THE BENZODIAZEPINE RECEPTOR AND RECEPTOR TOLERANCE PRODUCED BY CHRONIC TREATMENT OF DIAZEPAM

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Abstract—A characterization of the benzodiazepine receptor *in vivo* was carried out utilizing the up-and-down method for a quantitative determination of the maximal seizure threshold to diazepam. Wistar-Imamichi male rats of 21 days of age were used. The drug concentration in the brain at the steady-state was measured with radiolabeled diazepam. A kinetic analysis for the dose-response relation obtained gave 2.2 μ M and 1.6 for the K_d and n , respectively, in the Langmuir adsorption isotherm. The results suggested the existence of a hitherto undiscovered endogenous ligand(s) suppressing the binding of benzodiazepine to the receptor, as compared with those in the *in vitro* binding-assay reported by other investigators. A chronic pretreatment with diazepam once a day for 13 days (0.2 and 2 mg/kg, *s.c.*) produced receptor tolerance, but did not affect the amount of the seizure threshold unless the drug was acutely administered 10 min before the electric shock. These results supported the argument that two distinct entities exist for both GABA and benzodiazepine recognition sites which are closely related to each other in their functioning.

Three major actions of the benzodiazepines, anxiolytic, anticonvulsant and muscle relaxant activities, have been shown to have a good correlation with the binding ability of the drugs to the receptor (1, 2). By virtue of the discovery of the endorphins for the opiate receptor, one may inquire as to the possible physiological significance of the benzodiazepine receptors, and an endogenous ligand(s) for the receptor is being widely sought for a better understanding of the mechanisms underlying the drug-mediated and physiological effects (3–6).

Although much additional characterization of the receptor is required to establish a putative endogenous ligand for the benzodiazepine receptor as a true neurotransmitter or neuromodulator (4), the biological responses of intact neurons have been largely neglected in recent CNS pharmacology with the

emergence of ligand displacement methodology (7).

In the present study, *in vivo* evidence for the existence of the drug receptor for diazepam was provided with its desensitizing characteristics deduced from a quantitative determination of the maximal seizure threshold. A preliminary report has appeared elsewhere (8).

Materials and Methods

Animals: Wistar-Imamichi male rats (Institute for Animal Reproduction, Ohmiya) were used at 21 days of age when ontogenetic development of the benzodiazepine receptor is virtually complete (9, 10). ED₅₀ was determined on groups of 8 to 10 rats, which were formed from the large number of animals from the same farm necessary for one experiment and randomized for body weight

and their mothers fed. The mother rats were maintained on a laboratory chow (Oriental Yeast Co., Ltd.) and tap water ad libitum and were kept in a temperature-controlled room ($22\pm 3^{\circ}\text{C}$) with a lighting schedule of 14 hr light (06:00–20:00) and 10 hr darkness.

Chemicals: Diazepam was dissolved in a 5% ethanol solution so as to give a constant volume (0.02 ml/g body weight) and administered intraperitoneally 10 min before electric shock. For chronic treatment, the drug was given subcutaneously once a day for 13 days from the 8th to the 20th day after birth.

Measurement of seizure threshold: The maximal seizure threshold (MST) was adopted as a criterion, and the up-and-down method was utilized to determine the precise seizure threshold (MST₅₀). The details of the measurement are described in the previous study (11).

Measurement of serum calcium concentration: Immediately after judging the all-or-none type of seizure response, the animal was bled by cutting the juglar vein. The serum was treated with Lanthanum-TCA solution and centrifuged at $3,000\times g$ for 10 min. The supernatant was then used for atomic absorption analysis for determining the calcium concentration in the serum.

Measurement of radioactivity: One-hundred μCi of [N-methyl- ^3H]-diazepam (specific activity of 97 Ci/mmol, Amersham) was mixed with 0.9 mg of unlabeled diazepam (Takeda) and dissolved in 20 ml of 5% ethanol solution. The drug was administered intraperitoneally so as to make a constant volume (0.02 ml/g body weight). Blood samples were taken by decapitation and prevented from coagulation by adding 10 U of heparin. One hundred μl of whole blood was solubilized with 2 ml of NCS and decolorized with 1 ml of the benzoyl peroxide solution. Finally, 10 ml of PCS (Amersham) scintillator solution was added for radioactivity determination.

