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Diazepam Sensitizes Mice to FG 7142 and Reduces Muscimol-Stimulated $^{36}Cl^-$ Flux[1]

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LEWIN, E., J. PERIS, V. BLECK, N. R. ZAHNISER AND R. A. HARRIS. *Diazepam sensitizes mice to FG 7142 and reduces muscimol-stimulated $^{36}Cl^-$ flux*. PHARMACOL BIOCHEM BEHAV 33(2) 465–468, 1989.—Chronic treatment with benzodiazepine receptor agonists increases sensitivity to the convulsant action of FG 7142, an inverse agonist. We investigated whether or not changes in the number and function of GABA-gated chloride channels accompanies this increased sensitivity. Diazepam, 5 mg·kg^{-1}, was administered to mice daily for five days, and mice were then tested with a single injection of FG 7142, 40 mg·kg^{-1}, at several intervals thereafter. At 24 hours after the last diazepam dose, 10 of 15 mice had clonic seizures following FG 7142 and four of the remaining five had myoclonic jerks. At 48 hours, only one of six mice developed a clonic seizure, and none were observed in mice tested at 96 or 144 hours. Muscimol-stimulated chloride flux was reduced in cortical synaptosomes from diazepam-treated mice at 24 hours but not at 48 or 96 hours. However, the binding of [^{35}S]TBPS, a ligand closely associated with the chloride channel, was unchanged at 24 hours. These results suggest that a transient diminution in GABA-gated chloride channel function, unaccompanied by a reduction in channel number, may underlie the sensitization to the convulsant action of FG 7142 observed after withdrawal from chronic diazepam treatment.

Benzodiazepine Diazepam β-Carboline FG 7142 Chloride flux TBPS

THE repeated administration of the β-carboline FG 7142, an inverse agonist at the benzodiazepine receptor, results in chemical kindling, i.e., the development of seizures following doses which were initially insufficient to induce convulsant activity (8). The site of action of FG 7142 in causing kindling appears to be the benzodiazepine receptor since concurrent administration of the antagonist Ro 15-1788 prevents kindling (2). Paradoxically, it has been found that repeated administration of benzodiazepine agonists also increases the seizure sensitivity of mice to FG 7142 (5, 7, 9, 14). One explanation of these results is that the inverse agonist precipitates convulsions by inducing benzodiazepine withdrawal. On the other hand, it is possible that repeated administration of agonists or inverse agonists produces similar neurochemical changes which result in increased convulsions.

Recently, we found that both the function and number of $GABA_A$ receptors is decreased in the cortex of mice kindled with FG 7142 (6). This decrease in receptor number may explain the long-term nature of FG 7142 kindling. Therefore, in order to determine if repeated injections of FG 7142 or diazepam increase FG 7142-induced seizures by a similar mechanism, we measured the time course of diazepam "kindling" in addition to changes in muscimol-stimulated Cl^- flux and in the binding of [^{35}S]TBPS, a ligand closely associated with the chloride channel (15).

METHOD

Drug Administration

ICR mice, 6 to 10 weeks of age, were used. Diazepam, 5 mg·kg^{-1}, was injected IP once daily for five days. Control mice were injected with corresponding volumes of the vehicle (40% propylene glycol in water). At intervals after the last diazepam injection, mice were given either FG 7142, 40 mg·kg^{-1} IP, or Ro 15-1788, 2 mg·kg^{-1} or 10 mg·kg^{-1}, and observed for myoclonic jerks and clonic seizures or were sacrificed for measurements of muscimol-stimulated chloride flux or [^{35}S]TBPS binding. FG 7142 was suspended in water containing a drop of Tween 20 at a concentration of 4 mg·ml^{-1}. Ro 15-1788 was also suspended in water with Tween 20 at either 0.2 mg·ml^{-1} or 1 mg·ml^{-1}.

