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ACTION OF METRAZOL ON REGULATION OF THE $GABA_A$ RECEPTOR-CHANNEL COMPLEX

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Most of the epileptogenic activity of metrazol (pentylenetetrazol, PTZ) is due to its ability to inhibit selectively the Cl^- channel of the $GABA_A$ receptor complex, blockade of which is known to be one of the principal mechanisms of neuronal hyperactivity [1]. However, this conclusion is mainly drawn from indirect data. In the only study in which a direct method of determination of activity of the GABA channel-receptor complex was used, namely determination of the inflow of $^{36}Cl^-$ into synaptoneurosomes, the action of PTZ was not investigated in detail and its kinetic parameters were not determined [2].

In the investigation described below the kinetic parameters of inhibition of the muscimol-dependent inflow of $^{36}Cl^-$ into synaptosomes by PTZ were determined, slowing of desensitization of the $GABA_A$ receptor complex under the influence of PTZ was demonstrated, and the effect of PTZ on dependence of the effect of muscimol on concentration was analyzed.

EXPERIMENTAL METHOD

Synaptoneurosomes were obtained by the technique of Hollingsworth [5] with certain modifications: in particular, instead of expressing the brain tissue homogenate through a teflon filter with pore diameter of 10 μ with a syringe in order to separate cells that were not disintegrated, the more sparing procedure of successive filtration of the preparation through a series of kapron gauze strainers with decreasing mesh size was used.

Noninbred male albino rats weighing 180-200 g were decapitated, the cerebral cortex was isolated, and it was homogenized manually (five frictions) at 0-4°C in a glass homogenizer with teflon pestle in Krebs–Ringer medium

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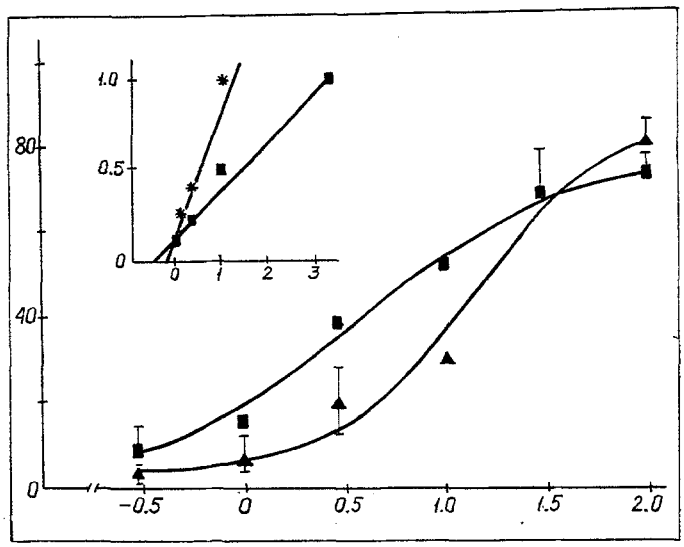

Fig. 1. Dose-response curve of inhibition of muscimol-stimulated $^{36}Cl^-$ inflow by PTZ. Synaptoneurosomes preincubated with PTZ (0.3-16 μM) for 20 min at 30°C before addition of muscimol (5 μM) and $^{36}Cl^-$ (0.5 μCi). Figure in top right corner – Hanes–Woolf coordinates for inhibition by PTZ (mM). I) Degree of inhibition, in % (not shown). Coefficient of correlation r = 0.9983. IC_{50} for PTZ 2.11 ± 0.25 mM and V_{max} for degree of inhibition was 94.1 ± 10.9%. Here and in Figs. 2 and 3, values shown are mean values of one experiment, with four parallel determinations, and repeated 3 times with the same results.

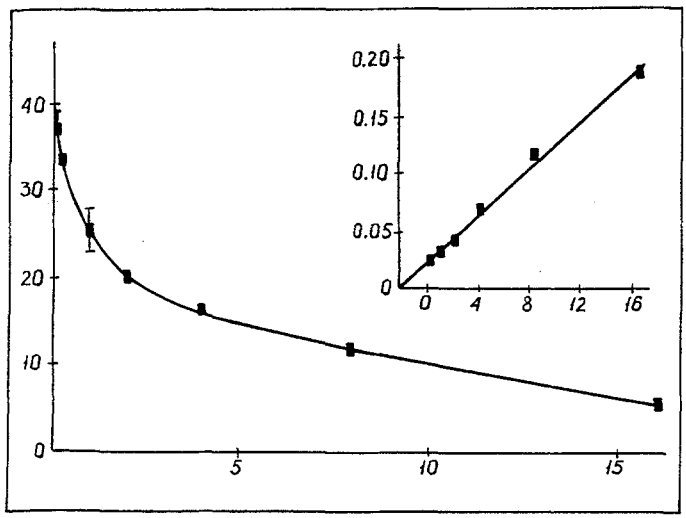

Fig. 2. Effect of PTZ on dose-response curve, reflecting stimulation of $^{36}Cl^-$ inflow by muscimol. Synaptoneurosomes preincubated with PTZ (10 mM) for 20 min at 30°C before addition of muscimol (0.3-100 μM) and $^{36}Cl^-$ (0.5 μCi). Top left figure shows double reciprocal coordinates, revealing a mixed (competitive-noncompetitive) type of inhibition. Coefficients of correlation were: in control (1) 0.9901 and in presence of PTZ (2) 0.0043. Values of V_{max} and EC_{50} were: 98.45 ± 10.13 nmoles/mg protein and 3.31 ± 0.42 μM, and 108.12 ± 14.7 nmoles/mg protein and 16.23 ± 4.27 μM respectively.

