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Repeated Administration of Diazepam Reduces [^{3}H]Ro 5-4864 Binding in Cerebral Cortex of Rats

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DIANA, G. AND M. MASSOTTI. *Repeated administration of diazepam reduces [^{3}H]Ro 5-4864 binding in cerebral cortex of rats.* PHARMACOL BIOCHEM BEHAV 42(2) 297–300, 1992. – Binding of [^{3}H]Ro 5-4864 to mitochondrial membrane preparations of rat cerebral cortex was measured after repeated (5 days) IV administration of diazepam (10 mg/kg daily) and clonazepam (2.5 mg/kg daily). The B_{max} value for [^{3}H]Ro 5-4864 was significantly reduced in rats treated with diazepam (–49%) but not in those treated with clonazepam. These findings suggest the involvement of peripheral-type binding sites in the development of rapid tolerance to the sedative effects of benzodiazepines. A downregulation of [^{3}H]Ro 5-4864 (–65%) was also observed after repeated administration of Ro 5-4864 (4 mg/kg daily), thus confirming that this compound behaves as an agonist at its own recognition sites.

Diazepam Clonazepam Ro 5-4864 Chronic administration [^{3}H]Ro 5-4864 binding

ALTHOUGH chronic administration of benzodiazepines induces tolerance to their sedative-hypnotic effects, no consistent changes have been found in central-type benzodiazepine receptor density [for references, see (6)]. However, diazepam (7,14) and flurazepam (16) have been reported to decrease the capability of GABA to stimulate [^{3}H]benzodiazepine binding in homogenate from rat brain cortex.

A distinct type of benzodiazepine binding site has been detected in peripheral organs, including kidney, liver, heart, lung, and, to a lesser extent, in central nervous tissue (5). These peripheral-type binding sites are located mainly in the outer membrane of the mitochondria (2). Specific peripheral site ligands are the chloride derivative of diazepam, Ro 5-4864 (putative agonist), and the isoquinoline carboxamide derivative, PK 11195 (putative antagonist) (4,8,10,12). Both drugs possess a very low binding capacity for central-type benzodiazepine binding sites. Diazepam – but not clonazepam – binds to peripheral-type binding sites. Both benzodiazepines, however, bind at central-type sites with high affinity (8).

A previous study showed that large doses of diazepam – but not of clonazepam – induce rapid tolerance to the electroencephalographic (EEG) synchronization and behavioral sedation (13). Tolerance is abolished when diazepam is coadministered chronically with PK 11195. In addition, in rats pretreated for 4 days with Ro 5-4864 (4 mg/kg, IV, once a day) the single administration of diazepam produced an EEG feature similar to that observed in diazepam-tolerant animals (1,13). Tolerance also develops to clonazepam when the drug is coadministered chronically with Ro 5-4864 (13). These data suggested that rapid tolerance to the sedative effects of benzodiazepines might be modulated by peripheral-type binding sites.

To further explore this possibility, we studied [^{3}H]Ro 5-4864 binding in mitochondrial membrane preparations from the cerebral cortex of rats given repeated administration of large doses of diazepam, clonazepam, and Ro 5-4864. The peripheral-type binding sites are mainly located in mitochondrial outer membrane; therefore, we found it necessary to concentrate the study in this subcellular fraction. The doses of the compounds and duration of the treatment were the same used in previous EEG studies (1,12).

METHOD

Animals

The experiments were carried out in male, adult Sprague-Dawley rats weighing 300–350 g at the time of surgery. Animals had free access to food and water. They were divided into eight groups, with five to six in each. Seven groups were operated and injected; one group was neither operated nor injected (control). Animal care and use followed the recommendations of the U.S. DHHS (17).

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Surgery

With the rat under equithesin (2.7 ml/kg, IP) anesthesia, a permanent polyethylene catheter was inserted 20 mm into the femoral vein and passed beneath the skin to exit midway in the back. Three to 4 days afterward, the drugs were injected through the catheter.