Chloride Uptake

Synaptosomes were prepared from cortices dissected from two

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mouse brains. Synaptosomes were isolated using Ficoll gradient separation as described by Fontaine *et al.* (3). The synaptosomes were removed from the Ficoll gradient, suspended in assay buffer (145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM D-glucose, 1 mM $CaCl_2$ and 10 mM HEPES adjusted to pH 7.5 with Tris base), and centrifuged at 15,000 × g for 15 minutes. The supernatant was removed, and the synaptosomes resuspended in assay buffer for determination and chloride uptake. Muscimol-stimulated $^{36}Cl^-$ uptake was measured using the method of Allan and Harris (1) with the modification that 0.1 mM picrotoxin was included in the quench and wash solutions. The uptake time was three seconds, and the assay temperature was 30°C.

Quantitative Autoradiography of [^{35}S]TBPS Binding

Mice were killed by decapitation. Brains were rapidly removed, frozen in dry ice and stored at −70°C. The method for measuring [^{35}S]TBPS binding has been described in detail elsewhere (6). Briefly, sagittal sections of 30 μm thickness were cut at a level including cortex, hippocampus, and cerebellum. Slide-mounted sections were transferred to slide mailers containing assay buffer (see above) at 25°C for 20 min to remove most of the endogenous GABA. Saturation curves were generated by incubating adjacent brain sections for 60 min in tissue buffer (25°C) containing 10 nM [^{35}S]TBPS (60 Ci/mmol), 0.1 mM bicuculline to inhibit any residual GABA, and one of nine concentrations (0.02–1000 nM) of unlabeled TBPS. Total binding was defined in the absence of unlabeled TBPS. Picrotoxin (0.1 mM) was used to define nonspecific binding. Sections were rinsed for 5 min in ice-cold buffer, followed by a dip in ice-cold distilled water and then dried at 50°C. Autoradiograms of the slide-mounted sections were prepared by exposing the sections, along with ^{14}C-containing plastic standards, to tritium-sensitive film (Hyperfilm, Amersham, Arlington Heights, IL) for 5 days at 25°C (12).

Quantitative densitometric analysis was performed on a Spatial Data systems EyeCom computer system using a nonlinear logistic function to construct the standard curve (11). Protein concentrations in the same brain sections were quantified (10) and binding values were expressed per mg protein. Scatchard transformations were used to calculate the K_d and B_{max} values from the saturation data. Differences between treatment groups were assessed using a two-way ANOVA.

Drugs and Chemicals

Diazepam was a gift of Hoffmann-La Roche (Nutley, NJ), FG 7142 was obtained from Research Biochemicals (Wayland, MA). $^{36}Cl^-$ was purchased from ICN (Irvine, CA), and muscimol from Sigma Chemicals (St. Louis, MO). [^{35}S]TBPS and unlabeled TBPS were from New England Nuclear (Boston, MA).

RESULTS

The seizure sensitivity of mice treated with diazepam to FG 7142 is shown in Fig. 1. At 24 hours, 10 of 15 mice had clonic seizures with loss of posture and four of the remaining five manifested myoclonic jerks. At 48 hours, only one of six mice developed clonic seizures; and no clonic seizures were observed in mice tested at 96 or 144 hours. Four of six mice at both 48 and 96 hours displayed myoclonic jerks but only one of six at 144 hours. Three of 16 vehicle-treated controls had clonic seizures following FG 7142 injection at 24 hours. No controls tested with FG 7142 at the longer intervals exhibited any type of seizure activity.

Ro 15-1788, either 2 mg·kg^{-1} (6 mice) or 10 mg·kg^{-1} (4 mice), was given 24 hours after the last diazepam injection in order to precipitate diazepam withdrawal if diazepam was still present. No myoclonic jerks or seizures were observed in any of these mice.

