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EXPERIMENTAL ARTICLES

An Effector Analysis of the Interaction of Propoxazepam with Antagonists of GABA and Glycine Receptors

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Abstract—Using effector analysis, we studied the mechanism of anti-seizure activity of the alcoxy-derived benzodiazepine (propoxazepam). In models of chemically induced seizures we determined the average molar and weight effective doses (ED_{50}) of propoxazepam as an antagonist of picrotoxin (4.10 ± 0.21 μmol/kg, 1.67 ± 0.09 mg/kg), pentylenetetrazole (2.24 ± 0.93 μmol/kg, 0.9 ± 0.04 mg/kg), and strychnine (40.33 ± 14.91 μmol/kg, 14.24 ± 0.47 mg/kg), which reflect the high activity level of the substance. On the basis of dose–effect curves, using comparative quantile analysis for chemoconvulsants with different mechanisms of action, we showed different stages of interaction of propoxazepam with GABA and glycine receptors under *in vivo* conditions. We evaluated the partial contribution of myoclonic and toxic components to the general structure of seizures induced by various chemoconvulsants. We believe that the results we obtained indicate that the anti-seizure action of propoxazepam is predominantly mediated by a GABAergic mechanism. Glycinergic components of the inhibition of strychnine-induced seizures by propoxazepam occur at doses that exceed the ED_{50} and seem to be an additional means of anti-seizure action.

Keywords: propoxazepam, chemoconvulsants, antiseizure action, GABA antagonists

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INTRODUCTION

The creation of innovative painkillers that affect the central components of the inhibition of pain syndrome (especially of neuropathic origin) is one of the important areas of modern pharmacology. A number of 3-substituted 1,4-benzodiazepines have been synthesized at the Physico-Chemical Institute of the National Academy of Sciences of Ukraine and their structure–activity relationships were studied. Their pharmacological effect was unusual, as, unlike most drugs in this class, in the models of nociceptive and neuropathic pain these substances showed significant analgesic activity; one of them, propoxazepam, 7-bromo-5-(*o*-chlorophenyl)-3-propoxy-1,2-dihydro-3H-1,4-benzodiazepin-2-one, is considered as a promising drug and is undergoing preclinical trials [1, 2]. Similar to gabapentin and pregabalin, which are well-known drugs used in general medical practice in the treatment of neuropathic pain [3], propoxazepam also has an anticonvulsant effect, which explains the analgesic component of the pharmacological spectrum.

Because the studied substance is related to benzodiazepine derivatives, the goal of this work was to determine whether the anticonvulsant effect of propoxazepam is limited only by the $GABA_A$ receptor (with the effector benzodiazepine site). In particular, it seemed appropriate to examine the effect of the compound on glycine receptors, which are evolutionarily related to the $GABA_A$ receptor and are also coupled to chlorine ion channels.

MATERIALS AND METHODS

In these experiments we used white mongrel mice of both sexes weighing 22–25 g that were obtained from the nursery of the Odessa National Medical University. The duration of the quarantine was 14 days. Animals were kept under standard conditions with free access to water and food. All experimental procedures were performed in accordance with the European Union Directive 2010/10/63 EU on animal experiments.

Pentylenetetrazole, strychnine, and picrotoxin (Sigma) were used in the study. Propoxazepam was synthesized according to the method described in [4]. The structure of the substance was determined by a complex of physicochemical methods (IR and mass spectroscopy, as well as X-ray diffraction analysis).

The anticonvulsant effect of propoxazepam was evaluated in experiments on mice as the relative number of surviving animals that were recorded 2 hours after the convulsant administration. The tested compound was administered intraperitoneally (in a Tween-80 emulsion) at doses whose limits were cho-