Drug Administration

All operated animals were injected once a day for 5 days. Groups of five to six animals were treated with single or repeated injections of vehicle, diazepam (10 mg/kg), clonazepam (2.5 mg/kg), or Ro 5-4684 (4 mg/kg). The single-dosed groups received the drugs on the fifth day, after repeated treatment with vehicle for 4 days.

All drugs were dissolved with 100 μl 10 N HCl and the pH of the solution was adjusted to 3–4 by adding 1 N NaOH. Drugs were injected by slow intravenous route (0.1 ml/1 min) in a volume of 2 ml/kg.

Membrane Preparation

On day 5, 30 min after drug injection, rats were killed by cervical translocation. The brain was immediately removed and the cortex rapidly dissected out on ice. The tissue was homogenized in 20 vol 0.32 M sucrose with a potter homogenizer. The suspension was centrifuged at 1,200 $\times$ *g* for 10 min. Then, the supernatant was collected and centrifuged at 45,000 $\times$ *g* for 20 min. Mitochondria were isolated by ultracentrifugation on a sucrose gradient according to the method described by Jones and Matus (9). The mitochondrial pellet was stored at -20°C. Before assay, the pellet was resuspended in 20 vol. distilled water and centrifuged at 0°C (45,000 $\times$ *g* for 20 min), then washed five times by centrifugation with 50 mM Tris-HCL buffer, pH 7.4

Binding Assay

Binding studies of [^{3}H]Ro 5-4864 were carried out according to the method of Marangos et al. (12). Briefly, the assay was performed at 0–4°C in a standard incubation medium of 50 mM Tris-HCl buffer, pH 7.4 (500 μl, final volume) containing 150–200 μg of proteins and five concentrations (0.55–12.83 nM, final) of [^{3}H]Ro 5-4864 (70.9 Ci/mmol). After an incubation time of 180 min, the reaction was terminated by rapid filtration on GF/B glass microfiber filters. Specific [^{3}H]Ro 5-4864 binding was defined as the difference between total binding and binding obtained in the presence of cold Ro 5-4864 (10^{-6}M, final). Protein concentrations were measured by the method of Lowry et al. (11).

Statistics

A one-way analysis of variance (ANOVA) and multiple *t*-test with Bonferroni's correction were used for intergroup comparisons. Ten comparisons (all groups vs. repeated vehicle; single diazepam vs. chronic diazepam; single clonazepam vs. chronic clonazepam; single Ro 5-4864 vs. chronic Ro 5-4864) were assumed to be relevant for the analysis.

RESULTS

The binding parameters for [^{3}H]Ro 5-4864 found in mitochondrial membrane fraction from cortices are reported in Table 1.

The B_{max} values were 657 $\pm$ 73 (means $\pm$ SEM) fmol/mg protein in control rats and 771 $\pm$ 59 fmol/mg protein in vehicle-treated rats. The overall ANOVA of the B_{max} values showed significant differences, $F(7, 37) = 11.65$, $p < 0.00001$; individual comparisons demonstrated significant differences from both vehicle-treated and acutely injected groups in animals injected for 5 days with diazepam and Ro 5-4864, but not with clonazepam (Table 1). A trend toward an increase of B_{max} values was found in animals receiving single injection of diazepam and Ro 5-4864; however, these changes did not attain statistical significance when compared to vehicle-treated animals (single Ro 5-4864, $p = 1.37$, ns; single diazepam, $p = 2.15$, ns) (Fig. 1).