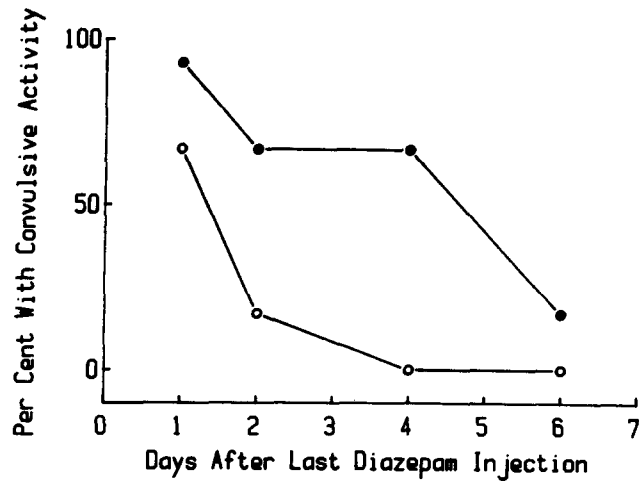


FIG. 1. Effect of FG 7142 in mice treated with five daily injections of diazepam, 5 mg·kg^{-1}. At 24, 48, 96 and 144 hours after the last diazepam injection, mice were given FG 7142, 40 mg·kg^{-1}. Percent of mice manifesting myoclonic jerks with or without subsequent clonic seizures (filled circles) and clonic seizures in addition to preceding myoclonic jerks (open circles) is shown, n = 15 at 24 hours; n = 6 at 48, 96 and 144 hours. The percent of mice having clonic seizures was significantly less at 48 ($p<0.05$), 72 ($p<0.01$), and 96 hours ($p<0.01$) than at 24 hours by chi-square. The percent having myoclonic jerks was significantly less than at 24 hours only at 144 hours ($p<0.01$).

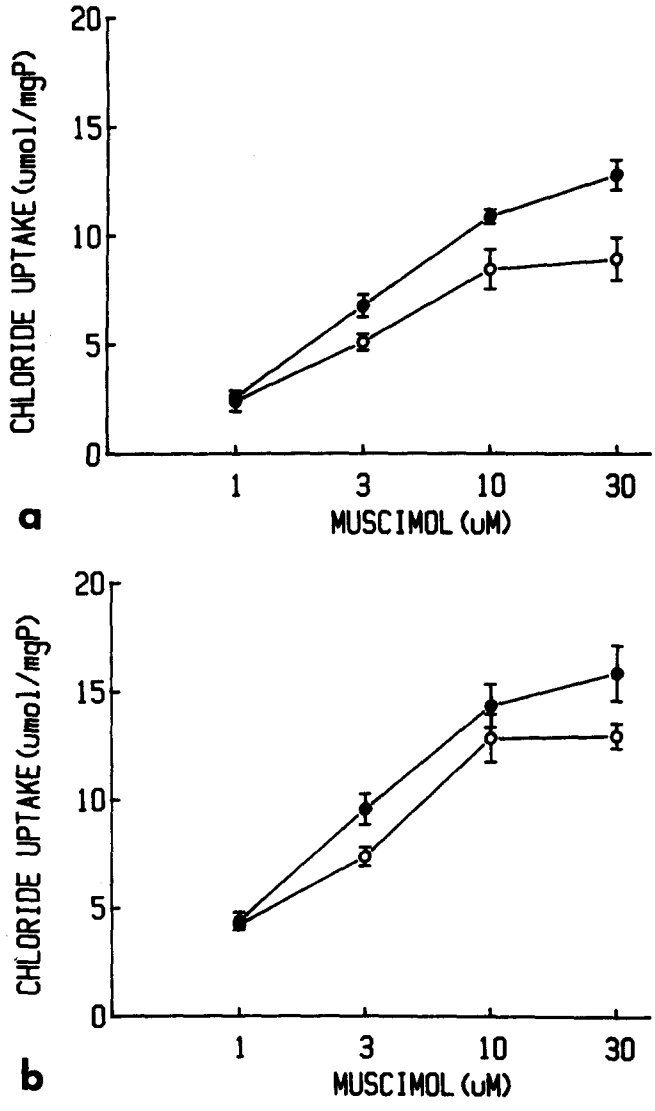
Results of muscimol-stimulated chloride uptake into synaptosomes prepared from the cortex of diazepam-treated and vehicle-injected mice are presented in Fig. 2. At 24 hours after the last of five daily diazepam injections (Fig. 2a), muscimol-stimulated uptake was decreased compared to controls receiving only vehicle. This difference increased at higher muscimol concentrations. At 48 hours (Fig. 2b) and at 96 hours (Fig. 2c) chloride uptake remained slightly lower in synaptosomes from diazepam-treated mice when compared with controls, but the difference was no longer significant.