Brain samples were dissected superficially to weigh about 50 mg for each region (cerebral cortex, hippocampus, cerebellar cortex and medulla) and washed out quickly for possible bleeding with physiological solution. The NCS solubilizer and PCS scintillator solutions were also used for the brain samples. The radioactivity counts were calibrated for the drug concentration by the preparative standard.

In the present study, the radioactivities measured at 10 min after intraperitoneal administration were approximated to be diazepam since no significant amount of the metabolites were expected to appear by that time based on the other experimental data in rats (12, 13).

Results

Diazepam concentrations in blood and brain after intraperitoneal administration: To establish the experimental conditions under which the steady-state pharmacokinetics of diazepam could be obtained, the time course of absorption and distribution of the drug were examined. After intraperitoneal injection of 0.9 mg/kg of diazepam with a tracer amount of radioactivity, samples of blood and brain tissues were taken from each animal at various time intervals.

Diazepam was absorbed very rapidly, and its concentration in the blood reached a peak in 10 min (Fig. 1A). The average concentration at 10 min was $0.45\pm 0.02\ \mu\text{g/ml}$ (mean $\pm$ S.E., $n=4$). Transfer of the drug from the blood to the brain was also very rapid, and already at 2 min after injection, an even higher concentration was attained in the brain than in the blood. The drug concentration in the brain became maximal in 6 min and this level continued for several min. The overall average concentration in the brain at 10 min was $0.70\pm 0.053\ \mu\text{g/g}$ tissue (mean $\pm$ S.E.M., $n=4$), which was 56% higher than that in the blood, suggesting a high affinity

Fig. 1. Time course of absorption and distribution of diazepam after intraperitoneal administration. The drug dissolved in 5% ethanol solution (0.9 mg/kg) was given with a tracer amount of [N-methyl-³H] diazepam. Ordinate: diazepam concentration in µg/ml or g tissue. Abscissa: time after injection in min. A, blood; B, brain tissue in which the filled squares are for the cerebral cortex; filled circles, the hippocampus; filled triangles, the cerebellar cortex; and open circles, the medullary region. The solid curve, a third power of polynomial regression. Each point represents the value from one animal.

of the drug for the brain tissues (Fig. 1A, B). Based on the result shown in Fig. 1, a 10 min wait after the drug administration was chosen in the following electric shock experiments for the steady-state pharmacokinetic analysis.

The dose-response relationship for anti-convulsant effect of diazepam: The MST₅₀ for the control rats (solvent alone) was 32.6±0.18 (n=4) mA with little variation among the groups. The MST₅₀ was elevated with an increase in the drug dose in a dose-

Fig. 2. A dose-response relationship for the anti-convulsant effect of diazepam. Ordinate: the maximal seizure threshold as determined by the "up-and-down method" in mA. Abscissa: logarithm of the intraperitoneally administered drug dose, except zero. Each point was obtained from 8 to 10 rats. The solid curve was drawn according to Eq. 1, where K_d and n values were obtained by the least squares method as described in the text. K_d, 0.86 mg/kg; n, 1.55.

dependent manner, and a sigmoid curve was attained in the dose-response relation (Fig. 2).

When diazepam was given beyond the maximum dose used in Fig. 2, the tonic convulsion that was a necessary sign for the maximal seizure was abolished to any large seizure current. When rats were given physiological solution alone, the MST₅₀ was 28.3 mA. These results indicate that the 5% ethanol solution (0.1% in the body weight) elevated the seizure threshold by 4.3 mA, which was 15% of the control.

Assuming that diazepam combines with the benzodiazepine receptor in a manner expressed by the Langmuir adsorption isotherm and that the elevation of the MST₅₀ is somehow proportional to the number of receptors occupied by the drug:

where n is the number of the drug molecules. At equilibrium.

$$Y = \frac{R_{\max} + R_{\min} \cdot K_d/D^n}{1 + K_d/D^n} \quad (1)$$

where $R_{\max}$ designates the maximum response; $R_{\min}$, the minimum response; and D , the concentration of the drug. By arranging Eq. 1, the K_d and n values were obtained by the least squares method from the intercept and the slope, respectively. The solid curve in Fig. 2 gave the best fit for the data with values of 0.9 mg/kg and 1.6 for K_d and n , respectively. The K_d value corrected for the brain concentration of the drug as obtained in Fig. 1 was 2.2 μ M.