An increased affinity for [^{3}H]Ro 5-4864 is seeming in cortices from animals subjected to repeated administration of

TABLE 1

EFFECTS OF CHRONIC BENZODIAZEPINES ON [^{3}H]Ro 5-4864 BINDING PARAMETERS IN THE MITOCHONDRIAL FRACTION OF RAT CEREBRAL CORTEX

Drugs				
Days 1–4	Day 5	*n*	B_{max}(fmol/mg protein)	K_d (nM)
–	–	6	657 $\pm$ 73	3.27 $\pm$ 0.67
Vehicle	Vehicle	6	771 $\pm$ 59	4.07 $\pm$ 0.69
Vehicle	Diazepam	6	891 $\pm$ 90	5.17 $\pm$ 1.00
Vehicle	Clonazepam	5	791 $\pm$ 57	3.89 $\pm$ 0.51
Vehicle	Ro 5-4864	5	922 $\pm$ 46	4.51 $\pm$ 0.30
Diazepam	Diazepam	6	390*‡ $\pm$ 90	2.72 $\pm$ 0.61
Clonazepam	Clonazepam	5	728 $\pm$ 52	3.81 $\pm$ 0.64
Ro 5-4864	Ro 5-4864	6	269†‡ $\pm$ 53	1.45 $\pm$ 0.32

B_{max} and K_d values are expressed as mean $\pm$ SE. *n* = number of rats.
*$p < 0.01$; †$p < 0.001$, significantly different from vehicle group.
‡$p < 0.0001$, significantly different from single-treated groups.

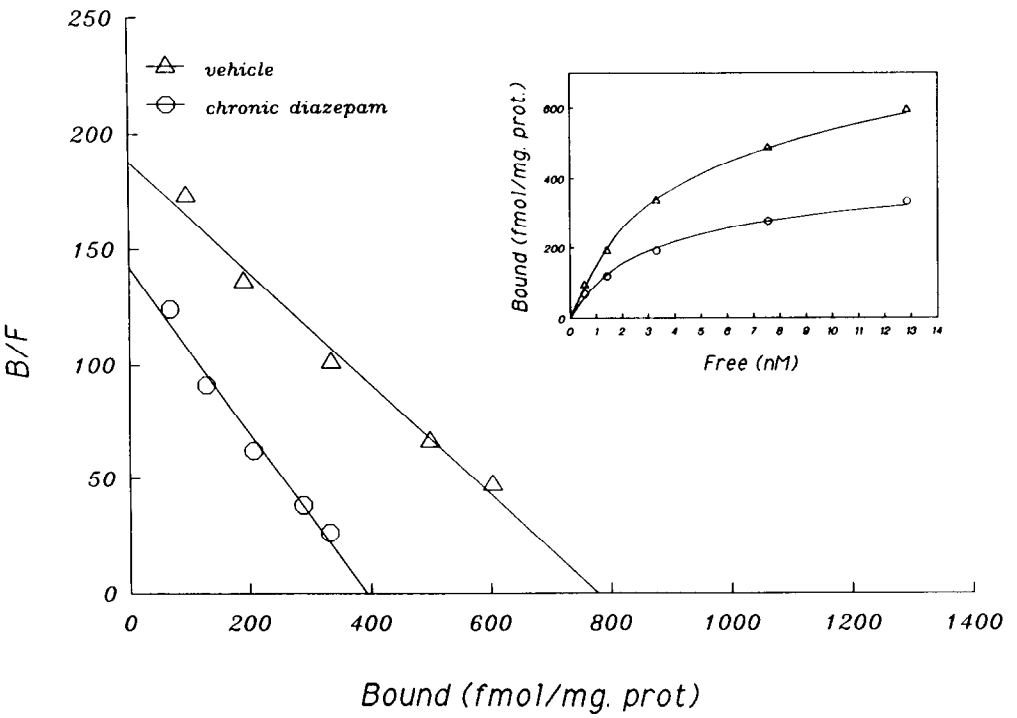

FIG. 1. Saturation curves and Scatchard plot analysis of [^{3}H]Ro 5-4864 binding (0.55–12.83 nM) in the cortex of animals treated with repeated administration of diazepam or vehicle. Each point is the mean of six experiments performed in triplicate. The r values of the linear regression analysis were the following: vehicle = 0.99; diazepam = 0.98. Note the decrease in the B_{max} value after repeated administration of diazepam.

diazepam and Ro 5-4864. However, despite the high F value to the ANOVA, $F(7, 37) = 3.26$, $p = 0.0086$, individual group comparisons with Bonferroni's correction showed that in no case the modification of K_d values attained statistical significance when compared with vehicle-treated animals; the reduction of the K_d values in the Ro 5-4864 chronic group only approached it ($p = 0.055$).

