leslie. S.W., Barr E., Chandlerol J., Farrar R.P. Inhibition of fast and slow-phase depolarization-dependent synapatosomal calcium uptake by ethanol // J. Pharmacol. Exp. Ther. 1983. Vol. 225. P. 571–575.
11. Lister R. Interactions of Ro 15-4513 with diazepam, sodium pentobarbital and ethanol in a holeboard test // Pharmacol. Biochem. Behav. — 1987. Vol. 28. P. 75–79.
12. Lister R.G., Nutt D.J. Is Ro 15-4513 a specific alcohol antagonist? // TINS. 1987. Vol. 10, No. 6. P. 223–226.
13. Lister R.G., Karanian J.W. Ro 15-4513 induces seizures in DBA/2 mice undergoing alcohol withdrawal // Alcohol. 1987. Vol. 4.P. 409–411.
14. Little H.J., Dolin O.H., Halsey M.J. Calcium antagonists decrease the ethanol withdrawal syndrome // Life Sci. 1986. Vol. 39. P. 2059–2065.
15. Lowry O.H., Rosebrough N.J., Farr A.L. Randall R.J. Protein measurement with the Folin phenol reagent // J. Biol. Chem. 1951. Vol. 193. P. 256–275.

16. Shavrin A., Zharkovsky A. The effect of nifedipine and Ro 15-4513 on potassium-evoked calcium influx into cortical synaptoneurosomes of ethanol-dependent rats // The II Baltic Meeting on Pharmacology and Clinical Pharmacology: Abstracts. 1990, P. 109.
17. Suzdack P.D., Glowa J., Crawley J., Schwarz R., Skolnick P., Paul S. A selective imidazobenzodiazepine antagonist of ethanol in the rat // Science. 1986. Vol. 234. P. 1243–1247.

EFFECT OF BACLOFEN AND NITRENDIPINE ON ETHANOL WITHDRAWAL IN THE RAT

Alexander Zharkovsky and Sandra E. File

Department of Pharmacology Tartu University,
202 400 Tartu, Estonia, and
Psychopharmacology Research Unit,
UMDS Division of Pharmacology, (London University),
Guy's Hospital, London SE1 9RT, UK.

Withdrawal of rats from chronic ethanol treatment (liquid Complan diet containing 10% of ethanol) resulted in an increased anxiety in both social interaction and plus-maze tests; this was accompanied by increased aggression and tremor. Baclofen (1.25–2.5 mg/kg) did not affect the behaviour of control animals but reversed the anxiogenic responses and reduced aggression and tremor scores in ethanol-withdrawn rats. A high dose (5.0 mg/kg) of baclofen reduced locomotor activity, the time spent in social interaction and the percent number of entries into and time spent on the open arms of the plus-maze in both control and ethanol — withdrawn rats. Nitrendipine (25–50 mg/kg) did not influence either anxiety or aggression in ethanol — withdrawn rats. At the high dose (100 mg/kg) nitrendipine produced sedation and reduced the time spent in the active social interaction and locomotor activity in both control and ethanol — withdrawn rats. This effect of the high dose of nitrendipine was accompanied by the reduction of tremor scores in ethanol — withdrawn rats. The results of the present study indicate that baclofen might be useful in the treatment of alcohol induced withdrawal reactions. It is proposed that baclofen interacting with GABA$_B$ receptors located on the presynaptic nerve endings, in contrast to nitrendipine, might induce a decrease in the intraneuronal calcium concentration and a consequent inhibition of the release of 5-HT and GABA, that might partly contribute to the reversal of anxiogenic responses and the inhibition of tremor in ethanol — withdrawn rats.

KEY WORDS: ETHANOL WITHDRAWAL, BACLOFEN, NITRENDIPINE, ANXIETY, TREMOR, AGGRESSION.

INTRODUCTION

Chronic exposure of animals or humans to ethanol results in the development of physical dependence that is manifested by a characteristic withdrawal syndrome upon the cessation of ethanol intake. The withdrawal syndrome in rats is characterized by increased anxiety, tremor, muscle rigidity and increased seizure activity [12, 13, 29]. Previous data have suggested that development of withdrawal syndrome after chronic exposure to ethanol might be explained by diminished GABA-ergic neurotransmission [27, 28,]. Variuos parameters of GABA-ergic neurotransmission are altered during ethanol withdrawal including an increase in K_D of the low affinity $GABA_A$ receptor sites [27], and reduced GABA concentrations in various regions of the brain [25]. Benzodiazepines and barbiturates are used as sedative drugs in the treatment of the ethanol withdrawal syndrome where they might compensate for the decreased GABA-ergic inhibition (Volicer, 1980). GABA receptors have been classified into $GABA_A$ and $GABA_B$ subtypes according to their pharmacological and anatomical properties [2, 3]. Previous studies have shown that the $GABA_A$-receptor agonists, GABA, muscimol and THIP injected intracisternally reduced the severity of audiogenic seizures but had not effect on tremor in ethanol-withdrawn rats [14]. Less is known about the effect of the $GABA_B$ receptor agonist baclofen on the signs of ethanol withdrawal, but in one study it slightly reduced the tremor in ethanol — withdrawn monkeys [ãž].

Current evidence suggests that voltage operated calcium channels also might be involved in the development of tolerance during chronic ethanol administration and ethanol withdrawal syndrome [õž]. Calcium channel antagonists nitrendipine and PN 200–110 prevented the development of tolerance to ataxic and the general anaesthetic actions of ethanol when they were given concurrently with ethanol [5, 6, 19]. Calcium channel antagonists nitrendipine and nimodipine abolished ethanol withdrawal convulsions [18] and inhibited apomorphine-induced aggressive reactions in ethanol withdrawn rats [23]. However, not all behavioural effects of ethanol withdrawal were affected by nitrendipine administration; it was without effect on the anxiogenic response in ethanol-withdrawn rats [12].

Recent studies show that baclofen and the dihydropyridines have some common mechanism of action. Nitrendipine at nanomolar concentrations inhibits binding of [^{3}H]baclofen to $GABA_B$ receptors [1]. Baclofen interacting with $GABA_B$ receptors, inhibits voltage dependent $[Ca^{2+}]_i$ rise in the rat brain synaptosomes [24] which, probably, accounts for the observed inhibitory effect of baclofen on GABA, 5-hydroxytryptamine and noradrenaline release [2,

17]. In the present study we compared the effects of baclofen and nitrendipine on the anxiogenic responses, aggression and tremor in ethanol-withdrawn rats.

METHODS

Animals. Male hooded Lister rats (Ollac, Bicester, UK) with initial weight 180–200 g, were housed in a room with a 13 h light: 11 h dark cycle (lights on at 06.00 hours). Animals were housed in groups of five until 5 days before testing, when they were housed individually. Three days prior to behavioural testing rats were allocated to test partners on the basis of their weight (±25 g).

Drugs. Baclofen (Ciba Geigy) was dissolved in warm distillated water, nitrendipine (Sigma) was suspended in distilled water with 2–4 drops of Tween-20 and injected intraperitoneally (ip) in a volume of 2 ml/kg body weight.

Ethanol treatment. Rats were randomly allocated to the ethanol and control diets. All the rats received 20% w/v liquid chocolate Complan diet; water was freely available in a separate bottle. The ethanol group received absolute ethanol additions to the Complan. This was 3% w/v on day 1 and was increased in 1% steps over the next 7 days. Rats were then maintained on a diet containing 10% ethanol for a further 4 weeks (giving a final daily dose 15.2±0.9 g/kg/day). The liquid diet intake of control group was restricted to maintain comparable weight gain to the ethanol-treated rats.

Apparatus. The social interaction test has been described in detail previously [10, 11].

The plus-maze was made of wood and consisted of two opposite open arms, 50x10 cm and two opposite enclosed arms, 50x10x40 cm with an open roof. The arms were connected by a central square, 10x10 cm, thus the maze formed the shape of plus sign. It was elevated to a height of 50 cm above the floor.

The holeboard is a wooden box 65x65x30 cm with four holes, each 6.5 cm in diameter, equally spaced on the floor. Head dipping was measured by infra-red cells located immediately beneath the edges of the holes; locomotor activity and rearing were measured by the number of times infrared beams located in the walls of box, 4.5 and 12.5 cm respectively from the floor, were broken.

Behavioural procedure. The Complan diet containing ethanol was replaced by equivalent amount of Complan without ethanol 7.5 h before testing. All behavioural tests were performed between 7 a.m. and 12 a.m. In the first experiment animals from both the control and the chronic ethanol diet received intraperitoneal (ip) injections of either vehicle or baclofen (1.25, 2.5 or 5.0 mg/kg, n=8–10/group) 30 min before the start of testing. The animals were then given

behavioural tests in the following order: social interaction test, plus-maze test and holeboard test. After testing, the rats were maintained on their previous ethanol or control liquid diets for a further 7 days. They were subsequently withdrawn again from their diets for another 7.5 h and received i.p. either saline or nitrendipine (25, 50 or 100 mg/kg) 30 min before testing. The animals were retested in social interaction test and plus-maze.

Social interaction test. The low light, familiar test condition was used, thus the rats were familarized with the test arena for 7.5 min on the 2 days prior to testing. The behaviour of rats was videorecorded to permit rescoring. Two categories of behaviour were scored separately: active social interaction (sniffing, following, grooming) and aggression (fighting, aggressive posture). Locomotor activity was measured by breaking of photobeams positioned 4.5 cm above the floor. At the end of each trial test arena was thoroughly cleaned.

Plus-maze test. Each rat was placed in the centre of the plus-maze, facing an anclosed arm. An observer who was blind to the drug treatment, scored the number of entries into open arms and the number into enclosed arms and times spent on the open and the enclosed arms of plus-maze. Each test lasted 5 min, and at the end of each trial the maze was thoroughly cleaned.

Holeboard test. Rats naive to the holeboard apparatus were placed singly in the centre of the holeboard and the following measures were taken during a 5 min trial: number of head dips, time spent head dipping, locomotor activity and rearing. At the end of each trial, any faecal boluses were removed and the box was wiped clean.

Ethanol withdrawal-induced tremor was evaluated using the method of Frye et al. [14]. Tremor scores were determined by lifting rats vertically by the tail and scoring the following reactions: a score of three was assigned to rats showing an immediate forelimb extension and violent generalized forelimb-whole body tremor; a score of two was assigned to rats that showed this reaction when they were rotated 180o around the axis of the tail; and score of one to rats that failed to display forelimb extension but showed clearly distinct forelimb tremor when lifted by the tail and rotated 180o. The rats were scored on two occasions, once immediately before and once after the social interaction test; a mean of these two scores was taken for the statistical analysis.

Statistical analysis. The data obtained in the behavioural experiments with the excepton of tremor data were assessed with analysis of variance (ANOVA) followed, where appropriate, with Duncan's multiple range test. The data of tremor scores were analysed using Kruskall-Wallis H test.

RESULTS

Experiment 1. **Effect of ethanol withdrawal.** Withdrawal from chronic ethanol treatment resulted in an increase of anxiety indicated by a reduction of time spent in active social interaction [$F(1,9)=9.8$, $P<0.01$], see Figure 1. An increasing anxiogenic response in social interaction test was accompanied by an increased score for the time spent in aggressive behaviours [$F(1,22)=22.7$ $P<0.001$], see Figure 2. Anxiogenic withdrawal response could also be detected in the plus-maze test. Thus, the rats withdrawn from chronic ethanol treatment showed a decrease in % number of entries into open arms of the plus maze [$F(1,20)=6.1$, $P<0.05$] and the % time spent on the open arms [$F(1,20)=10.5$, $P<0.01$] of the plus-maze but did not change the total number of entries in plus-maze (Figure 3). Withdrawal from chronic ethanol treatment increased rearing [$F(1,18)=4.3$, $P<0.05$], but did not affect other behavioural measures in the holeboard (Table 1). Withdrawal from ethanol induced strong tremor [$P<0.001$], see Figure 4.