Parameters of [^{35}S]TBPS binding in five brain areas of mice killed 24 hours after the last of five daily diazepam injections are presented in Table 1. No significant differences in either number or affinity of these binding sites between diazepam-treated mice and vehicle-injected controls were found in any of the regions studied.

DISCUSSION

Mice given diazepam for five days developed myoclonic jerks and clonic seizures when FG 7142 was injected 24 hours later. This seizure activity did not appear to result from the induction of diazepam withdrawal by the inverse agonist, as the benzodiazepine receptor antagonist, Ro 15-1788, did not induce seizures. Furthermore, brains from rats receiving diazepam, 5 mg·kg^{-1}, daily for three weeks were found not to contain significant concentrations of either drug or its metabolites when measured 24 hours after the last dose (4), and, therefore, a benzodiazepine receptor ligand could not induce diazepam withdrawal.

The sensitivity to FG 7142 in diazepam-treated mice diminished rapidly with time. This loss of sensitivity was in contrast to that produced by repeated injections of FG 7142, which persists indefinitely. Thus, although both drugs act on benzodiazepine



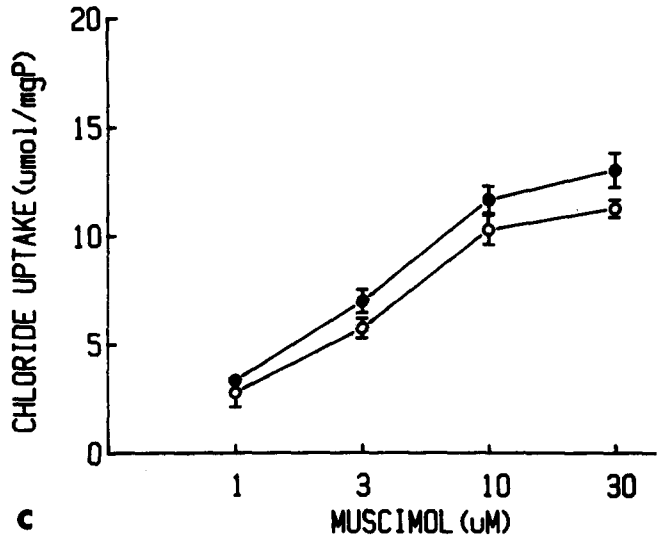


FIG. 2. Muscimol-stimulated $^{36}Cl^-$ flux into cortical synaptosomes of mice treated with five daily injections of diazepam, 5 mg·kg^{-1} (open circles) or vehicle (filled circles). Mice were sacrificed at 24 hours (a), 48 hours (b), and 96 hours (c) after the last diazepam injection. Each point is the mean ± S.E.M. of values obtained from paired cortices; n = 5 at 24 hours and 6 at 48 and 96 hours. At 24 hours the two curves are significantly different as determined by ANOVA for repeated measures. $F(1,8) = 9.24$, $p < 0.02$.

receptors, the mechanism by which they increase seizure susceptibility must differ in some way. In accordance with this postulate, muscimol-stimulated $^{36}Cl^-$ uptake was significantly diminished in cortical synaptosomes of diazepam-treated mice only at 24 hours after cessation of treatment, the time of maximal sensitivity to FG 7142, in contrast to the longer-lasting decrease found after FG 7142 kindling (6). Moreover, there was no evidence of a decrement in the number of convulsant binding sites in the cortex of diazepam-treated mice as opposed to that found in FG 7142-kindled animals (6). Thus, although both diazepam and FG 7142 appear to act at the same receptor site to enhance sensitivity to the convulsant effect of FG 7142, diazepam transiently uncouples the GABA response while FG 7142 produces a long-lasting reduction in GABA receptor number.