From the behavioral point of view, on day 1 and in single-dosed groups diazepam and clonazepam immediately induced marked sedation. Clonazepam-injected animals also exhibited wet-dog shakes. Rats injected with Ro 5-4864 showed clonic head jerks. On day 5, rats subjected to repeated diazepam administration were only mildly or not at all sedated. In contrast, animals receiving chronic clonazepam and Ro 5-4864 showed the same effects observed on day 1. However, the onset of the excitatory effects in Ro 5-4864-treated rats occurred earlier, from 60–90 s on day 1 to 20–40 s from day 2 or 3 to day 5.

DISCUSSION

Previous studies from our laboratory indicate that 5 days of treatment with large and equiactive doses of diazepam, but not clonazepam, can induce signs of EEG and behavioral tolerance. In rats subjected to repeated administration of Ro 5-4864, diazepam elicits an EEG feature similar to that found in tolerant animals (see above).

Comparison of the behavioral and biochemical effects observed on day 5 in our experiments, using the same schedule of treatment previously reported for EEG studies, indicates that the rapid tolerance to the sedative effect of diazepam is associated with downregulation of [^{3}H]Ro 5-4864 binding in mitochondrial fraction from rat cortex. Clonazepam, endowed with a poor binding capacity for peripheral-type binding sites (8), even at very large doses, failed to induce rapid behavioral tolerance or modify [^{3}H]Ro 5-4864 binding.

Our Ro 5-4864 findings appear to confirm the difficulty in understanding the physiological role of peripheral-type binding sites. The downregulation of [^{3}H]Ro 5-4864 binding occurring after repeated administration of Ro 5-4864 agrees with the hypothesis that this drug is a peripheral-type binding site agonist (4). Yet, the downregulation of [^{3}H]Ro 5-4864 binding is not associated to tolerance to EEG spike-and-wave complexes (13) and head jerks observed at the dose of 4 mg/kg Ro 5-4864. These excitatory effects might be due to the drug's ability to bind at t-butylbicyclophosphorothionate sites (15).

Since a single injections of diazepam did not significantly alter peripheral-type binding site density, changes in density after chronic injection of these drugs cannot be ascribed to drug residues in the membrane preparations. Similar results were found after single injection of the selective peripheral-type binding site ligand Ro 5-4864.

Recently, Weizman and Gavish (18) reported that repeated administration of diazepam (0.5 mg/kg daily) for 21 days increases the B_{max} of [^{3}H]PK 11195 (+19%) in crude membrane preparation from rat cerebral cortex. However, their report provided no details about the appearance of tolerance to the sedative effect of diazepam. In a recent article, we reported a significant decrease of [^{3}H]Ro 5-4864 binding and, consistent with the above-reported data (18), a significant in-

crease of [^{3}H]PK 11195 binding in mitochondrial fraction of rat cortices from animals receiving diazepam for 5 days (1). This would suggest that the different ligand used for binding assay might also be an important determinant in view of the demonstration of a peripheral binding site heterogeneity (3).

As in previous studies (13), no convulsions occurred after repeated administration of Ro 5-4864. This failure presumably depended on the speed of drug infusion (0.1 ml/min) since in subsequent experiments after a rapid IV infusion (0.4 ml/min) convulsions occurred on day 1 of treatment (unpublished data).

In conclusion, the present and previous studies (1,13) suggest the possible involvement of peripheral benzodiazepine binding sites in the occurrence of rapid tolerance to the sedative action of these compounds. However, the mechanism through which this effect can occur remains to be elucidated.

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