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Tofizopam Modulates the Affinity of Benzodiazepine Receptors in the Rat Brain

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SAANO, V. AND A. URTTI. *Tofizopam modulates the affinity of benzodiazepine receptors in the rat brain.* PHARMAC. BIOCHEM. BEHAV. 17(2) 367–369, 1982.—Tofizopam, a 3,4-benzodiazepine, lacks the sedative action common to 1,4-benzodiazepines, but has anxiolytic activity. In this study we administered tofizopam (50 mg/kg) to rats perorally twice a day for six days, and analyzed the binding of [^{3}H]flunitrazepam to benzodiazepine receptors of these drug-treated rats. The effect of tofizopam treatment was compared to that brought about by treatment with diazepam (12 mg/kg twice a day for six days) and to binding in controls treated with vehicle. Compared to the controls, the diazepam group had a marked decrease in binding of [^{3}H]flunitrazepam to benzodiazepine receptors both in the forebrain and in the hindbrain. As a result of the increased affinity of the receptors tofizopam slightly, but statistically significantly, enhanced binding. With both drugs the number of receptors was unaltered. The effect of tofizopam in the hindbrain was similar to that in the forebrain. The results of this study support our earlier finding from single-dose studies that tofizopam acts indirectly on benzodiazepine receptors.

Tofizopam Diazepam Benzodiazepine receptors

TOFIZOPAM, a 3,4-benzodiazepine, has been shown to have an anxiolytic effect [4, 10, 17]. In contrast to 1,4-benzodiazepines (e.g., diazepam, flunitrazepam, etc.), tofizopam does not cause sedation or muscle relaxation [12, 15, 17]. The therapeutic potency of various 1,4-benzodiazepines correlates positively with their ability to displace radiolabeled benzodiazepines from benzodiazepine receptors in the central nervous system [8]. Tofizopam does not displace tritiated flunitrazepam from receptors, but *in vitro* enhances the receptor-specific binding of flunitrazepam to rat brain and *in vivo* has been found to enhance this binding in rats after acute treatment [14]. The aim of the present study was to analyze the effects of tofizopam on benzodiazepine receptors after prolonged treatment. A small group of rats was treated with diazepam so that we could compare the effects of tofizopam to those caused by a 1,4-benzodiazepine; diazepam has been shown to occupy benzodiazepine receptors during acute and chronic treatment to the extent that is easy to identify *in vitro* using homogenates of brain tissue from drug-treated animals [6,11].

METHOD

Twenty-three male rats of inbred strain (BD IX/Kuo) weighing 220–340 g were housed in opaque plastic boxes under standard laboratory conditions: 10 hr dark/14 hr light cycle, air temperature 20±0.5°C, relative air humidity 55–75°. Before and during drug treatment, animals had free access to food and tap water.

Tofizopam and diazepam were suspended in 1% solution of Tween 80/water and were administered through a stomach tube in a volume of 5 ml/kg body weight. Drugs (or vehicle to controls) were administered twice a day at 12 hr intervals. Each dose of tofizopam was 50 mg/kg; each dose of diazepam was 12 mg/kg. [^{3}H]flunitrazepam was used as a ligand; it had a specific activity of 86.4 Ci/mmole and was purchased from New England Nuclear, Boston, MA. Tofizopam, diazepam, and non-radioactive flunitrazepam were generous gifts from the Research Center of Farmos Group, Turku, Finland.

Drug treatment continued for six days. Rats were decapitated 2 hr after they had received the last dose of drug. After decapitation the brain was removed immediately and the forebrain was separated from the hindbrain by an incision posterior to the occipital lobes and across the brain stem. The hindbrain (cerebellum + pons and medullar oblongata) and the forebrain were homogenized individually in 32 volumes of ice-cold Tris-HCl buffer (50 mM, pH 7.4). Aliquots of homogenate (400 μl) were added to a set of duplicate test tubes containing 6–9 concentrations of [^{3}H[flunitrazepam (range from 0.06 to 8.12 nM). For determination of nonspecific binding another set of tubes also contained 10 μM concentration of non-radioactive flunitrazepam. The tubes were incubated at 4°C for 30 minutes before the contents were filtered through Whatman GF/B filters which were then placed in 6 ml of Aquasol® scintillation liquid for at least 16 hr, after which the radioactivity was measured. The protein content of each homogenate was determined using the method described by Lowry *et al.* [7]. Maximum binding and the dissociation constant for [^{3}H]flunitrazepam binding were determined by Scatchard analysis as described by Bennett [2]. Statistical significance of the differences between values from drug-treated rats and those from control rats was tested using Student's *t*-test.