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sen after previous pilot studies and according to the requirements of statistical and calculation methods. Chemoconvulsant solutions (strychnine 2 mg/kg, picrotoxin 6.5 mg/kg, and pentylenetetrazole 20 mg/kg are doses that cause lethal effects in 95% of tested animals) were administered subcutaneously to animals (six to eight animals in each experimental group) 30 min after propoxazepam administration. The start of the experiment was the moment of the administration of the convulsive agent. During the follow-up period, we recorded the number of myoclonic tremors and generalized seizures in the form of a tonic extension, as well as the time to the onset of the lethal effect. To characterize the representativeness of each type of seizure, the data are presented as a relative number (partial contribution, $M \pm m$) of total convulsive episodes. The lethal effect was evaluated in an alternative form (presence or absence of effect). The protective effect of the substance (ED_{50}) was evaluated by the number of animals (the frequency of effect manifestation) that survived in each individual group. The ED_{50} values were calculated using the probabilities of effect development by the Kerber method (with the Barrens corrections) and by probit analysis [5]. The significance of the differences in indices of convulsive action between the control and experimental groups (after a preliminary analysis for compliance with the normal distribution law), as well as the final experimental data, were evaluated using the Student's t test or by nonparametric statistics (Wilcoxon–Mann–Whitney test) [6]. For a nonparametric estimation of the mutual distribution of data, we used quantitative analysis (the Q–Q analysis) with the calculation of the corresponding percentiles (with increments of 10) and further determination of the cumulative correlation coefficient of the linear regression [7, 8]. The data are presented as mean ± standard deviation ($M \pm m$), or as a median (first–third quartile), i.e., Me (Q1–Q3).

RESULTS AND DISCUSSION

Here, we made an attempt to elucidate the proportion (part) of the GABA and glycine receptors that participate in the process of formation of anticonvulsant action of propoxazepam in mice using effector analysis, which takes the mechanism of action into account. If in vitro studies, for example, radioligand, immunocytochemical, and electrophysiological studies, provide information only about the affinity of the ligand for the receptor and do not take important components such as barrier mechanisms (cellular, histohematic, and others) and the intrinsic activity of the compound into account; here, we analyzed the integral physiological response of the organism during the interaction of the agonist (propoxazepam) and antagonists (strychnine, picrotoxin, and pentylenetetrazole) with GABA and glycine receptors in experiments *in vivo*.

The choice of the therapeutic targets for propoxazepam is not accidental but was determined by the fact that its structure is related to 3-substituted 1,4-benzodiazepines and, consequently, the $GABA_A$-receptor is a suitable candidate involved in the mechanism of action of the compound. Taking the fact into account that every benzodiazepine-related drug has a different affinity (K_i, K_s, and GABA-shift) to GABA receptors and its own pharmacodynamic profiles, a new compound requires a similar description.

For the glycine receptor, its possible participation in the anticonvulsant action of benzodiazepines is still debated, at least, for the strychnine-sensitive subtype. Moreover, blockage of these receptors by strychnine strongly multiplies pain sensations, and their stimulation leads to a reduction in pain; this property is important for analgesic drugs, in particular, propoxazepam.

It is also known that many drugs interact with more than one therapeutic target, which leads to simultaneous changes in a number of biochemical signals [9]. These changes are facilitated by the fact that in the central nervous system certain neurons are able to produce and release in their synapses not a single but several neurotransmitters, including GABA and glycine [10]. Simultaneous activation of the receptors of two inhibitory neurotransmitters that affect each other may be manifested as mutual inhibition, enhancement of responses, and changes in their time course.

To study the interaction of propoxazepam with receptors, we used the pharmacological analyzers picrotoxin, corazol, and strychnine to evaluate its mechanisms of anticonvulsant action.

Picrotoxin is a noncompetitive antagonist because it acts inside the ion channel and not at the GABA binding site.

Strychnine is a classic competitive blocker of the glycine receptor, whose low doses promote excitation and large doses cause generalized tonic convulsions with severe pain syndrome, mainly of central origin [11].

Pentylenetetrazole is most widely used as a chemoconvulsant, which at low doses induces absence-like seizures. At moderate doses, pentylenetetrazole leads to the development of clonic, and, at high doses, tonic–clonic and generalized seizures (status epilepticus) and even death. Pentylenetetrazole belongs to the ligands of both GABAergic and glycinergic systems [12, 13], although its binding sites were identified in the chlorine channel of the GABA-receptor and its effect is often characterized as "non-competitive" with respect to 1,4-benzodiazepine derivatives, which exhibit an anticonvulsant effect [14, 15]. Consequently, this set of pharmacological analyzers may be used to evaluate the mechanisms of anticonvulsant action of propoxazepam.

