DIFFERENCES IN THE MECHANISM OF ANTIHYPOXIC ACTION OF BENZODIAZEPINE RECEPTOR AGONISTS AND MUSCIMOL

R. U. Ostrovskaya

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In the course of experimental study tranquilizers with a benzodiazepine structure were found to have antihypoxic activity [2]. Subsequent clinical trials of benzodiazepines (BDZ) confirmed that they are highly effective antihypoxants, although the mechanism of this effect is not clear. Recent investigations have shown that the brain and certain peripheral organs contain receptors for exogenous BDZ [5]. (From now on, BDZ binding sites will be called BDZ receptors). Classes of endogenous ligands of BDZ receptors which have been studied include purines (inosine, hypoxanthine), nicotinamide, and β-carbolines [7, 14]. An important step in the study of BDZ receptors was the discovery that they can undergo selective blockade [10]. The ability of BDZ to potentiate GABA-ergic inhibition in the cerebral cortex [15], cerebellum [8], and nigrostriatal system [9] is regarded as the result of interaction in the GABA — BDZ receptor system. Proof of the participation of this system in realization of the sedative and anticonvulsant action of BDZ has been obtained, and also, according to data obtained by some workers, the tranquilizing action of BDZ.

The aim of this investigation was to clarify the role of direct interaction of BDZ with receptors and the role of GAMA-ergic control in realization of the antihypoxic effect of BDZ. The following problems were chosen for study: What is the effect of blockade of BDZ and GABA receptors on the antihypoxic action of diazepam; do hypothetical ligands of BDZ receptors and GABA mimetics possess antihypoxic properties, and how do they interact with blockers of BDZ and GABA receptors under hypoxic conditions?

EXPERIMENTAL METHOD

In experiments on mice conditions of hypoxic hypoxia were created by keeping each animal in a separate airtight chamber with a volume of 250 ml, initially containing 8 vols. % of oxygen; soda lime was used to absorb carbon dioxide. The times of appearance of the first respiratory arrhythmias, the development of hypoxic convulsions, and the times of death of each animal in the airtight chamber were recorded. It was on this simple and highly reproducible model that the writer previously first demonstrated and analyzed the antihypoxic activity of DBZ of different structure, depakine, and other active antihypoxants [2, 3, 5]. In the present investigation diazepam was chosen as the BDZ. Of all the presumed ligands of BDZ receptors, those investigated were inosine, nicotinamide, and also one of the most probable ligands, namely the 3-carboxyethyl ester of β-carboline (3-carboxy-β-carboline), synthesized in the Institute of Pharmacology, Academy of Medical Sciences of the USSR by V. V. Zagorevskii and N. N. Novikova, and studied by Zhukov [1] from the standpoint of its tranquilizing action. In view of data showing the ability of dipyridamole to interact with BDZ receptors of peripheral type located in heart muscle [13], it was decided to investigate this substance also. All compounds in the doses indicated in Table 1 were injected 30 min before the animal was placed in the airtight chamber (except 3-carboxy-β-carboline which, because of the short duration of its effect, was injected 3 min before the beginning of hypoxia). Of the agonists of GABA receptors the following most typical representatives were tested: muscimol and the cetyl ester of GABA in doses of 1-3 and 10-20 mg/kg respectively. As specific blocker of

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TABLE 1. Effect of Diazepam and Analogs of Endogenous BDZ Ligands on Survival of Mice in Airtight Chamber (M ± σ)

Substance	Dose, mg/kg	Length of survival of mice, min
Control	—	20±1,2
Diazepam	3	32±4,2
	5	58±7,1
	10	65±8,2
Nicotinamide	100	25±3,1
	200	27±3,7
	1000	26±3,6
Inosine	50	35±4,2
	100	40±4,9
3-Carboxy-β-carboline	0,5	27±2,6
	1	18±2,2*
	3	16±2,1
Curantyl (dipyridamole)	50	31±4,1
	100	33±5,1
	200	30±5,2

Legend. Differences from control are statistically significant in all cases unless marked by asterisk.