Table 1

Effect of baclofen on locomotor activity in holeboard of control (CO) and chronic ethanol-withdrawn (EW) rats. The data are mean value ± SEM

Behavioural measure		Vehicle	Baclofen (mg/kg)		
			1.25	2.5	5.0
Time spent head-dipping	CON	27.8+5.0	27.7+5.7	21.8+2.7	2.0+0.6*
	EW	30.4+7.7	25.4+2.8	17.6+3.7	0.6+0.2**
Number of head-dips	CON	28.7+4.2	26.2+3.0	23.4+4.7	1.5+0.3*
	EW	33.9+6.9	28.8+4.3	24.5+6.9	0.5+0.2**
Locomotor activity	CON	427+162	465+173	338+87	61+21*
	EW	532+114	614+186	418+141	6+2**
Rearing	CON	14.6+2.9	11.3+3.1	7.7+1.2	1.0+0.5*
	EW	28.2+5.3*	33.3+8.7	19.2+4.3	0.5+0.2**

* – Significantly differed from vehicle control, ($P<0.05$); ** – significantly differed from ethanol withdrawn rats ($P<0.01$)

Effect of baclofen. Baclofen (1.25–2.5 mg/kg) did not change the behaviour of control animals. In ethanol-withdrawn rats baclofen (1.25 mg/kg) increased the time spent in active social interaction (Figure 1). There was a significant withdrawal x baclofen (1.25 mg/kg) interaction [$F(1,13)=20.4$, $P<0.01$]. This effect of baclofen disappeared at a dose of 2.5 mg/kg, and at a dose 5 mg/kg baclofen decreased the time spent in social interaction in ethanol-withdrawn animals (Figure 1). Baclofen dose dependently inhibited aggressive reactions in ethanol-withdrawn rats (Figure 2).

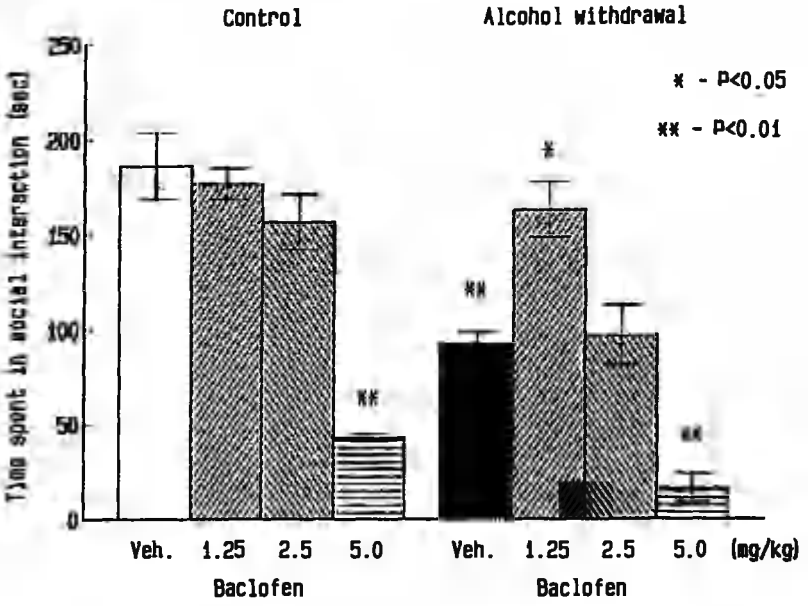

Figure 1. The time spent in social interaction by control and ethanol withdrawn rats 30 min after treatment with baclofen. Scores are means ± SEM. n = 5 per group. + – P<0.05 as compared to control group; * – P<0.05; ** – P<0.01 as compared to vehicle treated group.

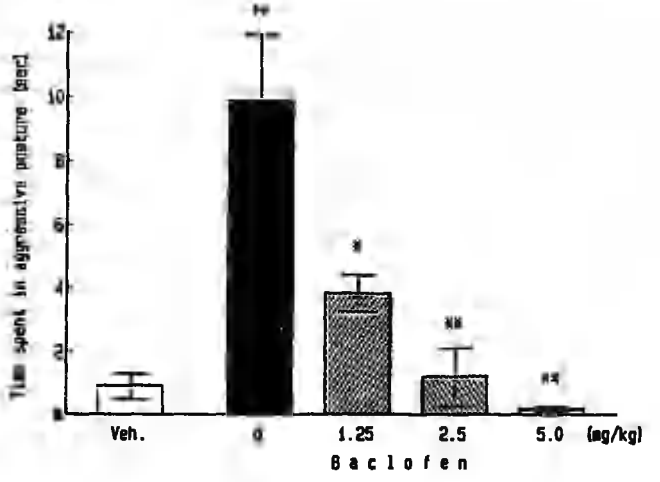

Figure 2. Effect of baclofen on aggression in ethanol withdrawn rats. Scores are means ± SEM. n = 5 per group. ++ – P<0.01 as compared to control group; * – P<0.05; ** – P<0.01 as compared to vehicle group.

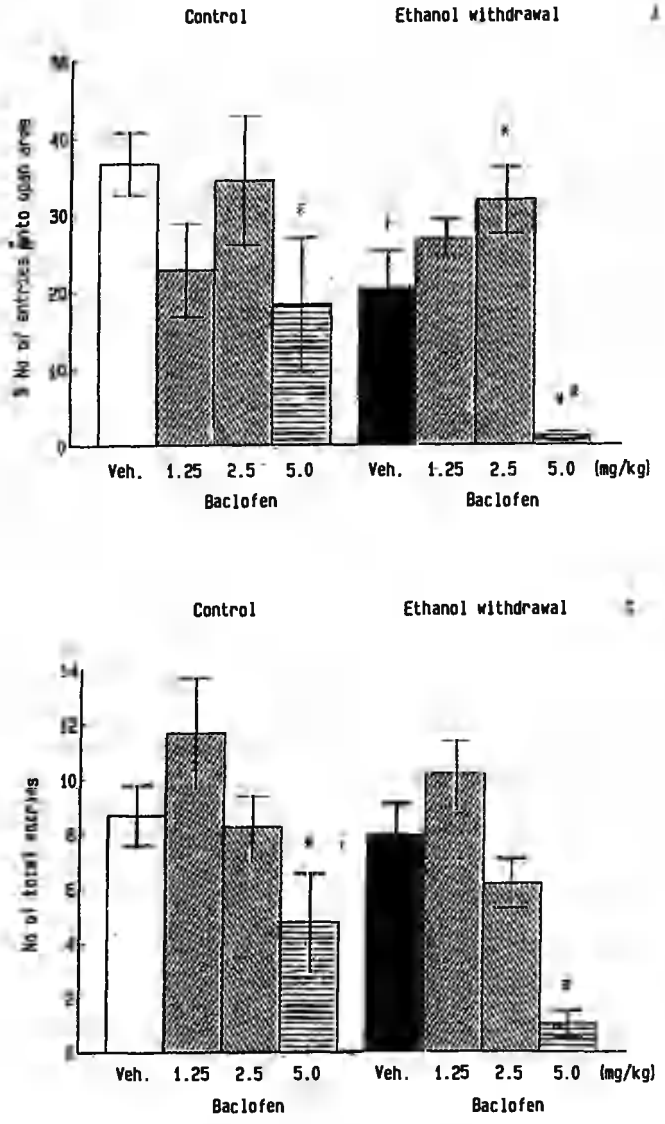

Figure 3. Mean (±SEM) percentage of number of entries into open arms (A), percentage of time spent into the open arms (B) and total number of entries (C) on the open and closed arms of the plus-maze by control and ethanol-withdrawn rats 30 min after administration of baclofen. n = 8–12 per group. + – $P<0.05$ as compared to control group; * – $P<0.05$; ** – $P<0.01$ as compared to vehicle group. ($P<0.05$).

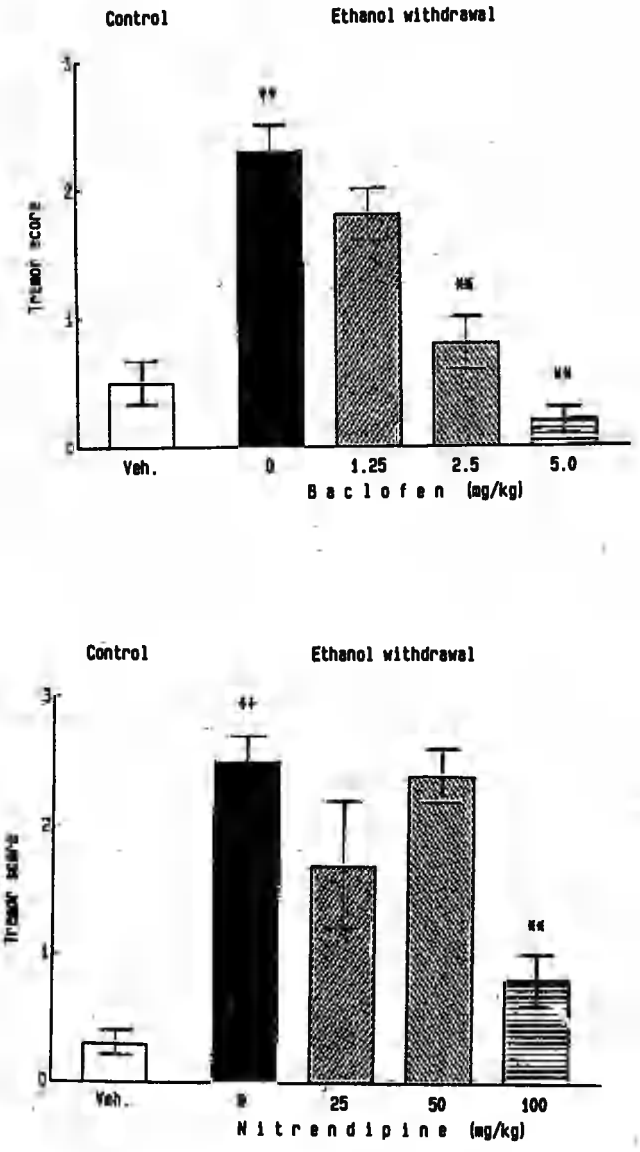

Figure 4. Effect of baclofen and nitrendipine on tremor in ethanol withdrawn rats. Scores are mean values ± SEM. n = 6–12 per group. ++ – $P<0.01$ as compared to control group; ** – $P<0.01$ as compared to ethanol-withdrawn group.

The reversal of anxiogenic response by baclofen in ethanol — withdrawn rats could be also detected in plus-maze test. Thus, at dose of 2.5 mg/kg baclofen increased % number of entries into, and at doses 1.25–2.5 mg/kg — % time spent on the open arms of plus-maze (Figure 3). The total number of entries on the plus-maze was not changed by baclofen (1.25–2.5 mg/kg) in either control or in ethanol-withdrawn rats. At a high dose (5 mg/kg) baclofen reduced the total number of arm entries in the plus-maze (Figure 3).

Low doses of baclofen did not affect orienting response and locomotion in holeboard of both control and ethanol-withdrawn animals (Table 1) and a high dose (5 mg/kg) significantly reduced these measures (Table 1). Ethanol-withdrawn rats were more sensitive than control to the inhibitory effect of high dose of baclofen (5 mg/kg) on locomotor activity in the holeboard test (Table 1). Analysis of covariance revealed that reduction of time spent in social interaction after 5 mg/kg of baclofen was dependent on the reduction in locomotor activity [$F(1,12)=40.0$, $P<0.001$].

Baclofen also suppressed tremor in ethanol-withdrawn rats. This effect was dose-dependent with maximum effect at 5.0 mg/kg (Figure 4).