Taking the neurochemical targets of action of the selected chemoconvulsants for the analysis of the anticonvulsant activity of propoxazepam into account, we used the following indices: (1) integral indices (char-

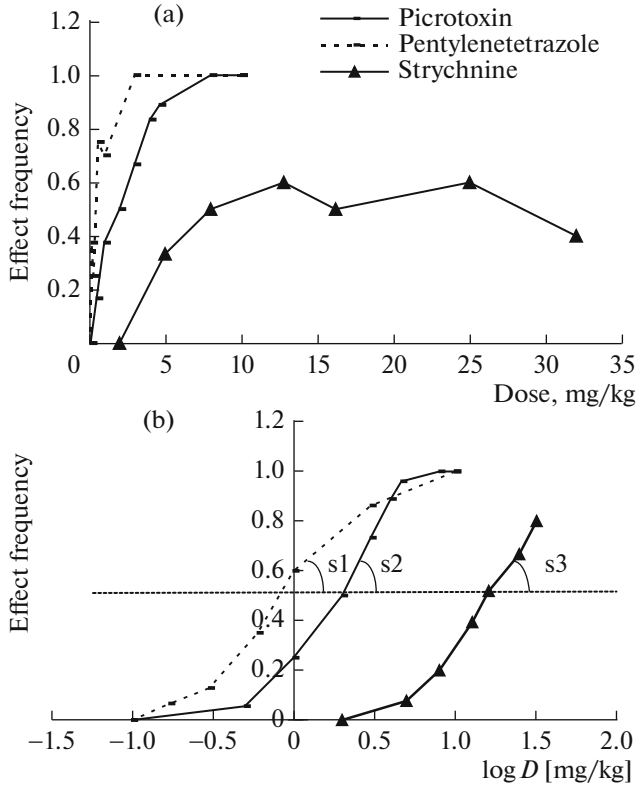

Fig. 1. The shape of the dose–response curves (proportion of survived animals) for primary experimental data (a) and data corrected using the Barrence method in semi-log coordinates (b) (s1, s2, and s3 are the curve slopes determined in the ED_{50} area).

acteristics of the dose–response curve), (2) indices that influence an increase in seizure readiness of the brain (myoclonic component of convulsive seizure), and (3) indices that form stable foci of paroxysmal activity (externally diagnosed by the development of tonic seizures).

The characteristics of the dose–response curves. The main index of the pharmacological activity of the compound is the dose at which the probability of effect development is at a maximum in 50% of the test animals (ED_{50}). However, the initial experimental data do not always correspond to this distribution, because it is a generalized characteristic of the response of the body. Thus, the initial indices of the protective effect of proproxazepam for antagonism with picrotoxin and pentylenetetrazole are characterized by a 100% effect, whereas for antagonism with strychnine, administration of even higher doses of propoxazepam (up to 30 mg/kg) did not lead to achievement of this result (Fig. 1a). Conversely, at higher doses of propoxazepam, an "inverse" paradoxical effect was observed.

The ED_{50} values for the protective effect of propoxazepam after administration of chemoconvulsants were determined after the correction of the experimental data via the Barrence method with subsequent representation in semi-log coordinates (Fig. 1b). The shape of the curves is close to the classical sigmoidal shape only for ligand antagonists of the GABA-receptor complex, whereas during antagonism with strychnine the maximum attainable protective effect was at the level of 70–80%.