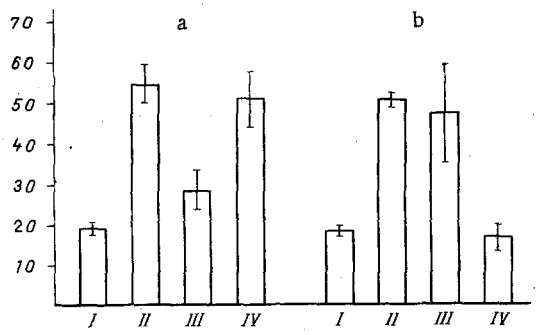

Fig. 1. Selective character of antagonism of diazepam and muscimol with blockers of BDZ and GABA receptors. Ordinate, duration of survival (in min) of mice kept in airtight chamber with initial oxygen concentration of 8 vols. %. a: I) Control; II) diazepam 5 mg/kg, 30 min before exposure in chamber; III) diazepam in same dose, followed 15 min later by Ro 15-1788 in a dose of 3 mg/kg (intraperitoneally), and 15 min later still, by exposure in airtight chamber; IV) diazepam in same dose, and 15 min later, bicuculline in dose of 5 mg/kg (subcutaneously). b: I) Control; II) muscimol 1.5 mg/kg (subcutaneously) 30 min before placing in airtight chamber; III) muscimol in same dose, 15 min later Ro 15-1788 in dose of 3 mg/kg, and 15 min later exposure in chamber; IV) muscimol in same dose, 15 min later bicuculline 4 mg/kg.

central BDZ receptors, ethyl-8-fluoro-5,6-dihydro-5-methyl-6-oxy-4-imidazole-1,4-benzodiazpine-3-carboxylate (Ro 15-1788), provided for study by Professor W. Haefely (from Hoffmann-La Roche), was investigated, and bicuculline was used to block GABA receptors. Both antagonists were injected 15 min after the agonists of BDZ and GABA receptors, i.e., 15 min before the animals were plced in the airtight chamber (in the case when 3-carboxy-β-carboline was given, immediately after it, and before the onset of hypoxia). Ro 15-1788 was injected in a dose of 3 mg/kg and bicuculline in a dose of 2-4 mg/kg (if bicuculline, in a dose of 2 mg/kg, did not weaken the antihypoxic effect of the agonist and if the presence of anticonvulsant activity of the agonist permitted the dose to be increased, bicuculline was injected in a dose of 4 mg/kg). All substances except muscimol and bicuculline were injected intraperitoneally, either separately or in the corresponding combinations, into a group of 12 animals. At the

beginning and end of each experimental day two control experiments were set up in a group of six mice, into which isotonic sodium chloride solution was injected. Altogether 480 mice were used in the experiments. The significance of differences was determined by Student's test.

EXPERIMENTAL RESULTS

Diazepam definitely delayed the times of development of respiratory arrhythmias and the appearance of hypoxic convulsions and increased the duration of survival of the animals. All the analogs of the presumed endogenous ligands of BDZ also exhibited some degree of antihypoxic action (Table 1). Incidentally, these compounds induced their antihypoxic effects in doses less than those for which a tranquilizing and sedative effect has been described [11]. 3-Carboxy-β-carboline exhibts antihypoxic activity in close to tranqualizing doses [1]. With an increase in the dose of this compound its antihypoxic effect is reversed, in agreement with views on the antagonism of this ligand (in high concentration) with BDZ receptors.

Compound Ro 15-1788, while not changing the length of survival of the animals exposed to hypoxia, considerably weakened the intensity of the antihypoxic effect of diazepam, so that the duration of survival of the animals approximated the control values (Fig. 1a). It also weakened the antihypoxic action of the presumed lignads of central BDZ receptors: inosine, nicotinamide, and the β-carboline which was studied. Bicuculline, which blocks GABA receptors on the other hand, did not weaken the antihypoxic action of diazepam, nicotinamide, or 3-carboxy-β-carboline (the effect of inosine was abolished by bicuculline). The selective character of antagonism between diazepam and the most probable ligands of BDZ with Ro 15-1788, a blocker of central BDZ receptors, demonstrated in these experiments suggests that the antihypoxic effects of BDZ are connected with direct interaction with these receptors.

The antihypoxic effect of dipyridamole in these experiments was not abolished by compound Ro 15-1788. This is further confirmation of views developed in the literature [10] regarding the absence of effect of Ro 15-1788 on peripheral BPZ receptors.