Experiment 2. Effect of nitrendipine. As in the experiment 1, withdrawal from ethanol treatment resulted in a significant decrease of time spent in social interaction [$F(1,8)=10.9$, $P<0.01$]. However, the scores obtained in the social interaction test in experiment 2, were lower for both control and ethanol-withdrawn groups than in experiment 1 (Figure 1 and Table 2). The reason for these differences in scores of two experiments is unclear, however it might be proposed that these differences in scores were due to the repeated testing. The tremor scores for the groups withdrawn from ethanol did not significantly differ in experiment 1 and 2. Nitrendipine (25–50 mg/kg) did not significantly affect the time spent in active social interaction (Table 2) or % number of entries into and the % time spent on the open arms of plus-maze (Table 3) of either control or ethanol-withdrawn rats. A high dose (100 mg/kg) of nitrendipine produced inhibition of the time spent in social interaction and a decrease of locomotor activity in social interaction test in both control and ethanol-withdrawn rats (Table 2). Analysis of covariance revealed that inhibition of social interaction by nitrendipine (100 mg/kg) was due to the decrease in locomotor activity [$F(1,8)=7.8$, $P<0.095$]. Similarly, nitrendipine at the dose of 100 mg/kg reduced % number and % time spent on the open arms of plus-maze which was accompanied by a decrease in total number of entries (Table 3). At doses of 25–50 mg/kg nitrendipine did not affect tremor scores in ethanol-withdrawn rats, but the dose of 100 mg/kg significantly inhibited tremor (Figure 4).

Table 2

Effect of nitrendipine on the time spent in active social interaction and the locomotor activity in social interaction test of control (CON) and ethanol withdrawn (EW) rats. The data are mean value ± SEM

Drug	dose (mg/kg)	Time spent in social interaction (sec)		locomotions	
		CON	EW	CON	EW
Vehicle		100+9	47+13	75+11	46+10*
Nitrendipine	25	86+23	32+9	66+20	48+6
	50	79+28	67+10	59+27	37+6
	100	37+14*	32+5	26+16*	12+3 **

+ – $P<0.05$ as compared to chronic control (CON). * – $P<0.05$; ** – $P<0.01$ as compared to vehicle. n = 4–5 pair of rats per group.

Table 3

Mean (±S.E.M.) percentage of number of entries into open arms, percentage of time or open arms and total number of entries into the open and closed arms of the plus-maze by control and ethanol-withdrawn (EW) rats 30 min after administration of nitrendipine

Group	Dose (mg/kg)	% Number	% Time	Total Number
Control: vehicle		20.4+5.2	21.2+6.4	12.4+1.5
Nitrendipine	25	20.4+5.1	18.1+6.1	8.7+1.9
	50	15.7+4.8	12.2+3.4	9.8+2.2
	100	13.1+4.7	4.4+1.5*	4.3+0.9*
EW: vehicle		15.1+3.9	8.9+2.6+	10.5+0.9
Nitrendipine	25	16.1+3.9	7.5+1.0	6.5+0.7
	50	9.0+4.4	4.6+1.2	4.6+0.9
	100	6.6+2.6*	3.3+1.8*	3.8+0.8**

n = 8–12 per group. + – $P<0.05$ as compared to control group; * – $P<0.05$; ** – $P<0.01$ as compared to vehicle group.

DISCUSSION

In agreement with our previous study [12] withdrawal from chronic ethanol treatment produced in rats a strong anxiogenic response, which could be detected in both tests of anxiety: social interaction test and plus-maze. The anxiogenic response was accompanied by the increased aggression and tremor, reflecting the state of withdrawal [21]. The data obtained in the present study show that

baclofen suppresses the anxiogenic response in ethanol-withdrawn rats. This effect of baclofen was only evident at the low doses 1.25–2.5 mg/kg. A higher dose of baclofen (5 mg/kg) produced a nonspecific reduction of all behaviours recorded in both control and ethanol-withdrawn groups. This is likely to reflect the sedative properties of this dose of baclofen [17]. Baclofen produced dose-dependent inhibition of aggression and tremor. This effect of baclofen could be observed not only at a high dose when it produced sedation but at the doses which did not induce sedative effect. On the basis of these data it might be proposed that baclofen given in relatively low doses at the withdrawal stage might reverse the anxiogenic response, aggression and tremor.

In contrast, the dihydropyridine calcium channel antagonist nitrendipine, failed to affect anxiogenic withdrawal responses. Only at a high dose of 100 mg/kg nitrendipine did produce an inhibition of social interaction which was associated with inhibition of locomotor activity and tremor in both: control and ethanol-withdrawn rats. These data confirmed the previously obtained data [12] and suggested that inhibitory effect of nitrendipine on the tremor in ethanol-withdrawn rats might be due to the sedative action of this dose of nitrendipine. In this study as well as in the others nitrendipine was used at the relatively high doses which obviously would have several peripheral effects, and therefore could not be employed for the treatment of abstinence signs in humans.

The mechanism of action of baclofen on the signs of ethanol withdrawal is not clear. Although there is no direct evidence indicating an involvement of $GABA_B$ receptor in the development of ethanol withdrawal syndrome one might propose that baclofen exerts this effect via $GABA_B$ receptors in the CNS. $GABA_B$ receptors are broadly distributed within CNS and involved in the regulation of GABA, 5-hydroxytryptamine and noradrenaline release [2, 17]. Previous studies have shown that activation of $GABA_B$ receptors reduced the inward calcium current [7, 8] and enhanced slow outward potassium current in dorsal root ganglion [7]. Baclofen also inhibits the rise of synaptosomal free calcium in rat brain synaptosomes with an IC_{50} in the low micromolar range [24]. The reduction of voltage-dependent calcium conductance and a decrease of $[Ca^{2+}]_i$ in the nerve endings is most likely involved in the inhibition of amine release after administration of baclofen [2, 15, 17]. An increase in Ca^{2+} sensitivity in the nerve terminals is associated with increased amine release as a result of chronic ethanol treatment [20]. Previous studies have demonstrated the importance of GABA-benzodiazepine receptor complex and 5-hydroxytryptamine in the regulation of anxiety [9]. It is possible that imbalance between these neurotransmitter systems is involved in the development of ethanol withdrawal anxiety also.

It might be proposed that baclofen, inhibiting calcium sensitivity and amine release, enhanced in ethanol-withdrawn rats, and thereby restores the balance between GABA and 5-hydroxytryptamine neurotransmitter systems and ameliorates signs of withdrawal. Recent studies have demonstrated an increase in the density of dihydropyridine binding sites after chronic ethanol treatment [5, 22]. But unlike baclofen, dihydropyridines have little if any effect on synaptosomal calcium uptake and are not involved in the regulation of amine release [4]. Also it is not excluded that baclofen and dihydropyridines affect different types of voltage sensitive calcium channels, which might explain the difference in the effects of these drugs on the ethanol withdrawal signs.

REFERENCES

1. Al-Dahan M.L, Thalman R.H. Effects of dihydropyridine calcium channel ligands on rat brain -aminobutyric acid$_B$ receptors // J. Neurochem. 1989. Vol. 53. P. 982–985.
2. Bowery N.G., Hill D.R., Hudson A.L., Doble A., Middlemiss D.N., Shaw J., Turnbull M. (–)-Baclofen decreases neurotransmitter release in the mammalian CNS by an action at a novel GABA receptor // Nature. 1980. Vol. 283. P. 92–94.
3. Bowery N.G., Hill D.R., Hudson A.L. Charactertistics of GABA$_B$ receptor binding sites on whole brain synaptic membranes // Brit. J. Pharmacol. 1982. Vol. 78. P. 191–206.
4. Daniel L.C., Barr E.M., Leslie S.W. $^{45}Ca^{2+}$ uptake into rat whole synaptosomes unaltered by dihydropyridine calcium antagonists // J. Neurochem. 1983. Vol. 41. P. 1455–1459.
5. Dolin S.J., Little H.J., Pagonis C., Littleton J. Increased dihydropyridine-sensitive calcium channels in rat brain may underlie ethanol physical dependence // Neuropharmacol. 1987. Vol. 26. P. 347–360.
6. Dolin S.J., Little H.J. Are changes in neuronal calcium channels involved in ethanol tolerance? // J. Pharmacol. Exp. Ther. 1989. Vol. 250. P. 985–991.
7. Dolphin A.C., Scott R.H. Inhibition of calcium currents in cultured rat dorsal root ganglion by baclofen // Brit. J. Pharmacol. 1986. Vol. 88. P. 213–220.
8. Dunlap K., Fishbach G.D. Neurotransmitters decrease the calcium conductance activated by depolarization of embrionic chick sensory neurones // J. Physiol. 1981. Vol. 317. P. 519–535.
9. File S.E., Hyde J.R.G. McLeod N.K.; 5,7-Dihydrotryptamine lesons of dorsal and median raphe nuclei and performance in the social interaction test of anxiety and in a home-cage aggression test // J. Affect. Disord. 1979. Vol. 1. P. 115–122.

10. File S.E. The use of social interaction as a method for detection anxiolytic activity of chlordiazepoxide-like drugs // J. Neurosci. Meth. 1980. Vol. 2. P. 219–238
11. File S.E. How good is social interaction as a test of anxiety // Animal Models of Psychiatric Disorders / Eds P. Simon, P. Soubrie, D. Wildlocher. Basel: S. Karger, 1988. Vol. 1. P. 151–166.
12. File S.E., Baldwin H.A. Hitchcott P.K.; Flumazenil but not nitrendipine reverses the increased anxiety during ethanol withdrawal in the rat // Psychopharmacol. 1989. Vol. 98. P. 262–264.
13. Freund G. Induction of physical dependence on alcohol in rodents // Biochemical Pharmacology of Ethanol / Ed. E. Majchrowics. New York: Plenum Press, 1975. P. 211–325.
14. Frye G.D., McCown T.J., Breese G.R. Differential sewnsitivity of ethanol withdrawal signs in the rat to aminobutyric acid (GABA) mimetics: blockade of audiogenic seizures but not forelimb tremors // J. Pharmacol. Exp. Ther. 1983. Vol. 226. P. 720–725.
15. Fung S.-C., Fillens M. Studies on the mechanism of modulation of [^{3}H]noradrenaline release from rat hippocampal synaptosomes by GABA and benzodiazepine receptors // Neurochem. Int. 1985. Vol. 7. P. 95–101.
16. Gandhi C.R., Ross D.H. Influence of ethanol on calcium, inositol phospholipids and intracellular signalling mechanisms // Experientia. 1989. Vol. 45. P. 407–412.
17. Gray J.A., Green A.R. GABA$_B$ receptor mediated inhibition of potassium evoked release of endogenous 5-hydroxytryptamine from mouse frontal cortex // Brt. J. Pharmacol. 1987. Vol. 91. P. 517–522.
18. Little H.J., Dolin S.J., Halsey M.J. Calcium channel antagonists decrease the ethanol withdrawal syndrome // Life Sci. 1986. Vol. 39. P. 2054–2065.
19. Little H.J., Dolin S.J. Lack of tolerance to ethanol after concurent administration of nitrendipine // Brit. J. Pharmacol. 1987. Vol. 92. 606 P.
20. Lynch M.A., Littleton J.M. Possible association of alcohol tolerance with increased synaptic Ca^{++} sensitivity // Nature. 1983. Vol. 303. P. 175–176.
21. Majchrowicz E. Induction of physical dependence upon ethanol and the associated behaviour changes in rats // Psychopharmacol. 1975. Vol. 43. P. 245–254.
22. Messing R.O., Carpenter C.L., Diamond L., Greenberg D.A. Ethanol regulates calcium channels in clonal neural cells // Proc. Natn. Acad. Sci. USA. 1983. Vol. 83. P. 6213–6215.
23. Pucilowski O., Kostowski W. Diltiazem suppresses apomorphine induced fighting and proaggressive effect of withdrawal from chronic ethanol or haloperidol in rats // Neurosci. Lett. 1988. Vol. 93. P. 96–100.
24. Stirling J.M., Cross A.J., Robinson T.N., Green A.R. The effect of GABA$_B$ receptor agonists and antagonists on potassium stimulated $[Ca^{2+}]_i$ in rat brain synaptosomes // Neuropharmacol. 1989. Vol. 28. P. 699–704.

