Effect of Muscimol on the Picrotoxin- and Bicuculline-Induced Delay in the Desensitization of the GABA Receptor/Cl^- Channel Complex

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Effects of picrotoxin and bicuculline on the muscimol-dependent $^{36}Cl^-$ entry into synaptoneurosomes of the rat cerebral cortex are examined as well as desensitization of $^{36}Cl^-$ entry at muscimol concentrations of 5 and 50 μM. At the 5 μM concentration (which is close to the muscimol IC_{50}), picrotoxin and bicuculline inhibited Cl^- entry into synaptoneurosomes and decreased the desensitization. At the 50 μM concentration, muscimol completely abolishes the bicuculline effects both on Cl^- entry and desensitization. Inhibition of Cl^- entry by picrotoxin is also abolished by 50 μM muscimol, whereas the picrotoxin-induced decrease in the desensitization rate is not. It is shown that both bicuculline effects result from inhibition of the GABA receptor, but the action of picrotoxin on the desensitization of Cl^- entry into synaptoneurosomes is not closely related to the functional activity of the GABA receptor/Cl^- channel complex.

Key Words: *desensitization; GABA receptor/Cl^- channel complex; muscimol; picrotoxin; bicuculline; $^{36}Cl^-$, synaptoneurosomes*

Desensitization of the GABA receptor/Cl^- channel complex is a process whereby the GABA-stimulated Cl^- conductivity is progressively decreased [3,4,6,9]. It is still unknown whether this desensitization represents a reduction in receptor sensitivity to GABA or a decrease in the conductivity of Cl^- channel. The difficulties encountered in seeking the correct answer to this question stem from the fact that it is not possible to study the Cl^- channel conductivity separately from activation of the GABA receptor. The only alternative that remains is analysis of how the desensitization is influenced by inhibitors and modulators of the GABA receptor/Cl^- complex with different mechanisms of action.

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This study continues and extends our previously initiated investigation [1,2] to compare the effects of the competitive inhibitor of the $GABA_A$ receptor bicuculline (BC) and of the Cl^- channel blockers picrotoxin (PT) and pentylenetetrazole on the desensitization of the GABA receptor/Cl^- channel complex.

MATERIALS AND METHODS

Synaptoneurosomes (SNS) were isolated using our modification [2] of a previously described procedure [7]. Randomly bred male rats (body weight 180-200 g) were decapitated, and their cerebral cortex was removed and homogenized at 0-4°C manually (5 frictions) in a glass homogenizer with a Teflon pestle in Krebs—Ringer's medium of the following composition (mM): 145 NaCl, 5 KCl, 1 $MgSO_4$, 1 $CaCl_2$, 10 glucose, and 10 HEPES (pH 7.4, 20°C) in a ra-

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tio of 1 g tissue per 15 ml medium. Each homogenate was filtered in sequence through nylon sieves (Rachmanovskaya Factory, Russia) with meshes of 300, 99, 60, and 27 μ. The filtrate was centrifuged at 2700 g for 5 min and the pellet was resuspended in the same volume of Krebs—Ringer's medium and centrifuged again as before. The pellet after the second centrifugation was so suspended in Krebs—Ringer's medium as to make the final SNS concentration equal to about 4 mg protein/ml. SNS were used immediately after their separation. The SNS suspension was dispensed in 100 μl aliquots (about 400 μg protein) into test tubes which were incubated for 30 min at 20°C after adding inhibitors in appropriate concentrations. Thereafter, 100 μl of Krebs—Ringer's solution containing 0.5 μCi of $^{36}Cl^-$ (Izotop, Russia) and muscimol in the required concentration were added to each test tube with vigorous agitation. After 5 sec, $^{36}Cl^-$ entry into SNS was stopped by filtration through GF/Cl fiberglass filters (Whatman) which were washed three times with 4 ml of PT-containing (100 μM) Krebs—Ringer's solution cooled to 0-4°C. The filters were then dried and placed in scintillation vials. Radioactivity was measured on a 1219 Rack-beta counter (LKB).