The results of the present investigation confirmed previous data on the antihypoxic activity of the original GABA mimetics, the cetyl ester of GABA (CEGABA) [3] and showed that muscimol has an even stronger protective action against hypoxia. The effect of muscimol is clearly dose-dependent: on injection (30 min before hypoxia) of 0.5 mg/kg of the compund the duration of survival of the mice in the chamber was increased on average by 30%, in a dose of 1 mg/kg by 90%, 1.5 mg/kg by 150%, and 2 mg/kg by 230% compared with the control. The protective action of muscimol and CEGABA was abolished by bicuculline, but not by compound Ro 15-1788 (Fig. 1b). This suggests that the antihypoxic effect of GABA mimetics is due to selective stimulation of the GABA-ergic fragment of the GABA — BDZ receptor complex.

In the modern view BDZ receptors exhibit considerable heterogeneity. Besides BDZ receptors whose state is modulated by GABA, there is a group of BDZ receptors that are not under the control of this amino acid [12]. Unlike the sedative, muscle-relaxing, and tranquilizing[4] action of BDZ, which is abolished by bicuculline and thiosemicarbazide, the antihypoxic effect of diazepam and of the BDZ ligands studied (except inosine — the least probable BDZ ligand from the point of view of specificity) was not weakened by bicuculline in the present experiments. This suggests that the antihypoxic effect of BDZ is linked with the system of BDZ receptors of the GABA (bicuculline-sensitive) type.

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ENDOGENOUS INHIBITORS OF SPECIFIC BENZODIAZEPINE BINDING IN THE BOVINE CEREBRAL CORTEX

A. V. Kladnitskii and A. G. Mukhin

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In the modern view the pharmacologic effects of benzodiazepines are realized through their interaction in the CNS with specific binding sites, known as benzodiazepine receptors [2, 10]. However, there is as yet no reliable proof of the existence of endogenous ligands for these binding sites, and consequently their physiological role is not yet clear. An intensive search for such ligands is now in progress [1, 3-5, 7-9, 11-13].

The writers have attempted to solve this problem by demonstrating the presence of low-molecular-weight inhibitors of specific binding of [^{3}H]diazepam in a homogenate of whole bovine cerebral cortex.

EXPERIMENTAL METHOD

Fresh bovine cerebral cortex was homogenized in 5 volumes of 0.01 HCl in a Waring blender at room temperature. After heating to 80°C for 30 min followed by cooling to 20°C the homogenate was centrifuged for 20 min at 1000g. The supernatant was neutralized with 0.1 M NaOH to pH 7.0 and filtered successively through Filtrak-88 (East Germany), GF/B (Whatman, England), and Twin-90 (Millipore, USA) filters. The filtrate was subjected to ultrafiltration through a filter consisting of hollow H10P5 fibers (nominal filtration limit 5000 daltons) on a DC-10 apparatus (Amicon, The Netherlands). The ultrafiltrate (about 40[illegible] liters from 12 g of original tissue) was frozen and lyophilized.

From 8 to 16 g of the freeze-dried products was dissolved in 20-25 ml of buffer A (24 mM NH_4HCO_3 pH 8.0), filtered through a GF/B filter, and applied to a column (4.4 × 80 cm) with Sephadex G-10, equilibrated with the same buffer. Elution was carried out with buffer A at the rate of 75 ml/h at 4°C, volume of fractions 12.5 ml. After determination of inhibitory activity, the fractions were pooled and freeze-dried.

Binding of [^{3}H]diazepam was carried out at 0.8-1.0°C for 45 min. The composition of the incubation medium was: 0.4 ml of a suspension of synaptic membranes, 0.1 ml of [^{3}H]diazepam (71 Ci/mmole, Amersham Corporation, England), giving a final concentration of 0.5 nM in the sample, and 0.5 ml buffer B (25 mM Tris-HCl, pH 7.4). The bound ligand was separated by filtration through GF/B filters. All solutions of the incubation mixture were made up in buffer B. To obtain the coarse fraction of synaptic membranes, the gray matter of the bovine cerebral cortex was homogenized in 10 volumes of 0.32 M sucrose by means of a Super 30 homogenizer (Virtis, USA); the residue obtained by centrifugation at 1000g for 15 min was resuspended in 10 volumes of buffer B and centrifuges at 30,000g for 30 min; the procedure was repeated 3 times and the residue resuspended in 5 volumes of buffer B, poured out in aliquots, and kept at −70°C. Before the experiment the membranes were diluted 8 times with buffer B and

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