Generally, repeated exposure of a given receptor to an agonist at that receptor results in uncoupling or down-regulation of that receptor and decreased function mediated by that receptor. In contrast, exposure to an antagonist results in compensatory up-regulation of receptor number or function. This effect is thought to be a compensatory mechanism invoked to combat the chronic stimulatory action of an agonist or inhibitory action of an antagonist. Since diazepam is an agonist for the benzodiazepine receptor, it acts in a predictable fashion according to this hypothesis and repeated exposure to diazepam decreases $GABA_A$-stimulated Cl^- flux. Because FG 7142 is an inverse agonist and initially inhibits $GABA_A$ receptor function, chronic exposure would be expected to increase receptor number or function, contrary to our previous findings (6). A number of hypotheses may

TABLE 1

BINDING PARAMETERS FOR [^{35}S]TBPS IN BRAIN REGIONS OF MICE 24 HOURS AFTER CHRONIC DIAZEPAM TREATMENT

	B_{max} (pmol/mg protein)	K_d (nM)
Cortex		
Diazepam-treated	1.2 ± 0.11	58 ± 15
Control	1.5 ± 0.27	50 ± 10
Cerebellum		
Diazepam-treated	2.2 ± 0.47	80 ± 18
Control	1.6 ± 0.46	56 ± 16
Hippocampus		
Diazepam-treated	1.2 ± 0.21	74 ± 22
Control	1.7 ± 0.46	64 ± 14
Superior colliculus		
Diazepam-treated	1.7 ± 0.18	77 ± 25
Control	1.9 ± 0.26	58 ± 10
Inferior colliculus		
Diazepam-treated	1.8 ± 0.37	80 ± 32
Control	2.3 ± 0.41	59 ± 10

Values are means ± S.E.M.; n = 6 for control and 5 for diazepam-treated mice. Mice were killed 24 hours after the last of five daily injections of diazepam, 5 mg·kg^{-1}, or vehicle.

be proposed as to why both benzodiazepine agonists and inverse agonists decrease receptor number and/or function. The simplest explanation is that FG 7142 has a toxic effect on cells possessing $GABA_A$ receptors and that the long-lasting decrease in receptor function is directly caused by the loss of receptors due to cell loss. Since the action of an inverse agonist ultimately results in increased cell depolarization, it is possible that FG 7142 and other inverse agonists may act similarly to kainic acid to cause cell death. On the other hand, the concept of inverse agonism is a relatively new one in pharmacology and neurochemical responses to inverse agonists have not yet been completely characterized. Thus, there is really no firm basis for the idea that repeated exposure to inverse agonists should have similar results as repeated exposure to antagonists and opposite results as repeated exposure to agonists.

Other studies have also found effects of repeated administration of benzodiazepines on receptor number and function. Two recent reports have included data on GABA-gated chloride flux in benzodiazepine tolerant animals. Yu *et al.* (16) found an increase in GABA-gated chloride flux in microsacs from the brains of rats given flurazepam in their drinking water for four weeks and then withdrawn for 12 hours before sacrifice. In rats studied 48 hours after withdrawal, no change was found. Miller *et al.* (13) observed a decrease in muscimol-stimulated chloride flux at 1 day but an increase at 4 days in mouse cortical synaptoneurosomes following removal of Alzet pumps containing lorazepam. In addition, the B_{max} of [^{35}S]TBPS binding to cortical membranes was reduced at one day but increased at 4 days, paralleling their chloride flux data. The differences in these results from ours may relate to dissimilar means of inducing tolerance, tissue preparation, and methodology. Also, these studies did not report seizure sensitivity at the time points used for neurochemical measures. The results in the present study and our previous report (6) demonstrate a clear temporal relationship between sensitivity to FG 7142-induced seizures and decreased GABA-gated chloride flux, regardless of whether seizure sensitivity is enhanced by a benzodiazepine agonist or inverse agonist thereby suggesting a causal relationship between seizure sensitivity and chloride channel function.

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