25. Sytinski I.A., Guzikov B.M., Gomanko M.V., Erenien V.P., Konovalova N.N. The gamma-aminobutyric acid (GABA) system in brain during acute and chronic ethanol intoxication // J. Neurochem. 1989. Vol. 25. P. 43–48.
26. Tarika J.S., Winger G. The effect of ethanol, pentobarbital, and baclofen on ethanol withdrawal in the rhesus monkeys // Psychopharmacol. 1980. Vol. 70. P. 201–208.
27. Ticku M.K. The effects of acute and chronic ethanol administration and its withdrawal on -aminobutyric acid receptor binding in rat brain // Brit. I. Pharmacol. 1980. Vol. 70. P. 403–410.
28. Tickn M.K. Ethanol and the benzodiazepine-GABA receptor-ionophore complex // Experientia. 1989. Vol. 45. P. 413–416.
29. Victor M. The alcohol withdrawal syndrome: theory and practice // Postgrad. Med. 1970. Vol. 47. P. 68–72.
30. Volicer L. GABA levels and receptor binding after acute and chronic ethanol administration // Brain Res. Bull. 1980. Vol. 5, Suppl. 2. P. 809–813.

CHRONIC TREATMENT WITH PERIPHERAL BENZODIAZEPINE LIGANDS AFFECTS BEHAVIORAND GABA/BENZODIAZEPINE RECEPTORS IN RAT

Lembit Rägo*, Veijo Saano, Aleksander Adojaan*,
Timo Auvinen, and Mauno M. Airaksinen

Department of Pharmacology and Toxicology,
University of Kuopio, P.O.Box 6, 70211 Kuopio,
Finland and *Department of Pharmacology,
Tartu University, Ülikooli 18, 202 400 Tartu, Estonia

Rats were treated for three weeks, twice daily, with peripheral benzodiazepine (PBD) receptor ligands Ro 5-4864 and PK 11195 (both 10 mg/kg, i.p.). After the first injection there were no differences between the drug-treated and control animals in behavioral tests. After 10 days treatment, the number of sniffings was increased in Ro 5-4864 treated rats. After the last injection, sniffings and ambulations were decreased in PK 11195 treated animals. The number of rearings and groomings remained unchanged throughout the treatment, and there were no changes in the results from elevated plus-maze test. Apparently these compounds are devoid of anxiolytic and anxiogenic effects at moderate doses. The effect of 72 h withdrawal from the above mentioned chronic treatment on PBD, central benzodiazepine (CBD), and GABA receptors was studied with receptor binding techniques using ^{3}H-Ro 5-4864, ^{3}H-flumazenil and ^{3}H-muscimol, respectively, as ligands. The number of GABA and CBD receptors was lower after Ro 5-4864 treatment, as was the effect of progesterone-induced stimulation of ^{3}H-muscimol binding. However, the GABA stimulated ^{3}H-flunitrazepam binding remained unchanged after withdrawal from both, Ro 5-4864 and PK 11195. The number of PBD receptors was decreased after Ro 5-4864 and PK 11195 treatments in olfactory bulb but not in cerebral cortex. The chronic treatment with PBD ligands Ro 5-4864 and PK 11195 produces different effects. Ro 5-4864, often presented as an agonist of PBD receptors, is behaviorally inactive. It affects, however, GABA/BD receptor complex linked to steroid binding sites. PK 11195, often considered to be an antagonist, develops sedative action during chronic treatment, but withdrawal from it does not produce changes in

GABA/BD receptor complex.

KEY WORDS: Ro 5-4864; PK 11195; chronic treatment; behavior; GABA and benzodiazepine receptors

INTRODUCTION

The existence of at least two different types of benzodiazepine (BD) receptors has been well documented. The central type BD receptors are present in the CNS [16] and a close correlation between the clinical potencies of BDs and their affinities for these binding sites indicates that they mediate the therapeutic actions of these drugs [25]. The peripheral type BD receptors are present both in peripheral tissues (adrenals, testis, kidney, heart, liver, spleen etc.) and CNS [for review see 22]. In spite of numerous studies, relatively little is known about the regulation and physiological role of the peripheral BD receptors. 4'-chlorodiazepam (Ro 5-4864) and the isoquinoline carboxamide, PK 11195 bind with high affinities to peripheral, but not to central BD receptors [15]. Ro 5-4864 induces convulsions and anxiety in rodents [6], displaces ^{35}S-TBPS from picrotoxinin binding sites [27] and antagonizes the depolarizing effect of muscimol on rat cuneate nucleus slices [23].

PK 11195 is known to antagonize some, but not all, of the effects of Ro 5-4864 [22]. Thus, Ro 5-4864 and PK 11195, are considered to be an agonist and antagonist of peripheral BD binding sites respectively.

The effects of chronic treatment with central BD ligands have been extensively studied. However, very little is known about the effects of chronic treatment with specific peripheral BD receptor ligands on behavior as well as on GABA and benzodiazepine receptors. Only recently it has been reported that two weeks treatment with PK 11195 reduces anxiety in humans [1].

The aim of the current study was to examine comparatively the effect of three weeks treatment with Ro 5-4864 and PK 11195, proposed agonist and antagonist of peripheral BD receptors, on behavior. Parallely the effect of withdrawal from chronic treatment on GABA and both types of BD receptors in the rat brain was studied.

METHODS

Animals and drugs. Male Kuo:Wistar rats (National Laboratory Animal Center, Kuopio, Finland, 220–250 g) were housed five animals per cage in a room with a lighting cycle 20.00 – 07.00 dark period and allowed free access to food and water. 4'-chlorodiazepam (Ro 5-4864, Fluka AG, Basle, Switzerland) and 1-(2-chlorophenyl)-N-

methyl-N-(1-methylpropyl)-3-isoquinoline carboxamide (PK 11195, Pharmuka Laboratories, Gennevilliers, France) were dissolved in saline with a drop of Tween-80. The drugs were injected twice a day (at 9.00 and 20.00) intraperitoneally 60 and 120 min before open field and elevated plus-maze respectively, in concentrations to give an injection volume of 5 ml/kg and dose 10 mg/kg. Saline containing the same amount of Tween-80 was used for control animals. The behavioral testings were carried out after the first daily injection.

Apparatuses and registration procedure. A white circular test arena (diameter 83 cm, divided into 19 equal areas, walls 40 cm) with 65 lux light was used for open-field. On the test day, the rats were transferred into the laboratory in the morning and allowed to habituate for at least 30 min. The behavior of rats was observed for 5 min directly by an experienced experimentator. The number of rearings, sniffings, self-groomings, ambulations (stepping across the lines on the floor) and the time of non-mobile exploration (rats sniffed and looked around while remaining in one place) were registered. The animals were tested in an open-field after the first injection on 1st, 11th and 21st day of treatment.

The elevated plus-maze method described by Pellow and File [17]. In brief, the plus-maze consisted of two open arms, 50 x 10, and two enclosed arms, 50 x 10 x 25, with an open roof, arranged so that the two arms of the same kind were opposite to each other. The central compartment of the plus-maze was an open square, 10 x 10 cm. During a 4-min test period the following observations were made: (a) number of open arm entries, (b) time spent in open and closed arms and, (c) total number of all arm entries. To begin the experiments, the animals were placed at the center of the plus-maze. The exploratory activity of rats treated with Ro 5-4864 and PK 11195 was studied in an elevated plus-maze on 2nd and 22nd day (after morning injection) of chronic treatment with these drugs.

The data of behavioral studies were manually transferred to a computer for automatic data handling (program by PC Soft Co, Joensuu, Finland).

In vitro binding studies. ^{3}H-flumazenil (Ro 15-1788, spec. act. 79 Ci/mmol, New England Nuclear, Boston, MA), ^{3}H-muscimol (spec. act. 22 Ci/mmol), ^{3}H-flunitrazepam (spec. act. 81 Ci/mmol, both Amersham Radiochemicals, England) and ^{3}H-Ro 5-4864 (spec. act. 89 Ci/mmol, New England Nuclear, Boston, MA) binding was carried out as described earlier [20,21]. Steroid hormone stimulated ^{3}H-muscimol binding was carried out as described by López-Colomó et al. [13] with minor modifications. The tissues were homogenized in 30 vol. 25 mM K_2HPO_4/KH_2PO_4 containing 50 mM KCl (pH 7.4) with Ultraturrax homogenizer (5 s, setting 6) and washed twice in the same buffer by centrifugation (48,000 x g for 20

min). The final pellets were stored overnight at – 20° C. After thawing they were rehomogenized and washed additionally four times in the same buffer for basal and steroid stimulated ^{3}H-muscimol binding. The reaction was initiated by the addition of tissue and terminated after incubation (30 min on ice) by rapid filtration over Whatman GF/B filters.

GABA (Sigma, St. Louis, MO), flunitrazepam (Hoffmann La-Roche, Basle, Switzerland) and PK 11195 (Pharmuka Laboratories, Gennevilliers, France) were used to determine the specific binding to GABA, central- and peripheral BD receptors respectively. Progesterone, 4-androstene-3,17-dione (androstenedione) and GABA (all from Sigma, St. Louis, MO) were used to stimulate ^{3}H-muscimol binding. Specific binding was calculated by subtracting the nonspecific from total binding. Protein content was measured by the Lowry et al. [14] method.

Calculations and statistics. Maximum binding (B_{max}) and affinity constants (K_D) were calculated using Scatchard analysis. The Scatchard plots were computed with the curve-fitting program Enzfitter. The significance of the differences between the binding data was calculated using two-tailed Student's t-test. The differences between control and treated animals in open-field and elevated plus-maze were assessed by Mann-Whitney U-test and ANOVA respectively. $P < 0.05$ was considered statistically different.

RESULTS

The effect of acute and chronical treatment with Ro 5-4864 and PK 11195 on behavior of rats in open-field. The acute treatment (first day, first injection) of rats with Ro 5-4864 (10 mg/kg) and PK 11195 (10 mg/kg) did not change any of the behavioral parameters studied in the open-field (Fig. 1,2). Treatment during 10 days (10 mg/kg, twice a day, behavior tested after 1st injection on 11th day) with Ro 5-4864 significantly increased the number of sniffings (Fig.1) but had only a slight tendency to increase rearings (data not shown) and ambulations (Fig.2). After 10 days of administration PK 11195 (10 mg/kg, twice a day) did not have any statistically significant effects on behavior (Fig. 1,2). However, after 20 days treatment (10 mg/kg, twice a day, behavior tested after 1st injection on 21st day) PK 11195 significantly reduced the number of sniffings and ambulations (Fig. 1,2). Ro 5-4864 lacked this effect of PK 11195, i.e. after 20 days of treatment it did not change either the number of rearings (data not shown) or sniffings or grooming (Fig. 1,2).

The effect of acute and chronic treatment with Ro 5-4864 and PK 11195 on exploratory activity of rats in an elevated plus-maze. Treatment with neither Ro 5-4864 nor PK 11195 caused any signifi-

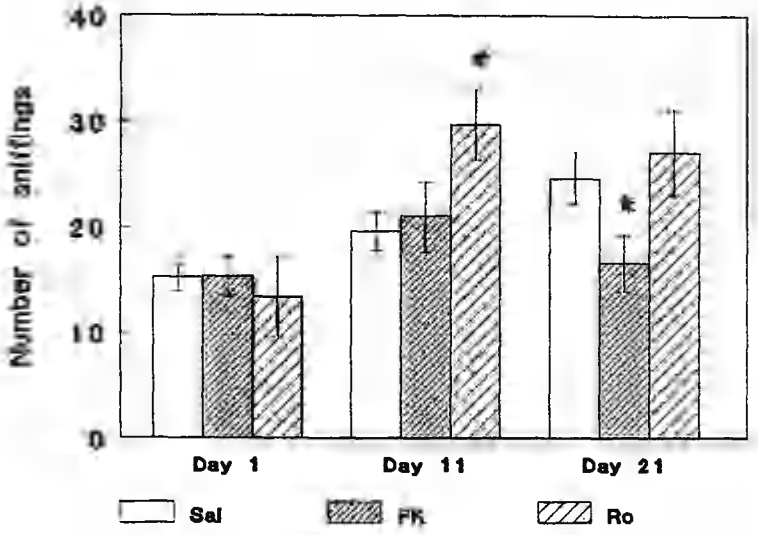

Figure 1. The effect of chronical treatment with Ro 5-4864 (10 mg/kg) and PK 11195 (10 mg/kg) on the number of sniffings in open field. The drugs were administered twice a day. The behavioral experiments were carried out after morning injection. The data presented are mean ± S.E.M. of 10 animals per group. * – $P < 0.05$ as compared to saline treated controls.