For the determination of stationary $^{36}Cl^-$ entry, the radioactive label was added to SNS without muscimol. The muscimol-stimulated $^{36}Cl^-$ entry into SNS, which characterizes the functional activity of Cl^- channel in the GABA-receptor complex, was determined as the difference between basal $^{36}Cl^-$ uptake by SNS and $^{36}Cl^-$ uptake in the presence of muscimol. In examining desensitization of the GABA receptor/Cl^- channel complex, muscimol was added to SNS without the isotope, followed by incubation for 5 sec; $^{36}Cl^-$ was then added, and the degree of Cl^- entry was estimated as described above. The degree of desensitization was determined as the percentage decrease in $^{36}Cl^-$ entry after preincubating SNS with muscimol.

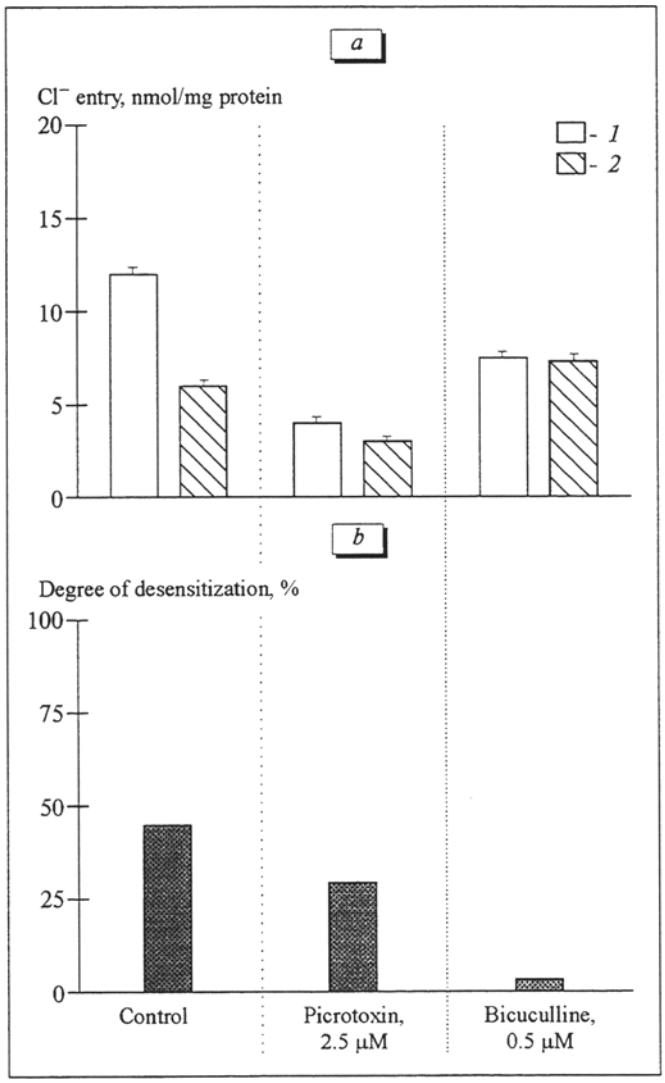

Fig. 1. Effects of picrotoxin (PT) and bicuculline (BC) on muscimol-stimulated (5 μM) $^{36}Cl^-$ entry into synaptoneurosomes (SNS) and on desensitization. Here and in Fig. 2: *a*) muscimol-dependent $^{36}Cl^-$ entry and desensitization in control tests and those using 0.5 μM BC or 2.5 μM PT; *b*) BC- and PT-induced changes in the degree of desensitization of the GABA receptor/Cl^- channel complex; *1*) muscimol-dependent $^{36}Cl^-$ entry when muscimol and $^{36}Cl^-$ were added to SNS together; *2*) muscimol-dependent $^{36}Cl^-$ entry when $^{36}Cl^-$ was added to SNS after their 5-second incubation with muscimol.