ED_{50} values (see Table 1) were significantly different ($p < 0.03$, Student's t test, [5]) between each other even in the case of picrotoxin and pentylenetetrazole. Propoxazepam had higher antagonism to corazol; it is likely that the GABAergic and partially glycinergic systems are involved [12]. For the model of pentylenetetrazole-induced seizures, the efficacy of the tested compound was 2.24 ± 0.93 μmol/kg (ED_{50}). With respect to picrotoxin, a competitive antagonist of the $GABA_A$-receptor, propoxazepam exhibited a smaller effect ($ED_{50} = 4.10 \pm 0.21$ μmol/kg). Apparently, this was due to the fact that the final result of competition between agonists and antagonists was determined by the ratio of molar concentrations, relative affinity to receptors, and the ratio of the relative intrinsic activity of agonists and antagonists [16].

For the model of strychnine-induced seizures, propoxazepam activity was almost an order of magnitude lower, 40.33 ± 14.91 μmol/kg, indicating little involvement of the glycinergic system in the anticonvulsant action of propoxazepam (see Table 1). This is confirmed by the higher slope of the dose–response curve (s3) (1.368 for strychnine-induced seizures); in this model, the protective effect of propoxazepam was observed in a broader range of doses, whereas the less steep slope of the corresponding curves (s1 and s2) for picrotoxin (0.789) and corazol (0.821) suggests a concentration-dependent character of the development of the effect. The ratio of the slopes for the picrotoxin/strychnine and pentylenetetrazole/strychnine curves (0.57 and 0.6, respectively) are close to the maximum of the detected anticonvulsant effect (Fig. 1a) of propoxazepam in the model of strychnine-induced seizures.

The dose–response curve may be satisfactorily described only in the range of the average effective dose by a distribution that is close to normal. However, in the range of administered dosages of propoxazepam, we observed significant deviations from a formally assumed symmetrical form with normal distribution characteristics, which also indicates different mechanisms of competitive antagonism of propoxazepam with respect to the chemoconvulsants we used. On the basis of the data of the dose–response curves (ED_{50} and slope) these changes cannot be detected and characterized but bear substantial information on the nature of the ligand–receptor interaction under in vivo conditions. Hence, we believe that it is more accurate to perform a comparative nonparametric Q–Q analysis of the complete curves by comparing the corresponding

Table 1. The indices of the pharmacological effect of propoxazepam (duration of the animal's survival time and average dose that blocks the lethal effect) for antagonism against seizure-inducing agents

Index		Picrotoxin	Pentylenetetrazole	Strychnine
Average effective dose ($M \pm m$), ED_{50}, μmol/kg (mg/kg)		4.10 ± 0.21 (1.67 ± 0.09)	2.24 ± 0.93 (0.9 ± 0.04)	40.33 ± 14.91 (14.24 ± 0.47)
Slope of "dose–response" curve		0.789	0.821	1.368
Time of lethal effect, min (Me(Q1–Q3))	Dose / Control	17.0 (16.25–17.5)	1.0 (1–1.75)	6.0 (4.25–7.00)
	0.1	19.5 (18.75–20)	8.0 (7–14.25)	–
	0.17	–	9.0 (7–13)	–
	0.3	–	5.0 (4–6)	–
	0.5	25.0 (24–32)	–	–
	0.6	–	15.0 (9–26)	–
	1	98.0 (37–98)	12.0 (9.5–17)	–
	2	36.0 (30.5–36.5)	–	11.0 (10.0–12.0)
	3	25.0 (24.5–25.5)	>120	–
	4	31.0 (31–31)	–	–
	4.7	36.0 (36–36)	–	–
	5	–	–	11.5 (10.00–12.25)
	8	>120	–	7.0 (6.5–23.0)
	10	–	>120	–
	12.8	–	–	16.5 (15.00–17.75)
	16.2	–	–	22.0 (19.5–34.5)
	25	–	–	18.0 (14.25–24.25)
	32	–	–	20.0 (15.25–36.75)

"–" Measurements were not performed at this dose.

pair quantiles of the frequencies of the effect of the protective action of propoxazepam (Fig. 2).