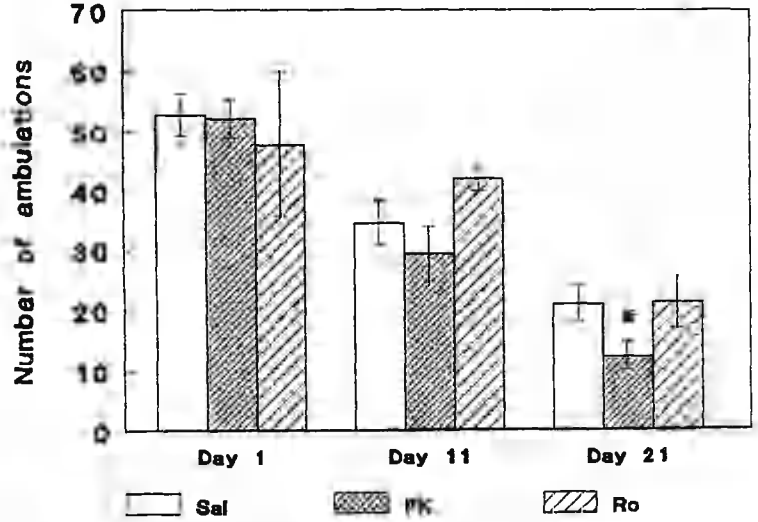

Figure 2. The effect of chronical treatment with Ro 5-4864 (10 mg/kg) and PK 11195 (10 mg/kg) on the number of ambulations in open field. The drugs were administered twice a day. The behavioral experiments were carried out after morning injection. The data presented are mean ± S.E.M. of 10 animals per group. * – $P < 0.05$ as compared to saline treated controls.

cant changes of the behavioral parameters registered in an elevated plus-maze. Hovever, in the beginning of the treatment only Ro 5-4864 had a tendency to decrease the proportion of time spent in the open arms. After the last injection (on 22nd day) the reverse was observed i.e. only PK 11195 seemed to lower the amount of time spent in open arms (Fig.3).

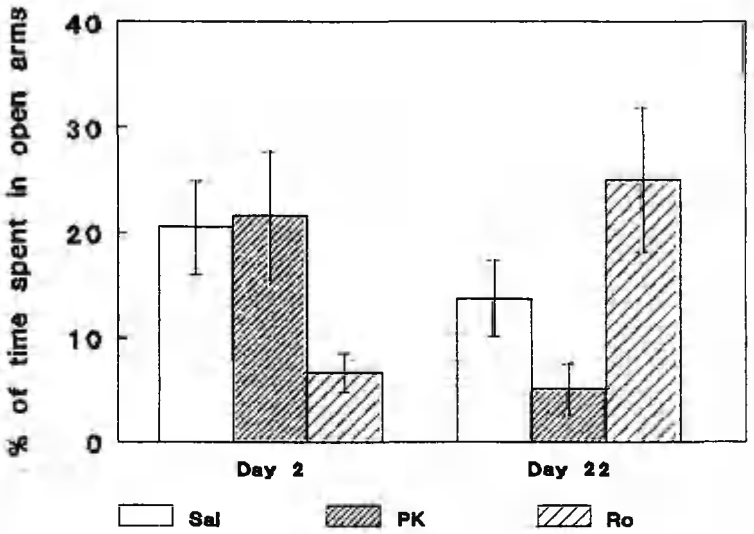

Figure 3. The effect of chronical treatment with Ro 5-4864 (10 mg/kg) and PK 11195 (10 mg/kg) on the % of time spent in the elevated plus-maze open arms. The drugs were administered twice a day. The behavioral experiments were carried out after morning injection. The data presented are mean ± S.E.M. of 10 animals per group.

The effect of withdrawal from chronic treatment with Ro 5-4864 and PK 11195 on central – and peripheral BD receptors. Chronic treatment with PK 11195 and Ro 5-4864 lowered the number of ^{3}H-Ro 5-4864 binding in olfactory bulb but not in cerebral cortex (Table 1). Only Ro 5-4864 lowered the the density of central BD receptors labeled by ^{3}H-flumazenil in cerebral cortex (Table 2). Chronic treatment with both of the peripheral benzodiazepine ligands did not change the affinity constants of neither ^{3}H-Ro 5-4864 nor ^{3}H-flumazenil (Tables 1,2).

The effect of withdrawal from chronic treatment with Ro 5-4864 and PK 11195 on ^{3}H-muscimol and GABA – stimulated ^{3}H-flunitrazepam. Chronic treatment with PK 11195 did not change either basal or progesterone and androstenedione stimulated ^{3}H-

Table 1

The effect of withdrawal (72 h) from chronic treatment with Ro 5-4864 and PK 11195 on ^{3}H-Ro 5-4864 binding in the rat cerebral cortex and olfactory bulb. The data presented are mean ± S.E.M. of 3–5 determinations each carried out in triplicate. Statistical comparisons were made using two-tailed Students t-test

Treatment, mg/kg twice a day	^{3}H-Ro 5-4864	
	B_{max} (fmol/mg)	K_D (nM)
	Cerebral cortex	
Saline	304 ± 56	3.67 ± 0.87
Ro 5-4864 10	446 ± 69	5.47 ± 1.08
PK 11195 10	177 ± 36	2.32 ± 0.71
	Olfactory bulb	
Saline	1672 ± 91	2.41 ± 0.25
Ro 5-4864	843 ± 81*	1.91 ± 0.28
PK 11195	897 ± 86*	2.18 ± 0.41

* – $P < 0.01$ as compared to saline controls

Table 2

The effect of withdrawal (72 h) from chronic treatment with Ro 5-4864 and PK 11195 on ^{3}H-flumazenil and ^{3}H-muscimol binding in the rat cerebral cortex. The data presented are mean ± S.E.M. of 3-5 determinations carried out in triplicate. Statistical comparisons were made using two-tailed Students t-test

Treatment, mg/kg twice a day	Binding characteristics	
	B_{max} (fmol/mg)	K_D (nM)
	^{3}H-flumazenil	
Saline	2151 ± 59	1.97 ± 0.30
Ro 5-4864 10	1782 ± 50*	1.81 ± 0.13
PK 11195 10	1896 ± 66	2.02 ± 0.18
	^{3}H-muscimol	
Saline	2530 ± 98	23.61 ± 1.82
Ro 5-4864 10	1823 ± 93*	17.12 ± 2.60
PK 11195 10	2305 ± 291	29.55 ± 3.55

* – $P < 0.05$ as compared to saline treated controls

muscimol binding characteristics (Table 2, Fig.4). Ro 5-4864 treatment lowered significantly the number of ^{3}H-muscimol binding sites in cerebral cortex (Table 2) and decreased the stimulation of ^{3}H-

muscimol binding caused by steroids (Fig.4). Chronic treatment with neither Ro 5-4864 nor PK 11195 changed the GABA stimulated ^{3}H-flunitrazepam binding in cerebral cortex (Fig.5).

DISCUSSION

Ro 5-4864, a proposed agonist of peripheral BD receptors, has anxiogenic effects that are not reversed by the suggested antagonist of these receptors, PK 11195 [7,8]. Interestingly, PK 11195 itself has also anxiogenic activity [8]. Additionally to anxiogenic effects Ro 5-4864 can cause convulsions that are reversed by PK 11195 pretreatment [5]. However, all the behavioral effects of peripheral BD ligands reported appear in the doses far higher (20–60 mg/kg and 30–90 mg/kg for Ro 5-4864 and PK 11195 respectively) than those needed to saturate these receptors [8,19]. Therefore, we decided to carry out a chronic treatment with a more moderate dose of Ro 5-4864 and PK 11195 (10 mg/kg) to examine comparatively the effects of acute and repeated treatment on behavior.

After the first treatment both of the drugs studied were without any significant behavioral effects in the open field test. After 10 days treatment with Ro 5-4864 only the number of sniffings was significantly increased. The number of ambulations also had a tendency to be increased after Ro 5-4864 treatment. However, after 20 days treatment these changes were not present any more. After 21 days of treatment, Ro 5-4864 had a tendency to increase the amount of time spent in the open arms of the elevated plus-maze. This may indirectly support the earlier observation of Zbinden and Randall [28] that Ro 5-4864 has a tranquilizing effect in humans. However, most probably the moderate behavioral effects of Ro 5-4864 observed in our studies reflect increased arousal. Electrophysiological studies indicate that Ro 5-4864 has both in vitro and in vivo effects opposite to those of the benzodiazepine tranquilizers [18,26].

In contrast to Ro 5-4864, chronic treatment with PK 11195 during 20 days resulted in a significant decrease of the number of sniffings and ambulations. The proportion of time spent in the open arms of the elevated plus-maze was also considerably (although not significantly) less than in controls. The development of these behavioral effects after chronic treatment with PK 11195 is difficult to explain. So far, it has only been demonstrated that no tolerance develops to anticonvulsant action of PK 11195 (against Ro 5-4864 induced convulsions) but rapid tolerance develops to its proconvulsant effect [5]. The behavioral actions of PK 11195 that develop during chronic treatment resemble either sedative or anxiogenic effects. In acute experiments no sedative action of PK 11195 has been reported. However, only a very high dose of PK

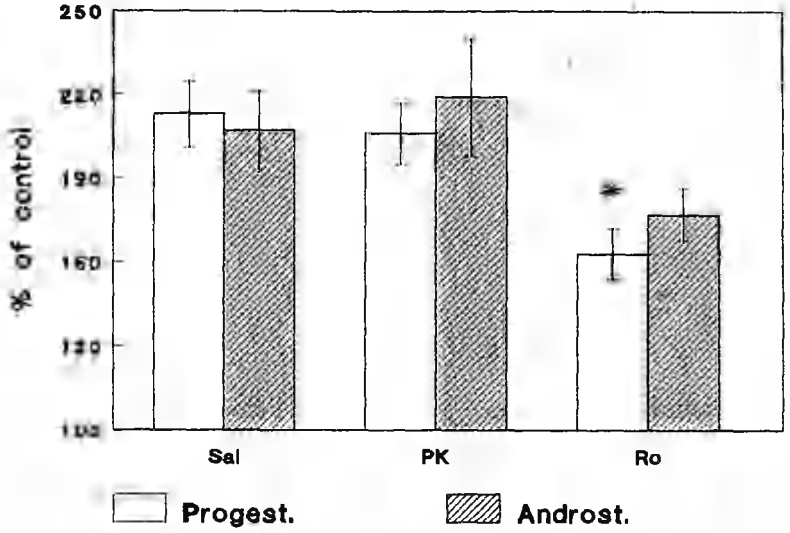

Figure 4. The effect of chronical treatment with Ro 5-4864 (10 mg/kg, twice a day) and PK 11195 (10 mg/kg, twice a day) on progesterone (30 μM) and androstenedione (30 μM) stimulated ^{3}H-muscimol binding in the rat cerebral cortex after 72 h of withdrawal. The data represent mean $\pm$ S.E.M. of 3–5 determinations each carried out in triplicate. * – $P < 0.05$ as compared to saline treated animals.