RESULTS

It has been shown that a rise in the concentration of GABA or enhancement of its effect by diazepam results in increased rate of desensitization [8]. Desensitization can be induced by agonists of the $GABA_A$ receptor (which is coupled to Cl^- channel) such as muscimol [9]. Conversely, inhibition of the GABA receptor by BC decreases the desensitization rate, as does blockade to Cl^- channel with PT or pentylenetetrazole [1,2]. The effect of BC as a competitive $GABA_A$ receptor inhibitor is possibly associated with a decrease in the number of receptors accessible for interaction and is thus analogous to the effect of a reduction in GABA concentration. On the other hand, the observation that PT and pentylenetetrazole delay desensitization strongly suggests that desensitization is linked directly either with an open state of the Cl^- channel or with accumulation of Cl^- ions in the cytoplasm of postsynaptic neurons. In the latter case the desensitization in not a true one, for it only involves a decrease in the transmembrane electrochemical Cl^- gradient. The delay of desensitization by Cl^- channel blockers should evidently depend not only on their concentration, but also on the GABA or muscimol concentration.

In order to determine how muscimol might influence the delay of desensitization of $^{36}Cl^-$ entry

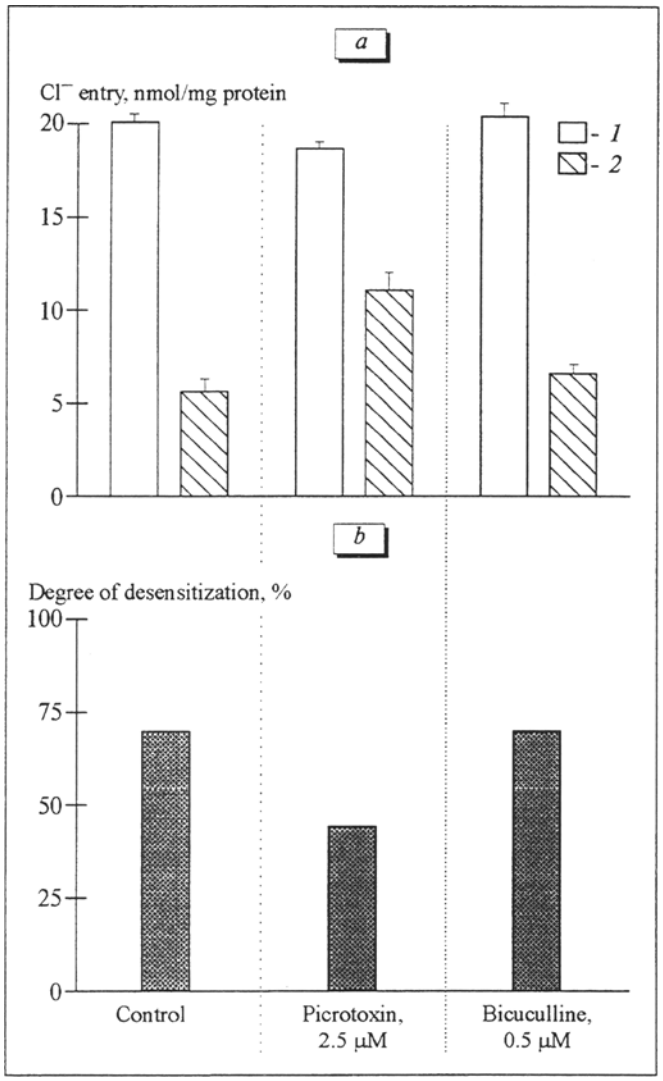

Fig. 2. Effects of picrotoxin and bicuculline on muscimol-stimulated (50 μM) $^{36}Cl^-$ entry into synaptoneurosomes and on desensitization.

into SNS caused by PT and BC, the effects of these two inhibitors were evaluated at two muscimol concentrations differing by an order of magnitude — 5 and 50 μM. The 5 μM concentration is close to the muscimol IC_{50} which equalled 3.3 μM under the experimental conditions we used [5] and is, therefore, an optimal concentration for the study of both inhibition and activation of the GABA receptor/Cl^- channel complex. The 50 μM muscimol concentration is at the beginning of the plateau on the dose—response curve. $^{36}Cl^-$ accumulation in SNS at that point reaches its peak and does not increase at higher muscimol concentrations.

