ANALYSIS OF SPECIFIC ^{3}H-DIAZEPAM BINDING IN THE BRAIN OF EMOTIONALLY DIFFERENT MICE

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Since the discovery of the benzodiazepine receptor complex (BDRC), consisting of a GABA receptor and Cl^--ionophore in 1977, sufficient experimental data have been obtained to prove its key role not only in the psychotropic effects of the benzodiazepines (BD), but also in regulation of the emotional state of the absence of pharmacological action [13].

A study of the behavior of inbred animals under conditions of emotional stress, of the biochemical parameters of the stress reaction, and effects of benzodiazepine tranquilizers, conducted in the writers' laboratory showed that the character of the response to stress and manifestation of the action of BD depend on hereditary factors [3]. The aim of this investigation was to study reception of ^{3}H-diazepam by brain cell membranes of C57BL/6 (B/6) and BALB/c (B/c) mice, which were used as models in the previous investigations.

EXPERIMENTAL METHOD

Experiments were carried out on male B/6 ahd B/c mice weighing 18-20 g. The animals were kept in the laboratory animal house for at least 2 weeks before the beginning of the experiments, on a standard diet, with 10 mice per cage, and with alternation of 12 h daylight and 12 h darkness.

To study binding of ^{3}H-diazepam (specific activity 71 Ci/mmole, Amersham International, England) the mice were decapitated and the brain quickly removed, the brain stem and cerebellum were detached, and the remainder was homogenized in 25 ml of 50 mM Tris-citrate buffer, pH 7.4, and centrifuged at 45,000g for 25 min of an L5-50 centrifuge (42.1 rotor, Beckman, USA). The residue was resuspended by rehomogenization in the same volume of buffer, and then centrifuged again. The washing procedure was repeated 3 times and the resulting residue resuspended in 70 ml of Tris-citrate buffer and used in a volume of 1 ml in experiments to study binding of ^{3}H-diazepam, which was added to the medium in a final concentration of 1 nM. In the series of experiments with previously frozen preparations, the residue was resuspended in the minimal volume of buffer and frozen for 24 h at −20°C. The sample was thawed and homogenized by centrifugation for 25 min at 45,000g, and then washed once. The residue was resuspended in 70 ml of buffer and used in radioligand binding experiments. The protein content in the samples was 150-200 μg. Nonspecific binding was determined in the presence of 10 μM unlabeled diazepam. Incubation was carried out for 30 min at 0°C and the reaction was stopped by rapid filtration through a GF/B filter (Whatman, England), followed by washing twice with cold buffer solution. The filters were placed in scintillation fluid: toluene–ethanol 7:3 by volume in the presence of 0.4% PPO and 0.01% POPOP). Radioactivity was measured on an LS-100C counter (Beckman). The counting efficiency was 45%. Protein was determined by a modified Lowry's method. The results were subjected to statistical analysis by Student's test.

EXPERIMENTAL RESULTS

No differences in diazepam reception were discovered during processing and incubation of the freshly isolated membrane preparations (FIMP) from the brain of the B/6 and B/c mice in Tris-citrate buffer (Fig. 1a). Addition of GABA to the incubation medium in concentrations of 1, 10, and 100 μM increased binding of the radioligand by FIMP from animals of the

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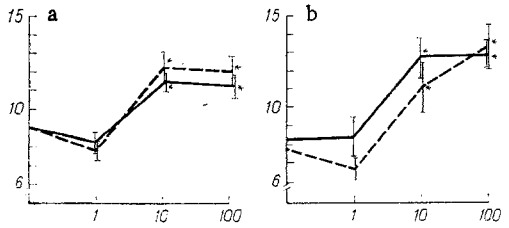

Fig. 1

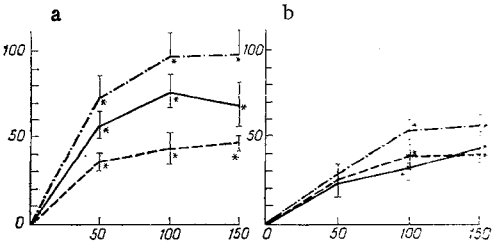

Fig. 2

Fig. 1. Effect of GABA on binding of ^{3}H-diazepam with mouse brain membrane fraction. Abscissa, GABA concentration (in μM); ordinate, binding of ^{3}H-diazepam (in cpm × 10^3/mg protein). a) FIMP; b) PFMP. Continuous line — B/6; broken line — B/c.