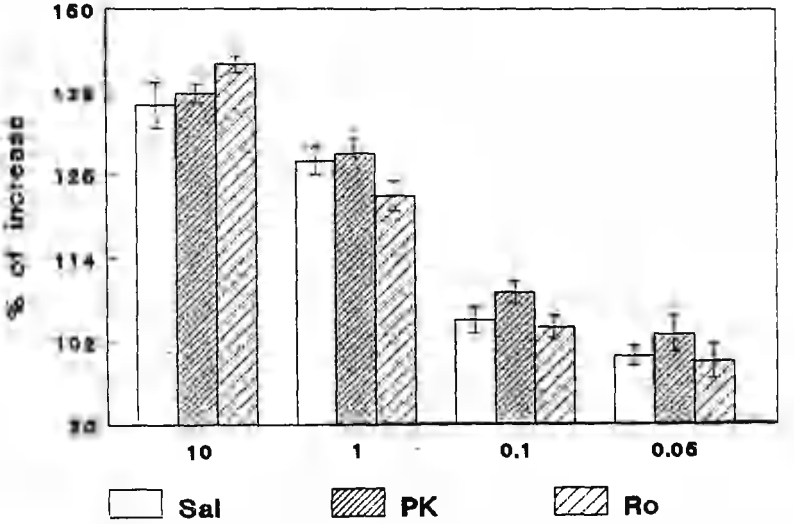

Figure 5. The effect of chronic treatment with Ro 5-4864 (10 mg/kg, twice a day) and PK 11195 (10 mg/kg, twice a day) on GABA (10 μM) stimulated ^{3}H-flunitrazepam binding in rat cerebral cortex after 72 h of withdrawal. The data represent mean $\pm$ S.E.M. of 3 determinations each carried out in triplicate.

11195 (90 mg/kg) reduces social interaction indicating a possible anxiogenic effect [8].

Withdrawal from chronic treatment with Ro 5-4864 resulted in a decreased number of GABA and central BD receptors in the rat cerebral cortex. In contrast, PK 11195 treatment did not cause any significant changes of these receptors. The findings with Ro 5-4864 seem to be in line with the recent data demonstrating that Ro 5-4864 is coupled functionally to a GABA-regulated chloride ionophore labeled by the cage convulsant ^{35}S-TBPS [9,10]. It has also been proposed that GABA/benzodiazepine receptor complex linked to a Ro 5-4864 binding site is allosterically coupled to the putative steroid recognition site [3].

Recently López-Colomó et al. [13] demonstrated the enhancement of ^{3}H-muscimol binding to brain synaptic membranes by progesterone and related pregnanes. In our study, withdrawal from Ro 5-4864, but not PK 11195, lowered the stimulation of ^{3}H-muscimol binding by progesterone and androstenedione. The two prototypic peripheral BD ligands, Ro 5-4864 and PK 11195, may not label identical populations of binding sites. Sites labeled by these two ligands have been shown to be differentially influenced by several agents, including arachidonate [24], detergents such as Triton X-100, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), and Tween 20 [3], and by chemical modification by phospholipase A_2 [11]. Nevertheless, in our studies both Ro 5-4864 and PK 11195, caused similar downregulation of ^{3}H-Ro 5-4864 binding in olfactory bulb but not in cerebral cortex.

After withdrawal from chronic treatment with agonist and antagonist one would expect different, rather than similar effects on the common receptor for these ligands. Similar effects may evidence that both these ligands can share agonistic properties. Indeed, in several studies it has been shown that PK 11195 can not antagonize all the effects of Ro 5-4864 [4,26] and in some cases can even mimic the effects of Ro 5-4864 [4,12]. The data obtained give evidence that even relatively high doses (10 mg/kg) of peripheral benzodiazepine ligands, both after single or chronical tratment, have very few, if any behavioral effects. However, chronic treatment with PK 11195 seems to cause mild sedation. Thus, if any therapeutic applications can be found for peripheral BD receptor ligands, these compounds would be without major unwanted behavioral side effects even during chronic treatment.

In conclusion, Ro 5-4864 and PK 11195, proposed agonist and antagonist of peripheral BD receptors respectively, have moderate but still different effects on behavior during chronic treatment. The chronic treatment with the peripheral BD ligands also affects GABA and central BD receptors differently but affects similarly peripheral

BD receptors in CNS.

Acknowledgements. This study was supported by the Academy of Finland (Medical Research Council) and Orion Corporation Research Foundation. We are greateful to Pharmuka Laboratories (Gennevilliers, France) for PK 11195.

REFERENCES

1. **Anssean M., Papart P., Cerfontaine J.L. Frenckell R. von.** Pilot study of PK 11195 (52028 RP), a selective ligand for the peripheral type benzodiazepine binding sites, among inpatients with anxious of depressive symptomatology // Therapie. 1990. Vol. 45. P. 291.
2. **Awad M., Gavish M.** Binding of ^{3}H-Ro 5-4864 and ^{3}H-PK 11195 to cerebral cortex and peripheral tissues of various species: species differencies and heterogenity in peripheral benzodiazepine binding sites // J. Neurochem. 1988 Vol. 49. P. 1407–1414.
3. **Belelli D., McCauley L., Gee, K.W.** Heterotropic cooperativity between putative recognition sites for progesterone metabolites and the atypical benzodiazepine Ro 5-4864 // J. Neurochem. 1990. Vol. 55. P. 83–87.
4. **Calogero A.E., Kamilaris T.C., Bernardini R., Johnson E.O., Chrousos P., Gold, P.W.** Effects of peripheral benzodiazepine receptor ligands on hypothalamic-pituitary-adrenal axis function in the rat // J. Pharmacol. Exp. Ther. 1990. Vol. 253. P. 729–737.
5. **File S.E.** Pro- and anti-convulsant properties of PK 11195, a ligand for benzodiazepine binding sites: development of tolerance // Brit. J. Pharmacol. 1984. Vol. 83. P. 471–476.
6. **File S.E., Lister R.G.** The anxiogenic action of Ro 5-4864 is reversed by phenytoin // Neurosci. Lett. 1983. Vol. 35. P. 9396.
7. **File S.E., Pellow S.** The anxiogenic action of Ro 5-4864 in the social interaction test: effect of chlordiazepoxide, Ro 15-1788 and CGS 8216 // Naunyn-Schmiedeberg's Arch. Pharmacol. 1985a. Vol. 328. P. 225–228.
8. **File S.E., Pellow S.** The effects of PK 11195, a ligand for benzodiazepine binding sites, in animal tests of anxiety and stress // Pharmacol. Biochem. Behav. 1985b. Vol. 23. P. 737–741.
9. **Gee K.W.** Phenylquinolines PK 8165 and PK 9084 allosterically modulate [^{35}S]-t-butylbicyclophosphorothionate binding to a chloride ionophore in rat brain via a novel Ro 5-4864 binding site // J. Pharmacol. Exp. Ther. 1987. Vol. 240. P. 747–753.
10. **Gee K.W., Brinton R.E., McEwen B.S.** Regional distribution of a Ro 5-4864 binding site that is functionally coupled to the gamma-aminobutyric acid/benzodiazepine receptor complex in rat brain // J. Pharmacol. Exp. Ther. 1988. Vol. 246. P. 803–812.
11. **Havondjian H., Cohen R.M., Paul S.M., Skolnick P.** Differential sensitivity of "central" and "peripheral" type benzodiazepine receptors to phospholipase A_2. // J. Neurochem. 1986. Vol. 46. P. 804–811.

12. Larcher J.-C., Vayssiere J.-L., Le Marquer F.J., Cordeau L.R., Keane P.E., Bachy A., Gros F., Croisat P. Effects of peripheral benzodiazepine upon the O_2 consumption of neuroblastoma cells // Eur. J. Pharmacol. 1989. Vol. 161. P. 197–202.
13. López-Colomé A.M., McCarthy M., Beyer C. Enchancement of ^{3}H-muscimol binding to brain synaptic membranes by progesterone and related pregnanes // Eur. J. Pharmacol. 1990. Vol. 176. P. 297–303.
14. Lowry O.H., Rosebrough N.J., Farr A.L., Randall R.S. Protein measurement with the folin phenol reagent. // J. Biol. Chem. 1951. Vol. 193. P. 265–275.
15. Marangos P.J., Patel J., Boulenger J.-P., Clark-Rosenberg R. Characterization of peripheral-type benzodiazepine binding sites in brain using ^{3}H-Ro 5-4864 // Mol. Pharmacol. 1982. Vol. 22. P. 26–32.
16. MFhler H., Okada, T. Benzodiazepine receptor: demonstration in the central nervous system // Science (Wash. DC). 1977. Vol. 198. P. 849–851.
17. Pellow S., File S.E. Anxiolytic and anxiogenic drug effects on exploratory activity in an elevated plus-maze: a novel test of anxiety in rat // Pharmacol. Biochem. Behav. 1986. Vol. 24. P. 525–529.
18. Pieri L., Polc P., Bonetti W., Burkard W., Cumin R., Scherschlicht R., Haefely W. Some pharmacological effects of Ro 5-4864, a specific ligand of the peripheral type of benzodiazepine binding sites // Naunyn-Schmiedeberg's Arch. Pharmacol. 1983. Vol. 322, Suppl. 1. P. 337.
19. Price G.W., Ahier R.G., Hume S.P., Myers R., Manjil L., Cremer J.E., Luhtra S.K., Pascali C., Pike V., Frackowiak R.S. In vivo binding to peripheral benzodiazepine binding sites in lesioned rat brain: comparison between ^{3}H-pk 11195 and ^{18}H-PK 14105 as markers for neuronal damage // J. Neurochem. 1990. Vol. 55. P. 175–185.
20. Rägo L., Kiivet R.-A., Harro J., Põld, M. Central- and peripheral-type benzodiazepine receptors: similar regulation by stress and GABA agonists, Pharmacol. Biochem. Behav.. 1989. Vol. 32. P. 879–883.
21. Rägo L., Kiivet R.-A., Adojaan A., Harro J., Allikmets L.H. Stress-protective action of β-phenyl(GABA): involvement of central and peripheral type benzodiazepine binding sites // Pharmacol. Toxicol. 1990. Vol. 66. P. 41–44.
22. Saano V., Rägo L., Räty M. Peripheral benzodiazepine binding sites // Pharmacol. Ther. 1989. Vol. 41. P. 503–514.
23. Simmonds M.A. Interactions of the benzodiazepine Ro 5-4864 with GABA receptor complex // Brit. J. Pharmacol., Proc. 1984, Suppl. 82. 198 P.
24. Skowronski R., Beaumont K., Fanestil D.D. Modification of the peripheral-type benzodiazepine receptor by arachidonate diethylpyrocarbonate and thiol reagents // Eur. J. Pharmacol. 1987. Vol. 143. P. 305–314.
25. Tallman J.F., Paul S.M., Skolnick P., Gallager D.W. Receptors for the age of anxiety: the pharmacology of benzodiazepines // Science (Wash. DC). 1980. Vol. 207. P. 274–279.

26. Weissman B.A., Cott J., Hommer G., Paul S.M., Skolnick P. Electrophysiological and pharmacological actions of the convulsant benzodiazepine Ro 5-4864 // Eur. J. Pharmacol. 1984. Vol. 97. P. 257–263.
27. Weissman B.A., Cott J., Jackson J.A., Bolger G.T., Weber K.H., Horst W.D., Paul S.M., Skolnick P. Peripheral-type binding sites for benzodiazepines in brain: relationship to the convulsant actions of Ro 5-4864 // J. Neurochem. 1985. Vol. 44. P. 1494–1499.
28. Zbinden G., Randall L.D. Pharmacology of benzodiazepines: Laboratory and clinical correlations // Adv. Pharmacol. 1967. Vol. 5. P. 213–292.