Figure 1 illustrates the effects of PT and BC on the permeability of SNS membranes for Cl^- and on desensitization of the receptor-ionophore complex at the 5 μM muscimol concentration. Both BC (0.5 μM) and PT (2.5 μM) inhibited the muscimol-dependent $^{36}Cl^-$ transport (by 30.9% and 58.8%, respectively) and decreased the degree of desensitization caused by the 5-second preincubation of SNS with muscimol (to 3.2% and 26.8%, respectively, *vs.* 44.1% in the control tests). These results agree with those obtained earlier in experiments with high concentrations of these preparations [6].

The much higher muscimol concentration of 50 μM resulted in complete inhibition of $^{36}Cl^-$ entry into SNS by BC (0.5 μM) and PT (2.5 μM) (Fig. 2). Also, there was virtually no difference between desensitization rates in the control tests and those using 0.5 μM BC, the degree of desensitization being 68.8% and 67.0%, respectively. In the presence of 2.5 μM PT, however, the desensitization remained delayed (its degree amounted to only 43.6%).

Muscimol in a concentration an order of magnitude higher than its IC_{50} thus abolished almost completely the inhibition of $^{36}Cl^-$ entry into SNS by PT and BC. In the case of BC this result can be readily explained by its competition with muscimol for binding to the GABA receptor. As regards PT, the observed effect of this Cl^- channel blocker appears unexpected. However, the conclusion that PT does not interact with the GABA receptor was based on data obtained in experiments where ligand-receptor interactions were evaluated using synaptic membranes isolated by a special procedure. When the effects of GABA (100 μM) and diazepam (100 μM) on ^{3}H-α-dihydropicrotoxin binding by a synaptic membrane preparation and by cultured cortical neurons were compared [10], it was found that GABA and diazepam had little effect on the amount of bound ^{3}H-α-dihydropicrotoxin in the synaptic membrane preparation, but displaced the ^{3}H-α-dihydropicrotoxin almost completely in the cortical neuron culture. The properties of the GABA/benzodiazepine receptor complex in isolated synaptic membranes are therefore very much different from its properties in live neurons. SNS, on the one hand, incorporate synaptic membrane fragments and, on the other hand, are closed membrane structures retaining the plasma membrane potential, the potential of intrasynaptoneurosomal mitochondria, and the metabolism of second messengers [4]. The occurrence of active metabolism in SNS suggests that the functional state of their receptor complexes is the same as in live neurons. Hence, our finding that the inhibitory effect of PT was abolished by muscimol in high concentration well agrees with the results obtained for cultured neurons.

Of special interest is the observation that the high (50 μM) muscimol concentration eliminated the delay of desensitization by BC but not its delay by PT. The ability of muscimol in high concentration

to abolish both BC effects (i.e., inhibition of Cl^- entry and delay of desensitization) warrants the conclusion that its effect on desensitization is an indirect one and results from inhibition of muscimol binding to the receptor. The preservation, under similar conditions, of the PT-induced delay of desensitization (when Cl^- entry was not inhibited by PT under the conditions used) indicates that PT and BC act on desensitization by different mechanisms.

Although it is still difficult to tell what is the precise mechanism of this phenomenon shown by PT, some inferences can be made. Thus, the effect of PT on desensitization is not a simple consequence of the Cl^- entry inhibition by this compound. Also, there is no rigid relationship between the degree of desensitization and the number of Cl^- anions that pass through the Cl^- channel. Furthermore, a decrease in the transmembrane electrochemical Cl^- gradient is not a cause of the observed decrease of Cl^- entry into SNS. It may therefore be concluded that desensitization of the GABA receptor/Cl^- channel complex represents a reduction of Cl^- channel conductivity rather than a reduction of receptor sensitivity, although it is possible that these two processes proceed concurrently as a result of conformational changes in the structure of the complex.

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