Curves of the paired quantiles of the protective effect of propoxazepam differ markedly depending on the convulsant agent and the elimination of seizures caused by it. Propoxazepam effectively reduced the development of lethal effects when antagonized by the competitive antagonist of the GABA receptor complex picrotoxin compared with the noncompetitive antagonist pentylenetetrazole (the picrotoxin (^xQ_i)–pentylenetetrazole (^yQ_i)) curve and an antagonist of the other inhibitory system strychnine (picrotoxin (^xQ_i)–strychnine (^yQ_i)), which results in the concave shape of these relationships. However, the efficiency of the protective effect of propoxazepam for antagonism with pentylenetetrazole was stronger compared to strychnine (pentylenetetrazole (^xQ_i)–strychnine (^yQ_i)), which also confirms the predominant interaction of propoxazepam with the $GABA_A$ receptor.

Quantitative evaluation of the interaction density of the protective effect of propoxazepam via antagonism against these convulsive agents was made on the basis of the cumulative correlation coefficient (Fig. 3). Note that despite the high values of this index (0.95–0.98) there are significant differences in the possible mechanisms of action of propoxazepam: selective

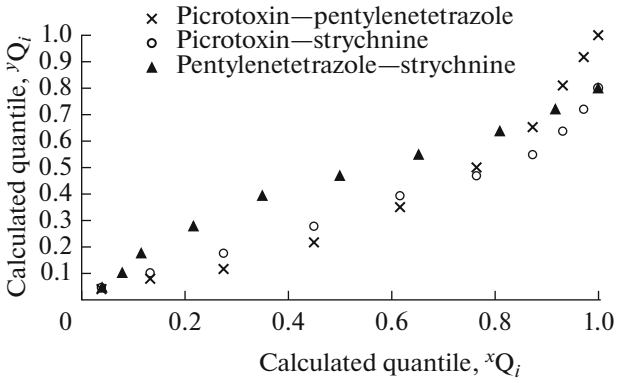

Fig. 2. The probability plot of paired quantiles (^xQ_i–^yQ_i) of frequencies of the protective action of propoxazepam for antagonism with seizure-inducing agents.

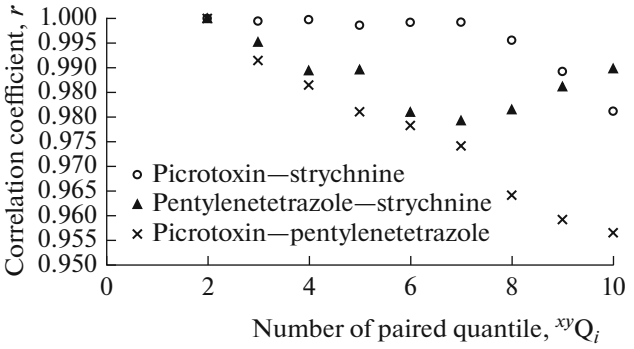

Fig. 3. Changes in the cumulative correlation coefficient for calculated paired quantiles of frequencies of the protective action of propoxazepam.

competition with picrotoxin appears to be limited to a narrow dose interval (in contrast to nonspecific antagonism with pentylenetetrazole). As a result, a moderate slope of dose–response curve for antagonism with corazol (Fig. 1) reflects the slow development of the inhibitory effect of propoxazepam.

The change in the correlation coefficient for antagonism with corazol and strychnine (Fig. 3) is biphasic with a pronounced increase in the protective effect of propoxazepam in relation to strychnine in comparison with pentylenetetrazole and a subsequent increase in the inhibition due to activation of GABAergic system by high doses of propoxazepam.

A comparative analysis of changes in paired quantiles for picrotoxin and strychnine shows that their change is linear in the initial portion of the dose–response curves (more than 70% of these values of the protective effects of propoxazepam vary proportionally for these convulsants) (Fig. 3). A partial increase in the protective effect was observed only at high doses of propoxazepam with respect to antagonism with picrotoxin. Taking the fact into account that the picrotoxin is a competitive $GABA_A$ receptor antagonist, it may be assumed that propoxazepam in the dose range of 2–16 mg/kg in the presence of strychnine has a similar mechanism of action, whose effectiveness, however, is not concentration-dependent at higher doses. This hypothesis is also supported by the fact that the protective effect of propoxazepam via antagonism with strychnine does not exceed 65–70% (Fig. 1).