THE SIGNIFICANT ROLE OF LOCAL CONFORMATIONAL CHANGES IN FUNCTIONING OF TRANSMEMBRANE PROTEINS (Na, K-ATPase)

M. Zilmer, T. Kullisaar. K. Zilmer,
T. Vihalemm, R. Kask, L. Tähepõld
Department of Biochemistry and Laboratory of Enzymology of Tartu University

This article develops our earlier conception [10, 14, 16], which suggest that the changes (domenic changes) in conformation of transmembrane proteins (Na, K-ATPase) play a crucial role in the functioning of these proteins. It means, that the realization of some functions of these proteins is quaranteed already by local of changes (alteration) of their conformation. On the occasion of Na-pump such kind changes underlay on regulation of functioning of Na, K-ATPase with several effectors, of the fulfillment of partial reactions etc. Although we analyze this conception by investigation of Na-pump, it seems that such conception is valid for several membrane proteins, including receptors.
KEY-WORDS: Na-pump; lipid-protein interactions; conformations; brain tumors

INTRODUCTION

Na-pump is an integral plasmatic membrane protein, which protomer consists af α- and β-subunits [18]. With molecular cloning of cDNAs the complete amino acid sequences of these subunits (SU) from several species has been detected [3, 17]. Na-pump is characterized by cooperative interactions with Na^+ and K^+ [6, 13] and membrane lipids are engaged in the regulation of the functions of the Na, K-ATPase [2, 20, 21]. Under the pathological states the activity and some of the properties of Na-pump undergo alterations [4, 16, 22].

It is known, that the major subunit of Na, K-ATPase (α-SU) has 7–8 membrane-segments, very little extracellular structure, and two large cytoplasmatic domains [8]. The first is located near the amino terminus (Fig. 1) and may be promote the exchange, the second region evidently mediates phosphorylation, ATP binding,

and, probably the ATPase activity. The single extracellular loop is connected with ouabain binding. The β-SU (the smaller SU) consists of a single membrane spanning segment [3, 8], a short cytoplasmic domain, and relatively large extracellular sequence (Fig. 1). The last part contains some glycosylation sites.

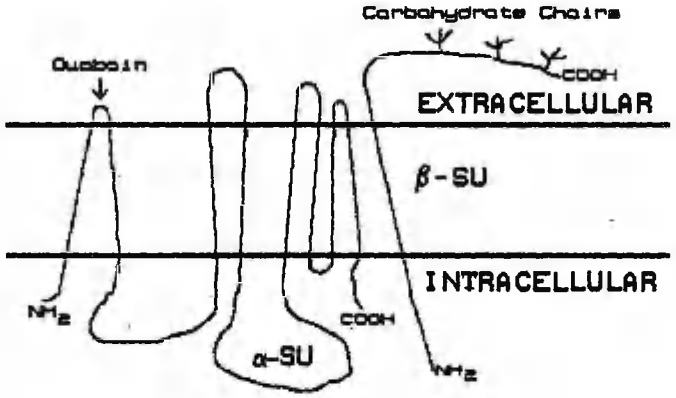

Figure 1. Localization of Na, K-ATPase subunits according to Brown et al. [ ] Jorgenson [ ].

Although valuable information of the structure of the Na-pump has been collected, nevertheless there is not a great deal known about the relations between the changes of conformation and functions of this pump. The decoding of these relations, no doubt, is very important and it considerably enlarges our knowledge about functioning of other transmembrane proteins (e.g. receptors) on the whole. In this article we analyze data which prove our earlier suggestion [10, 11, 14, 16] that the local changes of conformation (LCC) play the primary role in the functioning of the Na-pump of the brain. It means, that the fulfillment of some processes is completely realized by of the LCC of Na, K-ATPase system.

METHODS

The enzyme preparations (EP) of Na, K-ATPase from normal brain tissue of adult Wistar rat (NBT), of young (7–11 days) Wistar rats (YR), of human normal brain tissue (HNBT) and of human tumorous brain tissue (HTBT) were isolated according to our method of Karelson et al [9]. The Hill's coefficients (n_H), treatment with desensibilizators and inhibition of enzyme with pCMB and MCCD were performed as described earlier [15, 16, 24]. The flourescence measurements (quenching of tryptophan fluorescence) were

made according to the procedure of Tyson and Steinberg [19], using Perkin-Elmer spectroflourometer LS-5. Fluorescence quenching was analyzed after to the modified Stern-Volmer eguation and values of f_a and K_a (the fractional accessible flourescence and the quenching constant, respectively) were calculated [12, 19]. The temperature inactivation of Na-pump was studied range in 45–52o C anf K_T (the constant of termoinactivation rate) and sS (change of entropy) were calculated [7]. The hormone binding ability (HBA) of subunits of Na, K-ATPase was determined then in presence or absene of ATP (5mM), NaCl (100mM), and $MgCl_2$ (4mM), and phosphatidylinositol (30 M) by radioimmunoaassay (RIA) method using commercial kits (CEA-IRE-SORIN for ACTH, PROL; The Bioorganic Institute of the BSSR for CORT). The HBA was expressed as a percentage of total activity of the labelled hormone. Attempting to avoid unspecific binding several control-tests were used. The subunits of Na, K-ATPase were separated by PAGE-system and after removing from slices of gels were concentrated by ultrafiltration (Diaflo Ultrafilters Type YM-10). The protein content was determined by the modified method of Lowry et al. [1] and the specific activity of Na, K-ATPase was expressed in μmol P_i per mg protein per minute.

RESULTS AND DISCUSSION

Our previous studies [10, 14, 15, 16] demonstrated that in case of Na-pump from NBT (or HNBT) on the Arrhenius plot the typical break at 20–22oC appeared and that n_H for Na^+ and K^+ were 1,7–1,8 and 1,4, respectively. On the occasion of EP from HTBT, this break was not revealed at 20–22oC, but appeared at 27,5–30,5oC. At the same time the n_H for Na^+ with Na-pump from HTBT was only 0.9, but cooperative binding of K^+ was preserved (n_H = 1.3). On the basis of these data we can suggest, that the break at 27.5–30.5oC is, probably, associated with LCC of protein component of Na-pump. Our suggestion has been supported by the temperature dependence experiments which show that the nonlinearity of Arrhenius plot above 20oC is delipidation-independent and therefore is not conditioned by alterations of the membrane itself, but is associated with confo mational changes of enzyme protein [5].

A further evidence for the role of LCC in Na-pump is also obtained by the experiments with blocators of functional groups of Na-pump. In Table 1, it is shown that the protective effects of ATP against the both blocators is depending on testtemperature. A more remarkable temperature-dependent difference the protective effect of ATP was noticed in case of blocator SH-groups (pCMB) and especially clear difference was detected between EP fromm NBT and HTBT at both 37oC and 28–29,5oC (Table 1.). Obviously, the

temperature interval from 27,5o to 30,5oC is associated with the certain alterations in the LCC of Na-pump, which leads to some changes in the SH-groups packing. This assumption is supported by the detection of the break on Arrhenius plot in case of NBT at 27.5–30.5oC after treatment EP with agents which cause most significant alterations in the SH-groups packing [15, 16].

Table 1

Protective effects of ATP (5mM) against 1,4–1,5 μM pCMB and 1,0–2,0 mM MCCD (data for MCCD are represented in the parenthesis)

Temperature	37oC		28–29,5oC	
Enzyme preparations	NBT	HTBT	NBT	HTBT
Inhibition of Na, K-ATPase, %	44±2,0 (52±8,3)	49±3,5 (42±7,3)*	52±4,0 (48±7,3)	60±9 *(49±5,6)*
Protective effect of ATP, %	92±4,0 (50±7,3)*	21±4,1 (25±6,0)*	60±5,8 (65±4,8)*	No detectable (10±3)*

* The results are expressed as M±SEM (n = 5–6); the preincubation was 10 and 75 min. for pCMB and MCCD, respectively.

Proceeding from above-mentioned conception we investigated also the cooperativity of Na^+ and K^+ to Na-pump at different temperatures. We found, that n_H at 37oC was 1,8±0,07 and 1,4±0,06 and at 25.5–30.5oC 1,3±0,06 and 1,3±0,08 for Na^+ and K^+, respectively. Therefore, the definite decrease of n_H for Na^+ ($p<0.005$ vs 37oC), but not for K^+ is in good agreement with the suggestion, that the cooperativity for Na^+ (e.g. allosteric properties for Na^+) eliminates more easily than the cooperativity for K^+ [16, 20], while the maintenance cooperativity for K^+ does not need interprotomeric interactions. That all, together, corroborated with LCC of Na-pump.

It is known, that the differences in the thermodynamic parameters are conditioned by alterations of protein's conformations [7[]. Termoinactivation of Na-pump indicated, that K_T was 1.69 and 1.29 at 52oC for enzymes from normal brain tissue and from tumorous brain tissue, respectively. At the same time, in case of NBT ΔS^* was 59,8 cal/(mol*grade) and in case of HTBT ΔS^* = 113,1 cal/(mol*grade) at 55 oC. Consequently, these data reflect the conformational differences between Na-pump from NBT and HTBT and particularly, that the Na, K-ATPase in the tumorous brain tissue has more stable conformational state than in the normal brain tissue. This viewpoint is also supported by stability of the cooperativity for K^+ [23].

Our following experiments (quenching of tryptophan flouresсence) give the results, which are in favour of the abovementioned comprehensions. As is shown in Table 2 the f_a and K_a for both NBT (without or with Na^+) and for HTBT (without or with Na^+) have a remarkable difference, (it is particularly clearly revealed in the presence of Na^+). At the same time the values of K_a in the presence of K^+ in case of NBT and HTBT are not so different and K_a for both HTBT and HTBT+K^+ are practically similar.

Table 2

Quenching parameters of Na, K-ATPase determined from modified Stern-Volmer plots (F_0/F_0-F versus [acrylamide]M^{-1})

Temparature	28,5oC	
	f_a	K_a
NBT	0,92±0,020	4,3±0,49
+ 20mM Na^+	0,70±0,034	9,4±0,65
+ 10–20mM K^+	0,70±0,009	11,9±0,96
HTBT	0,75+0,016	7,7±0,89
+ 20mM Na+	0,93±0,019	4,8±0,39
10–20mM K^+	0,71±0,014	8,5±0,40

* The results are expressed as M±SEM (n = 3–4)

It must be pointed out that especially clearly are LCC expressed in parameters of Na-pump, associated with Na^+. This is in good agreement with our experiments, which demonstrated that the HBA for ACTH could be never detected in the case of protomer ($\alpha\beta$) and subunits (α or β) of Na, K-ATPase from HTBT. At the same time the HBA for ACTH in all experiments increased (10–30%) after the addition of Na^+ in the presence of Mg^{2+}, ATP, phospholipid in spite of sources (NBT, HNBT or YR) of protomer and subunits of Na-pump.

On the basis of the above-mentioned facts and assumptions it should be concluded, that LCC (domenic conformational changes) of transmembrane brain Na-pump play an important, perhaps, a crucial role in the regulation and functioning of this pump.

In conclusion, attention should be called to some aspects in the respect the whole complex (protein + lipids + carbohydrates) of Na, K-ATPase:

1) The data, presented in this article do not eliminate the important role of the membrane lipids in the regulation of the functioning of brain Na, K-ATPase (see 21, 23); 2) The abovementioned data and our new, unpublished facts (ouabain-binding, interaction with peptides etc.) allow to suggest that in tumorous brain tissue the

Na-pump is, probably, in "K^+-opportune conformation"; 3) The disturbance of the cooperativity for Na^+ in EP from HTBT belongs to the group of phenomena which are connected with intensive growth of tumorous cell [23]. We suppose that the general basis of all these phenomena arises from the alterations of conformation (and localization) of α-SU in the membrane. The latest, probably, remarkably depends on changes in the carbohydrate chains of β-Su (Figure 1). The investigation of this problem (e.g.) the role of carbohydrate chains in functioning of Na-pump) is just in progress.