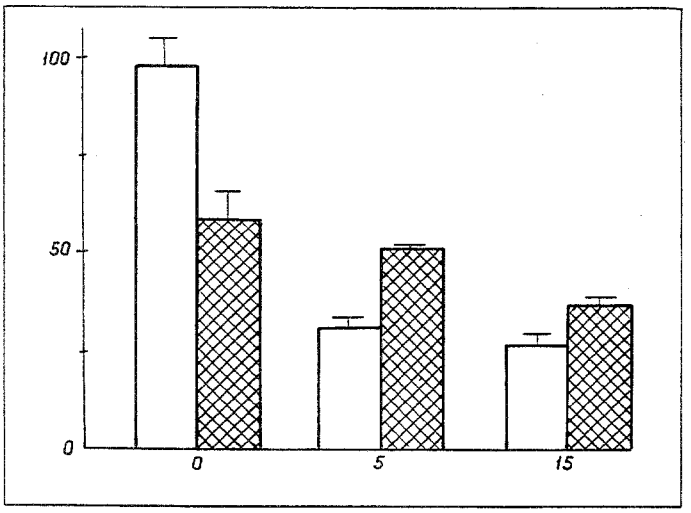

Fig. 3 Slowing of desensitization of muscimol-stimulated inflow of $^{36}Cl^-$ under the influence of PTZ. Synaptoneurosomes preincubated with PTZ (10 μM) for 20 min at 30°C, followed by addition, first, of muscimol alone (final concentration 20 μM), and 5 or 15 sec later, of muscimol (20 mM) and $^{36}Cl^-$ (0.5 μCi) unshaded columns indicate $^{36}Cl^-$ inflow in control, shaded columns – in presence of PTZ. Numbers below columns indicate time (in sec) of preincubation of synaptoneurosomes with muscimol and without isotope; 0 sec means that muscimol and $^{36}Cl^-$ were added simultaneously.

of the following composition, in mM: NaCl – 145, KCl – 5, $MgSO_4$ – 1, $CaCl_2$ – 1, glucose – 10, HEPES – 10, pH 7.4 (20°C) in the proportion of 1 g tissue to 15 ml medium. During successive iiltration of the homogenate kapron sieves (Rakhmanovskii Combine) with 300, 99, 60, and 27 μ mesh were used. The filtrate was centrifuged for 5 min at 2700*g* and the residue was resuspended in the same volume of Krebs–Ringer medium, and recentrifuged under the same conditions. The residue after the 2nd centrifugation was suspended in Krebs–Ringer medium so that the final concentration of synaptoneurosomes as protein was about 4 mg/ml. The synaptoneurosomes were used immediately after isolation, for the quality of the preparation began to deteriorate after 2-2.5 h.

Estimation of the Cl^- conductivity of the membrane, controlled by the GABA/benzodiazepine receptor complex was carried out by measuring the change in the rate of inflow of $^{36}Cl^-$ into the synaptoneurosomes [4]. The suspension of synaptoneurosomes was poured in aliquots of 100 μl into test tubes, PTZ was added in the necessary concentration, and the sample was preincubated for 20 min at 30°C. Next, 100 μl of Krebs–Ringer solution containing 0.5 μCi of $^{36}Cl^-$ (Izotop, Russia) and muscimol in the necessary concentration, was added to each sample, with vigorous mixing. After 5 sec the inflow of $^{36}Cl^-$ into the synaptoneurosomes was stopped by filtration in vacuo through GF/C glass-fiber filters ("Whatman," England) and washed 3 times, each time with 4 ml Krebs–Ringer solution, containing 100 μM picrotoxin, cooled to 0-4°C. To determine the basal inflow of $^{36}Cl^-$ the radioactive label was added without muscimol. To study desensitization of the $GABA_A$-receptor complex, muscimol (final concentration 20 μM) was added to the synatoneurosomes without the isotope, and incubated for 5 or 15 sec, after which muscimol in the same concentration and $^{36}Cl^-$ were added. Radioactivity was measured on a "RackBeta" 1219 counter (LKB, Sweden).