Fig. 2. Effect of NaCl on binding of ^{3}H-diazepam with mouse brain membrane fraction. Abscissa, NaCl concentration (in mM); ordinate, binding of ^{3}H-diazepam (in % of control). Line of dots and dashes represent F_1 hybrids. Remainder of legend as in Fig. 1.

two strains equally, confirmed data in the literature [14]. A different result was obtained when the regulatory influence of Cl^- ions was studied. It was found that the level of reception of FIMP from B/6 mice in the presence of NaCl was increased by a lesser degree than in B/c and (B/6 × B/c)F_1 hybrids (Fig. 2a). Interlinear differences were thus established in the sensitivity of BDRC to the stimulating effect of Cl^- ions on reception of ^{3}H-diazepam.

Since both FIMP and previously frozen membrane preparations (PFMP) were used to study receptor binding of BD, similar experiments also were carried out on PFMP from mouse brain. In this case the basal level of binding of the radioligand was found to be lowered, evidently on account of removal of GABA from the membrane preparation during freezing and thawing [1]. This suggestion is confirmed by the stronger stimulating effect of GABA (Fig. 1b).

Addition of NaCl to PFMP caused a much smaller increase in binding than in the experiments with FIMP (Fig. 2b). These results are in agreement with those of investigations [11] which showed a decrease in the sensitivity of BDRC to Cl^- after the freezing–thawing procedure. Characteristically, in the experiments with PFMP no interlinear differences were found in ^{3}H-diazepam reception.

Taken as a whole, the results of these experiments provide an explanation for the contradictions present in the literature on the study of genetic control of BD reception.

In experiments on B/6 and B/c mice and on Maudsley Reactive and Maudsley Nonreactive rats, which also differ emotionally in the open field test, it was shown [7, 8] that binding of labeled diazepam differs in the brain of the animals of these strains. However, in Roman High Avoidance and Roman Low Avoidance rats, which are similar in their open field behavior, were used in similar experiments, no such differences could be discovered [9, 10].

Analysis of these studies shows a significant difference in the conditions of isolation and incubation of the membrane preparations. Robertson et al. [7], for instance, used a medium with a high concentration of Cl^- and of ions of bivalent metals, whereas Shephard et al. [9, 10] used Tris-buffer of low molarity and previously frozen membranes. Consequently, interlinear differences in reception are found under conditions of an adequate Cl^- concentration in the incubation medium for FIMP, which is in full agreement with our own data and confirms that the Cl^--ionophore plays a role in the formation of hereditary differences in BD reception.

The physiological importance of these results must be assumed on the basis of existing views regarding the function of the Cl^--ionophore, which is to regulate membrane transport of Cl^- and thus to correct the transmembrane potential [15]. It has been shown that picrotoxin, an inhibitor of the Cl^--ionophore, induces convulsions, and some workers consider that metrazol [15] has a similar mechanism of its convulsant action. Anxiogenic effects of certain compounds, including the structural analog of BD, R-05-3663 [5], are currently being linked with the Cl^--ionophore. It can be tentatively suggested that the unequal interaction of Cl^- with the benzodiazepine receptor in B/6 and B/c mice is one element in the mechanism of formation of differences in reactions to emotional stress and to BD that are specific for the animals of these strains.

Meanwhile the charge on the membrane is known to correlate with its viscosity, its phospholipid composition, and the level of its free-radical processes [12]. These last factors, in turn, determine the functional state of BDRC [6] and are essential for the formation of the effects of the benzodiazepine anxiolytics and anticonvulsants [2]. It can therefore be tentatively suggested that genetic control of regulation of BDRC, dependent on the Cl^--ionophore, discovered in the present investigation, is based in unequal, charge-determined membrane structural changes that are responsible for different conformational changes in BDRC and, as a result of this, a specific pattern of physiological effects linked with it.

The existance of these mechanisms, consolidated during evolution, suggests that they can be regarded as the target for psychopharmacological action and it explains the presence of psychotropic properties, similar to those of BD, in a series of membrane-active compounds that do not bind directly with the benzodiazepine receptor [4].

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EFFECT OF PYRROLIDONE-2 ON THE CEREBRAL CIRCULATION

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Our ideas on the role of the principal inhibitory neurotransmitter of the brain, namely GABA, are continually being widened and deepened through the discovery of additional facts [5, 9, 14]. Many studies of its role in the regulation of blood pressure (BP) have now been published [15]. However, as long ago as in 1964, it was discovered that GABA can stimulate the cerebral circulation and, at the same time, raise the partial pressure of oxygen in brain tissues [2]. GABA promotes the formation of several active substances in the brain, many of which give rise to physiological and pharmacological effects [4].

The discovery of pyrrolidone-2 [10], a cyclic derivative of GABA, and that linear and cyclic GABA derivatives differ in their action [6, 7] prompted the authors to undertake further investigations.

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