Based on the data we obtained using the comparative nonparametric Q–Q analysis of the paired dose–response curves, it may be concluded that the protective (anticonvulsive) effect of propoxazepam in the low-dose strychnine seizure model (up to ED_{50}) involves mechanisms that also function for its antagonism with picrotoxin. This suggests a significant contribution of the GABAergic system in this range of doses. The glycinergic components that reduce strychnine seizures are activated after administration of propoxazepam at doses exceeding ED_{50}, presumably because of the small relative abundance of the combined GABA–glycine synapses. Note that despite the similar nature of the interaction of picrotoxin and pentylenetetrazole with the GABA–receptor complex, the protective effect of propoxazepam against these agents differs. This is due to differences in the mechanisms of these blockers (picrotoxin is a direct, and pentylenetetrazole is an indirect antagonist).

Generalization of excitation as a characteristic of the interaction of $GABA_A$ergic and glycinergic systems in the inhibitory effect of propoxazepam. The development of excitation in the central nervous system and the further achievement of lethal effects is the final stage of antagonistic interactions between the activating and inhibitory systems. This period also includes separate intermediate stages, which are reflected on the physiological level in the form of arbitrary contractions of skeletal muscles.

During the experiment, we recorded components of convulsive seizures that correspond to different degrees of excitation generalization: myoclonic tremor (shaking of the head, limbs or body) and clonic seizures in the form of alternating tremor of large amplitude, episodes of tonic convulsions (sudden tensions caused by simultaneous contraction of antagonist muscles), and the survival times of the experimental animals.

Analysis of the structure of the experimental data showed that they do not correspond to the normal distribution law and are characterized by large differences in the asymmetry and kurtosis. Only the survival time of animals showed a dose-dependent effect (see Table 1), whereas the time and intensity of the myoclonic and tonic component of seizures are parabolic. However, after administration of different doses of propoxazepam, a significant ($p \le 0.02$, Wilcoxon–Mann–Whitney test [5]) increase in the survival time was observed. In general, propoxazepam significantly increased (by 2.5–3 times) the survival time of animals after the administration of convulsive agents (3 mg/kg for pentylenetetrazole and 8 mg/kg for picrotoxin) and had a protective effect at high doses.

As mentioned above, the protective effect of propoxazepam did not exceed 70% in the model of strychnine-induced seizures. This blockage of glycine receptors at the level of the spinal cord leads to an increase in the inflow of excitatory impulses into the brain, because the GABAergic system (whose participation may be estimated at 65–70%) does not provide proper control and suppression of incoming signals. At the physiological level, this is manifested by the fact that the myoclonic component in the control group of animals is practically absent and single myoclonic spasms almost immediately lead to the development of a tonic component with further paralysis of the respiratory muscles; on the basis of this, an animal's survival time index may be recognized as a characteristic