Receptor tolerance induced by chronic pretreatment of diazepam: Diazepam dissolved in a 5% ethanol solution was chronically administered to test rats (0.2 and 2 mg/kg, s.c.) once a day for 13 days from the 8th to the 20th day after birth, and the thereby-produced changes in the seizure responses were examined on the 21st day for the pretreated rats in Fig. 3. When diazepam was acutely given to the pretreated rats, the MST_{50} was greatly suppressed in a manner dependent upon the dose of the pretreatment (1, 0; 2, 0.2; 3, 2 mg/kg). In contrast, when the solvent alone was given acutely, the MST_{50} was not altered significantly for each group (1, 2 and 3) compared to the untreated control (not shown).

Fig. 3. Receptor tolerance induced by chronic pretreatment with diazepam. Pretreatment: 1, 5% ethanol; 2, 0.2 mg/kg diazepam; 3, 2 mg/kg diazepam. Ordinate: the MST_{50} in mA. Abscissa: logarithm of the acutely administered diazepam. Each point was derived from 8 to 10 rats. The solid curve was the same as that obtained in Fig. 2.

Namely, the physiological function of the relevant neurons including synaptic events was not affected by the chronic treatment, but the drug-mediated function was largely affected in terms of epileptogenic activity. These experimental facts strongly supported the argument that there exist two distinct entities for both GABA and benzodiazepine recognition sites which are closely related or are part of the same macromolecular complex (5, 14).

The drug tolerance as revealed in Fig. 3 is probably a "receptor" tolerance rather than a "metabolic" tolerance since the latter type of phenomenon is found not to occur in the 10 min after the drug administration the time, at which the seizure threshold was determined (13, 15, 16). There is another possibility that some metabolites derived from the pretreatment behaved antagonistically to acutely administered diazepam, taking into account the relatively long biological half lives of the drug and its metabolite(s) (13, 15, 16). This possibility was, however, excluded by the experiment described below.

No residual effect of diazepam and its metabolites on the maximal seizure threshold: Three groups of rats were used, and diazepam was administered subcutaneously to each group of rats once a day for 1, 2 and 3 days before the electric shock at the 21st day after birth. None of these pretreatments exerted any influence on the MST_{50} determined where 0.8 mg/kg diazepam was acutely given (Fig. 4). The result also demonstrated that the receptor tolerance was a relatively slow process that required at least 3 days for induction.

No significant alteration in serum calcium concentration by diazepam: Since an increase in serum calcium concentration raises the maximal seizure threshold (11), a possible change in the calcium concentration by diazepam was explored. However, the serum calcium concentration had not been altered

Fig. 4. The maximal seizure threshold after a short term (1–3 days) pretreatment with diazepam. The drug at 2 mg/kg was subcutaneously given once a day as in Fig. 3, starting at the day shown on the abscissa, and the MST_{50} in mA was measured at the 21st day (arrow) on the pretreated rats to the acutely-given diazepam of 0.8 mg/kg. Filled circle, with no chronic pretreatment. Each value shows the ED_{50} for the maximal seizure threshold obtained from 8–10 animals.

significantly by the pretreatment at the 21st day when the receptor tolerance was produced. A short term pretreatment for 1, 2 and 3 days as in Fig. 4 also did not change the serum calcium concentration at the 21st day. When diazepam (2 mg/kg) was given for 6 days from the 8th to the 14th day after birth, no significant change in the serum calcium concentration was observed. All these results were summarized in Table 1. Calcium ions could not be causally related to the receptor tolerance observed in Fig. 3.

Discussion

After intraperitoneal administration, diazepam was absorbed very rapidly so as to be transferred from the blood to the brain, and it nearly attained a steady-state concentration between 6 and 12 min after the injection (Fig. 1). The maximal seizure threshold was quantitatively determined utilizing the "up-and-down method" in its steady-state (10 min after administration), and the sigmoid curve in the dose-response relation was obtained for anti-convulsant effect of

Table 1. Serum calcium concentrations after diazepam administration

Treatment	Periods (day)	Serum calcium concentration (mg/dl)
Saline	8–20	11.4 ± 0.31 $n=8$
5% ethanol	8–20	11.1 ± 0.26 $n=4$
Diazepam		
0.2 mg/kg	8–20	11.0 ± 0.38 $n=4$
2.0 mg/kg	8–20	11.6 ± 0.17 $n=13$
Saline	19–20	11.7 ± 0.20 $n=6$
5% ethanol	19–20	11.2 ± 0.09 $n=7$
Diazepam		
0.2 mg/kg	19–20	11.4 ± 0.31 $n=6$
2.0 mg/kg	17–20	10.8 ± 0.23 $n=7$
*5% ethanol	8–14	11.1 ± 0.23 $n=10$
*Diazepam		
2.0 mg/kg	8–14	11.4 ± 0.22 $n=10$

Diazepam was given subcutaneously once a day for each period shown, and the serum calcium concentrations were determined at the 21st day except the cases indicated with an asterisk (*) which were measured at the 15th day.

diazepam as shown in Fig. 2.