REFERENCES

1. Bailey J. Methods of proteins Chemistry. Moscow, 1965. P. 265–266 (in Russian).
2. Boldyrev A.A., Lopina O.D., Prokopieva V.D. Membrane lipids as regulators of protein-protein interactions // Neurochemistry. 1986. Vol. 17. P. 80–95 (in Russian).
3. 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.
4. De Wardener H.E., Mac Gregor G.A. Le role d'un inhibiteur circulant du transport de sodium dans l'hypertension assentielle // Nouv. Presse Med. 1980. Vol. 9. p. 1984–1988.
5. Esmann M., Skou J.C. Temperature dependencies of various catalytic activities of membrane-bound Na, K-ATPase // Biochim. Biophys. Acta. 1988. Vol. 944. P. 344-350.
6. 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.
7. Johnson F.H., Eyring H., Stover B.L The theory of rate processes in biology and medicine New York, 1974. P. 1–250.
8. Jorgensen P.L. The Na, K-ATPase. Part A: molecular aspects / Eds J.C. Skou, J.G. Norby, A. Maunbach et al., NY: Alan R. Liss, 1988. P. 19–38.
9. Karelson E.L, Zilmer M.K., Tähepõld L.J. Prostaglandin E_2 as a lipophilic modulator of Na-pump // Quest. Med. Chem. 1985. Vol. 31. P. 84–87 (in Russian).
10. Kullisaar T.E., Salum T.T., Zilmer M.K., Tähepõld L.J. Differences of the oligomeric structure and features of the Na, K-ATPase from different sources // Abstr. of 12th All-Union meeting of ATPase. Irkutsk, 1987. P. 12 (in Russian).
11. Kullisaar T., Zilmer M., Salum T., Tähepõld L. Cooperative interactions od essential cations with Na, K-ATPase in normal and tumorous brain tissue // Abstr. of 14th Int. Congr. of Biochem. Prague, 1988. P. 111.
12. Lakowicz J.R. Principles of Flourescence. New York and London: Plenum Press. 1983. P. 262–305.

13. Paesalu E.L, Tarve U.S., Tähepõld L.J., Zilmer M.K. About structural and functional features of Na, K-ATPase activity allosterical regulation in brain // Quest. in Neurochemistry, Leningrad, 1977. P. 61–70 (in Russian).
14. Salum T.T., Vihalemm T.E., Zilmer M.K., Tähepõld L.J. The protein-lipid interactions and the functions of Na, K-ATPase // Abstr. of 12th All-Union meeting of ATPase. Irkutsk, 1987. P. 18 (in Russian).
15. Salum T.T., Zilmer M.K., Kullisaar T.E., Vihalemm T.E., Tähepõld L.J. Cooperative interactions of Na, K-ATPasewith Na^+ and K^+ and it's oligomeric structure // Ukrainian Biochem. J. 1988. Vol. 60. P. 47–52 (in Russian).
16. Salum T.T., Zilmer M.K., Kullisaar T.E., Vihalemm T.E., Tähepõld L.J., Randvere T.O., Sinisalu V.N. The temperature dependence of Na, K-ATPase from normal and tumorous brain tissue // Ukrainian Biochem. j. 1989. Vol. 61. P. 65–69 (in Russian).
17. Schull G.E., Schwartz A., Lingrel J.B. Amino acid sequence of the catalytical subunit of the $(Na^+ + K^+)$-ATPase deduced from a complementary DNA // Nature. 1985. Vol. 316. P. 691–695.
18. Stahl W.L. The Na, K-ATPase of nervous tissue // Neurochem. Internat. 1986. Vol. 8. P. 449–476.
19. Tyson P.A., Steinberg M. Accessibility of tryptophan residues in Na, K-ATPase // J. Biol. Chem. 1987. Vol. 262. P. 4644–4648.
20. Vihalemm T., Salum T., Zilmer M., Laasik J. Protein-lipid interactions and the functioning of brain Na, K-ATPase // Abstr. of 14th Int. Congr. of Biochem Prague, 1988. P. 111.
21. Vihalemm T., Zilmer M., Salum T., Kengsepp A.-T., Tähepõld L. Allosteric properties of Na, K-ATPase is controlled by membrane lipids // Abstr. of 19th FEBS Meeting of Biochem. Rome, 1989. TH 358.
22. Zilmer M., Salum T., Kullisaar T., Haug J. A possible relationship between pathological stages and allosterical features of the Na, K-ATPase // Abstr. of 19th FEBS Meeting of Biochem. Rome, 1989. TH 108.
23. Zilmer M., Salum T., Vihalemm T., Kullisaar T., Karelson E., Tähepõld L. Oligomerization level — a regulatory factor in functioning of transport (and receptor) proteins // Transactions of Tartu University. 1989. Vol. 866. P. 90–98.
24. Zilmer M., Tarve U. Isolation of active preparation of Na, K-ATPase from cattle brain and study of the role of carboxyl, sulphydryl and hydroxyl groups in its activity // Ukrainian Biochem. J. 1975. Vol. 47, P. 458–464 (in Russian).

CO-OPERATION OF Na-PUMP AND Na^+ /H^+-EXCHANGER IN CASE OF CANCER (HYPOTHESIS)

M. Zilmer
Department of biochemistry of Tartu University

KEY-WORDS: Na-pump; Na-proton-exchanger; brain tumors

Several studies have shown elevated glycolysis in the various tumor cells, particularly in the cancer cells, and the increased role of glycolysis in the generating of cell ATP pool [see for example, 4]. The enhancement of the glycolysis is in accordance with the development of hypoxia and is accompanied with intensive production of H^+.

At the same time it is known that intracellular ratio of Na^+ and K^+ (Na_i/K_i) is connected with transcription, i.e. the certain increase of Na_i plays a definite role in the intensive growth of tumour cells [5]. Therefore, in case of the cancer cells the effective system for elimination of excess H^+ with simultaneous creation of suitable Na_i/K_i should exist. The aim of the present paper is to put forward a hypothesis how the co-operation of Na-pump and Na^+/H^+-exchange can resolve these problems.

Numerous studies have reported that several factors (growth factors, oncoproteins) activate Na^+/H^+-exchange, evidently by inositol lipids pathway [see for instance 3]. Although, the activation of Na^+/H^+-exchange is accompanied with elevation of transport of H^+ from cells, we suppose, that in case of very intensive release of H^+ (see above) in the tumour cells an additional transport of H^+ from cells should take place. This is the transport by Na-pump, i.e. the co-transport with Na^+ (Fig. 1.) Our hypothesis could be grounded on the following circum-stances. The Na-pump works in the membrane of the tumour cell quite intensively due to abundance of glycolytical ATP. At the same time the normal active transport of H^+ is to a certain extend diminished whereas some properties of the Na-pump from the tumorous tissue are altered [7, 8, 10]. This diminishing is proba-bly an adaptation phenomenon in case of cancer (at least in case of glioma) whereas one part of H^+ is transported by Na-pump. This co-operation between Na-pump and Na^+/H^+-exchange in cancer cells resolve the problems which are

associated with intracellular pH and Na^+/K^+ (Fig. 1). Numerous facts are in accordance with our hypothesis: 1) The affinity for H^+ to the Na-pump is considerably higher than the affinity for Na^+ [9], 2) in the acidic medium the H^+/K^+-exchange and ATPase activity also exist without Na^+ [2], 3) The intracellular pH in case of cancer cells is, to a certain extend, lower than in the cells of normal tissue, 4) Na^+ can substitute H^+ and vice versa as a coupling ion [7]. It is very likely , that they also substitute each other to a certain extend (in certain conditions) in case of Na-pump. The co-transport effectivity depends of $[H^+]$ and $[Na^+]$ in the cell, 5) At saturating cytoplasmic $[Na^+]$ the ratio of the Na^+ transported per ATP in the acidic medium decreases to 1.5:1 [1], 6) The functionality of the Na-pump, i.e. the transition between the Na-from (a deprotonated from) and K-form (a protonated form) [6] is also in accordance with our hypothesis (see Fig. 1).

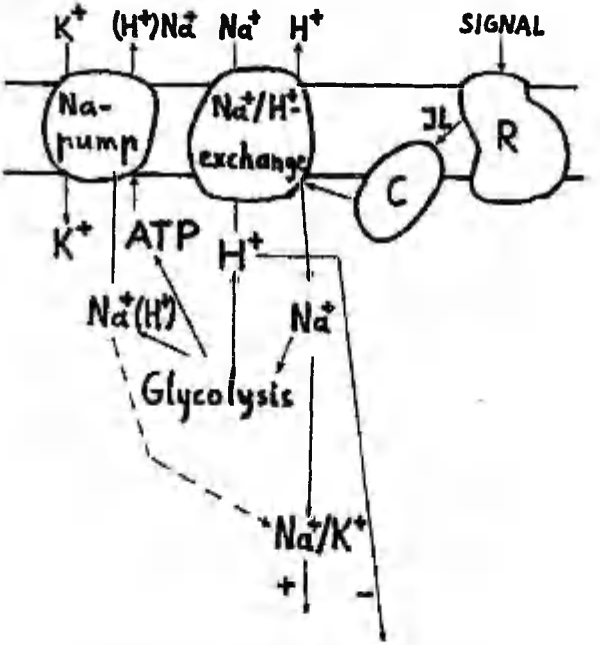

Figure 1. Co-operation between Na-pump and Na/H-excange in case of cancer (IL – inositool lipids, R – receptor, C – proteinkinase C, Signal – growth factors, oncogenes)

REFERENCES

1. Cornelius F. Variable stoichiometry in reconstituted shark Na, K-ATPase engaged in uncoupled efflux // Biochim. Biophys. Acta. 1990. Vol. 1026. P. 147–152.
2. Hara Y., Yamada J., Nakao M. Proton transport catalyzed by the sodium pump. Ouabain-sensitive ATPase activity and the phosphorylation of Na, K-ATPase in the absence of sodium ions // J. Biochem. 1986. Vol. 99. P. 531–539.
3. Michell B. Oncogenes and inositol lipids // Nature. 1984. Vol. 308. P. 770.
4. Pedersen P.L. Tumor mitochondria and the bioenergetics of cancer cells // Progress in experimental tumor research / Ed. D.P.H. Wallach. Basel: Karger, 1987. Vol. 22. P. 109–274.
5. Pool T.P., Cameron J.L., Smith N.K., Sparks R.L. Intracellular concentration of sodium and regulation of the growth: Comparison of normal and transformed cells // The transformed cell / Eds. J. Cameron and T. Pool. 1981. P. 362–382.
6. Skou J.C. The effect of pH of ATP and of modification with pyridoxal-5-phosphate on the conformational transition between the Na^+-form and the K^+-form of the Na, K-ATPase // Biochim. Biophys. Acta. 1982. Vol. 688. P. 369–380.
7. Zilmer M., Salum T., Vihalemm T., Kullisaar T., Karelson E., Tähepõld L. Oligomerization level -a regulatory factor in functioning of transport (and receptor0 proteins // Acta et commen. Univ. Tartuensis / Ed. L. Allikmets. 1989. Vol. 866. P. 90–98.
8. **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. 47–52ю
9. **Скулачев В.П.** Энергетика биологических мембран // Ред. О.Л. Оболонская. М.: Наука, 1989. С. 300–432.
10. **Цильмер М.К., Тяхепыльд Л.Я., Салум Т.Т., Силлард Р.Г., Каск Р.Р.** О роли конформационных изменений в функционировании Na, K-АТР-азы мозга // Цитология (in press).

Transactions of Tartu University
N 929
MOLECULAR PHARMACOLOGY OF RECEPTORS IV
In English
Tartu University
EV 202 400 Tartu, Ülikooli Street, 18
Vastutav toimetaja L. Allikmets.
Paljundamisele antud 13.06.1991.
Formaat 60×90/16.
Kirjutuspaber.
Kiri: roman. Rotaprint.
Arvestuspoognaid 5.92.
Trükipoognaid 5.5.
Trükiarv 350.
Tell. nr. 325.
Hind rbl. 5.–.
TÜ trükikoda. EV 202 400 Tartu, Tähi, 78.