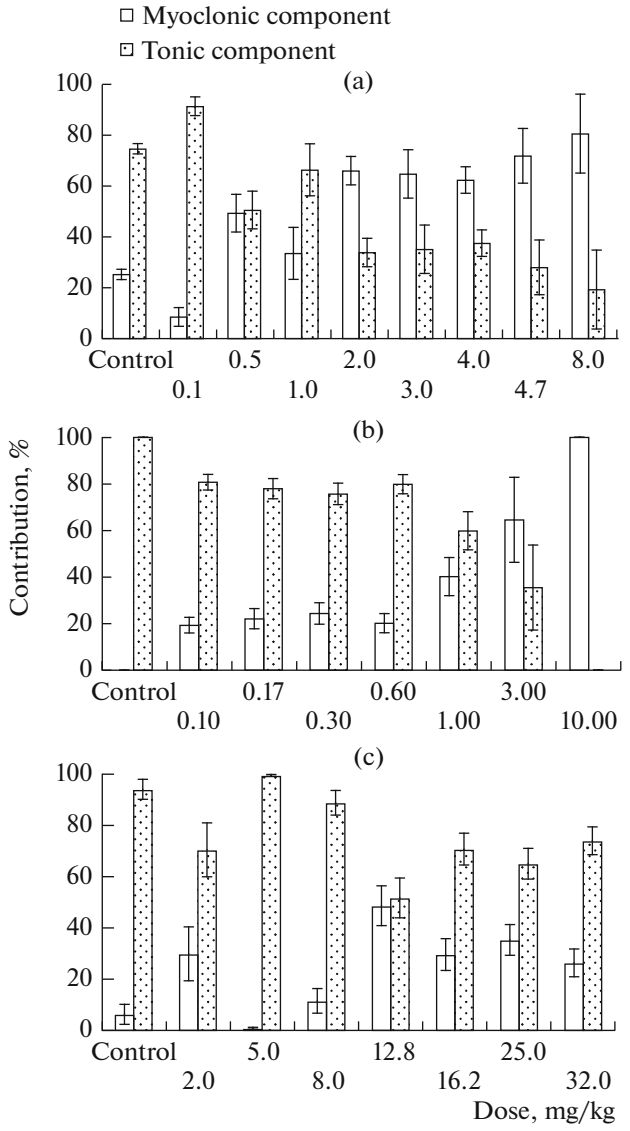

Fig. 4. Changes in the partial contribution of separate components in the structure of the total seizure induced by seizure-inducing agents ((a) picrotoxin (6.5 mg/kg), (b) pentylenetetrazole (120 mg/kg), (c) strychnine (2 mg/kg), subcutaneously) after administration of different doses of propoxazepam (mean ± standard deviation).

that reflects the interaction of GABA and glycine receptors.

The normalized indices of the partial contribution of myoclonic and tonic components (as a ratio between the number of each type of seizure and their total number) in the structure of convulsive seizure caused by various chemoconvulsants also differ (Fig. 4). Thus, at low doses of propoxazepam, the tonic component predominates in the structure of the seizures of animals, whose intensity (after administration of picrotoxin or pentylenetetrazole) decreases proportionally to the administered dose of propoxazepam, reflecting a preferential increase in the intensity of inhibitory processes in the CNS and a decrease in the formation of foci of paroxysmal activity. Conversely, when blocking the glycine system with strychnine, the tonic component plays the leading role in the pathological process of the generalization of excitation in these groups of animals and is not reduced below 50%, even after the administration of high doses of propoxazepam. Nevertheless, the data only reflect the relative proportion of each component and an actual increase in the number of tonic seizures may result from an increase in the survival time of animals at high doses of propoxazepam.

The characteristics of the experimental models of pain do not make it possible to determine the mechanism of analgesic action of propoxazepam using the proposed technique. Nevertheless, it may be assumed that at least for neuropathic pain with paroxysmal components the mechanisms of action of propoxazepam are identical to those of anticonvulsants.

CONCLUSIONS

We performed an effector analysis of the protective effect of the innovative compound of the 1,4-benzodiazepine derivative (propoxazepam) on antagonism to various chemoconvulsants, which makes it possible to elucidate the involvement of the GABA and glycine receptors in this process. For the models of chemically induced seizures, the mean mole and weight effective doses (ED_{50}) of propoxazepam were determined for antagonism with picrotoxin (4.10 ± 0.21 μmol/kg, 1.67 ± 0.09 mg/kg, pentylenetetrazole (2.24 ± 0.9 μmol/kg, 0.9 ± 0.04 mg/kg), and strychnine (40.33 ± 14.91 μmol/kg, 14.24 ± 0.47 mg/kg), which indicate a high activity of the substance.

These data suggest that the inhibition of the development of pathological excitation by propoxazepam occurs primarily through GABAergic mechanisms. The use of selective chemoconvulsants also suggests the involvement of mixed GABA/glycine synapses, whose contribution to the overall effect, however, doess not exceed 70%.

The redistribution of the ratio of the myoclonic and tonic components of seizures that were induced by picrotoxin and pentylenetetrazole with an increase in the administered dose of propoxazepam also indicates the predominant participation of the GABAergic system in the mechanisms of its action. In the structure of seizures caused by strychnine, the leading component is the tonic component (over 50%), which indicates negligible involvement of glycine receptors in the pharmacological action of propoxazepam.

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