It should be noted that the ED_{50} so far determined for the anti-convulsant effect of a drug was rather arbitrary as can be seen in Fig. 2. In other words, there exist a number of ED_{50} values depending on the amount of seizure current given, which was empirically determined.

The apparent K_d value estimated from the dose-response curve in Fig. 2 was $2.2 \mu M$, which was strikingly larger than that obtained in the binding-assay in vitro (1, 2). A lower temperature used in the binding-assay may explain the 5 fold difference in binding affinity of the benzodiazepine receptor (17), though there still remains a 200 fold difference

in the K_d values between the two, strongly suggesting the existence of a hitherto undiscovered endogenous ligand(s) suppressing the binding of benzodiazepine to the receptor.

The binding-assay *in vitro* demonstrated a single binding site for the benzodiazepine receptor in the kinetic analysis, i.e., $n=1$ in Eq. 1 (1, 2). In the present *in vivo* study, however, a larger value than unity for the n in Eq. 1, 1.6, was obtained. This may suggest a possibility that part of the receptors has multiple binding sites. In this connection, it is noteworthy that a heterogeneity of benzodiazepine receptors has repeatedly been reported (18–23). There is another possibility that a full pharmacological response was expressed by a small percentage of the receptors occupied or a significant response was initialized after a considerable amount of receptor occupancy by the drug. The solid lines (A and B) in Fig. 5 illustrate presumed dose-response relations for these two cases

Fig. 5. A simulation of the dose-response relations for the presumed two cases of $n=1$ in Eq. 1. The solid curve is the same as that in Fig. 2. A, for the case of a full pharmacological response produced by unsaturated receptors; B, for the case of initiation of a significant pharmacological response after a considerable amount of receptor occupancy. See text for the details of calculation. The dose-response relations were shifted along the dose axis as to cross the second (A) and 9th (B) points in Fig. 2, which resulted in a rather arbitrary treatment of the data, but it was still useful to estimate a possible range for the potency of the presumed endogenous inhibitor(s).

in which n was assumed to be unity, and the doses were calculated as to give the corresponding responses obtained in the experiment (Fig. 2). The K_d values estimated were $11 \mu\text{M}$ and $1.1 \mu\text{M}$, respectively. Therefore, the presumed endogenous ligand(s) would have a potency for suppressing the benzodiazepine receptor by a factor of 70 to 600 times. This information may be helpful for a further search for the endogenous ligand(s).

Another interesting finding in the present study was the receptor tolerance induced by chronic treatment with diazepam. In the previous study, a build-up of tolerance for benzodiazepines was suggested on epileptic seizures in *Papio papio* on a rather qualitative basis (24). The data reported so far in the *in vitro* binding-assay are not unequivocal and rather confusing (6): After long-term treatment with benzodiazepines, no changes were found with moderate or high doses (25, 26); other authors reported a decrease of the benzodiazepine receptor density with very high doses (27). It is unlikely that the receptor tolerance was produced by an increase in calcium concentration, leading to a stabilization of the excitable membranes (28), since neither acute nor chronic administration of diazepam produced any significant change in the serum calcium concentration (Table 1).

A consistent accumulation of demethylated metabolites in the white matter structures and in subcortical areas following a repeated administration was discussed for the mechanism of action of diazepam in the course of chronic treatment in man (29). None of the metabolites of diazepam, however, could be a deceptive cause for the receptor tolerance observed in the present study (Fig. 3) as shown in Fig. 4. It is not known whether or not any metabolite of the drug is causally implicated in the occurrence of the receptor tolerance.

The effect of the receptor tolerance was not manifested on the seizure threshold

unless diazepam was present (Fig. 3). If this effect were on the GABAergic postsynaptic receptor, the seizure threshold should have been altered in Fig. 3 since the GABAergic synapses must be functioning during the maximal seizure (30). Thus the receptor tolerance in Fig. 3 provided in vivo evidence for the existence of its own receptor for benzodiazepine.

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