EXPERIMENTAL RESULTS

PTZ inhibited muscimol-stimulated inflow of $^{36}Cl^-$ into synaptoneurosomes to a degree dependent on its concentration (Fig. 1). The action of PTZ was detected when muscimol was present in a concentration of 5 μM – close to its EC_{50} value. Since the result of interaction of PTZ with the GABA–receptor complex is to reduce the rate of $^{36}Cl^-$ inflow, to determine the kinetic parameters of the action of PTZ, the graph in Fig. 1 was transformed into a graph of the degree of inhibition of $^{36}Cl^-$ inflow as a function of PTZ concentration. The degree of inhibition of $^{36}Cl^-$ inflow was determined as the difference, expressed in per cent, between values of $^{36}Cl^-$ inflow in the control and in the presence of PTZ. The graph was linearized by the method of Hanes and Woolf [3]. The experimental points fitted well into a straight line (the coefficient of correlation was 0.9993), evidence that the results are in full agreement with the theoretical binding isotherm. Calculation of the kinetic parameters of action of PTZ gave the following results: IC_{50} = 2.11 ± 0.25 mM and V_{max} = 94.1 ± 10.5% (relative to the degree of inhibition). PTZ, within the concentration range tested, had no effect on the basal inflow of $^{36}Cl^-$ into the synaptoneurosomes. These results are close to values for the half-maximal action of PTZ, obtained in experiments to study radioligand binding of ^{35}S-TBPS [8, 9] and they are in good agreement with the results of measurement of PTZ concentration in the mouse brain during seizures caused by its preliminary intraperitoneal injection [10]. All this confirms the view that the epileptogenic action of PTZ takes place through inhibition of the $GABA_A$-receptor complex.

With an increase in the muscimol concentration the degree of inhibition of $^{36}Cl^-$ inflow into the synaptoneurosomes by PTZ (10 mM) was reduced virtually to zero, in the presence of 100 mM muscimol. The graph in Fig. 2 shows that PTZ shifts the concentration curve of muscimol to the right, i.e., it in iact reduces the sensitivity of the $GABA_A$–receptor complex to muscimol (Fig. 2). Under these circumstances PTZ increased EC_{50} for muscimol by 4.9 times (from 3.31 ± 0.43 to 16.23 ± 4.27 μ) without any great change in V_{max}. On the basis of graphs linearized by the method of double reciprocal coordinates, it can be concluded that inhibition was of the mixed (competitive–non-competitive) type. As was pointed out above the prolonged (on a physiological scale) action of GABA and its agonists on the $GABA_A$-receptor leads to weakening of its functional activity (desensitization). In our system desensitization was manifested as a decrease in the muscimol-dependent inflow of $^{36}Cl^-$ after preincubation of the synaptoneurosomes with muscimol (20 μM) for 5 or 15 sec before addition of the isotope (Fig. 3). It will also be clear from Fig. 3 that under the influence of PTZ, muscimol-induced desensitization of the $GABA_A$-receptor complex was greatly retarded. Thus PTZ inhibits not only transport of Cl^- ions through the chloride channel of the $GABA_A$–receptor complex, but also desensitization of the complex by muscimol. For instance, preincubation of membrane vesicles with muscimol for 5 sec led to loss of activity of the $GABA_A$–receptor complex by about 70%, whereas in the presence of 10 mM PTZ, the loss was only 10%. Thus after preincubation of synaptoneurosomes with muscimol for 5 sec, muscimol-dependent Cl^- transport was increased under the influence of PTZ by about 50% compared with the control. Lengthening the preincubation time to 15 sec led to weakening of this effect of PTZ, i.e., it also led to desensitization, but more slowly than in the control.

A theoretically possible mechanism of inhibition of Cl^- transport through the membrane by PTZ could be an increase in the rate of desensitization of the $GABA_A$–receptor complex and, as a result of it, reduction of the integral Cl^- inflow throughout the duration of action of the mediator. However, the results are evidence that the reverse is the case: PTZ slowed the rate of desensitization of this receptor complex. With an increase in the duration of incubation of the synaptoneurosomes with muscimol a paradoxical situation may arise: reversal of inhibition of muscimol-dependent $^{36}Cl^-$ transport by PTZ into its "activation" (Fig. 3). This unique phenomenon is evidently due to slowing of desensitization of the channel–receptor complex in the presence of PTZ. Evidence in support of this hypothesis is given by an increase in the rate of desensitization of the $GABA_A$–receptor complex under the influence of diazepam, an activator of GABA-dependent Cl^- transport [7]. The data showing an increase in the rate of desensitization with an increase in the GABA concentration can be regarded from the same standpoint [6]. Dependence of the rate of desensitization on the degree of activity of the $GAEA_A$-channel–receptor complex evidently reflects

regulation of the complex by the negative feedback principle. So far as the mechanism of desensitization is concerned, inhibition of the receptor/channel complex both by the agonist (through its receptors with lower affinity) and by an increase in the intracellular Cl^- concentration is possible. This last suggestion is a fundamentally new idea on the possible mechanism of desensitization. The point is that desensitization may probably be determined by the intracellular Cl^- concentration: the higher $[Cl^-]_i$, the greater the degree of desensitization of the $GABA_A$-receptor complex. Thus desensitization is determined not only by the GABA concentration, but also by negative feedback from the inflowing and intracellular Cl^-. Biologically and functionally this mechanism is valid. If the inflow of Cl^- and, correspondingly, the degree of increase of $[Cl^-]_i$ is reduced (as, for example, under the influence of PTZ), the rate of desensitization of this receptor complex also is reduced. To elucidate the concrete mechanisms of this internal negative feedback a special study is needed.

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