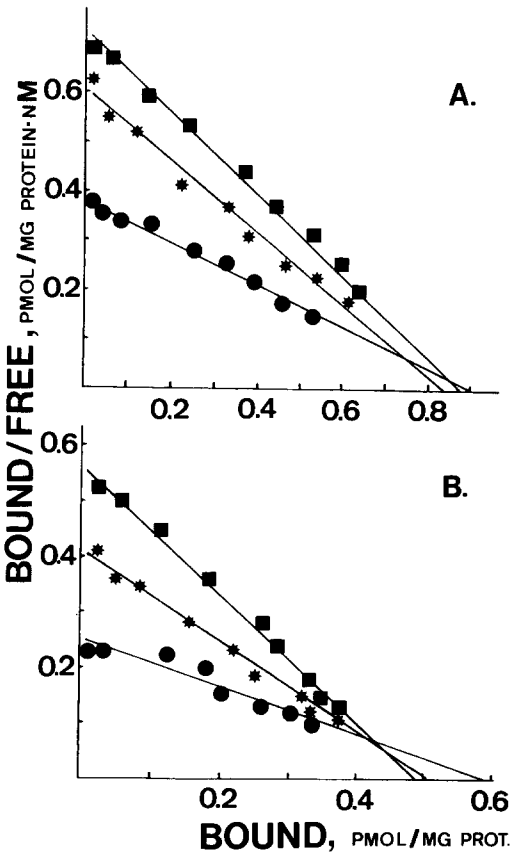

FIG. 1. (A). Scatchard analysis of specific [^{3}H]flunitrazepam binding in the forebrain of rat treated for six days with (squares) tofizopam or (dots) diazepam. Stars indicate binding in control rat. A linear correlation coefficient (r) of the plots was determined by linear regression: tofizopam, r=0.993; diazepam, r=0.978; control, r=0.967. (B). Scatchard plots of [^{3}H]flunitrazepam binding in the hindbrain of rats. Methods and symbols as in A. Tofizopam, r=0.985; diazepam, r=0.855; control, r=0.990. Results are typical examples from a series of experiments, see Table 1.

RESULTS

In both the forebrain and the hindbrain similar changes in binding could be seen (Table 1). Treatment with diazepam increased K_d values, indicating occupation of benzodiazepine receptors; and treatment with tofizopam lowered K_d values showing increased affinity for [^{3}H]flunitrazepam. Both drugs left the B_{max} values unaltered; the number of binding sites was not changed by treatment.

Scatchard analyses produced linear, close fitting curves in both the tofizopam group and the control group; with diazepam, however, the fit was poorer for data points from the hindbrain, but the decrease in the specific binding at subsaturating radioligand concentrations can be seen clearly (Fig. 1). Linear correlation coefficients (r) of Scatchard plots were determined using linear regression. For tofizopam group r was $\geq$0.956 (forebrain, n=9 experiments) and $\geq$0.897 (hindbrain, n=11); for controls r was $\geq$0.957 (forebrain, n=9) and $\geq$0.933 (hindbrain, n=9); for the diazepam group r was $\geq$0.968 (forebrain, n=3) and $\geq$0.837 (hindbrain, n=3).

TABLE 1

EFFECTS OF DIAZEPAM AND TOFIZOPAM ON BINDING OF [^{3}H]FLUNITRAZEPAM TO BENZODIAZEPINE RECEPTORS IN THE RAT BRAIN

Brain area and treatment	Maximum binding (fmoles/mg protein)	Dissociation constant (nM)
Forebrain		
controls, n=9	899.8 ± 23.8	1.62 ± 0.10
diazepam, n=3	927.9 ± 27.1	2.78 ± 0.47*
tofizopam, n=9	884.2 ± 34.8	1.13 ± 0.05*
Hindbrain		
controls, n=9	541.0 ± 22.3	1.31 ± 0.19
diazepam, n=3	759.8 ± 126.7	3.78 ± 1.31*
tofizopam, n=11	493.1 ± 24.8	0.88 ± 0.11†

Values are means ± S.E.M. from Scatchard analyses of the binding of [^{3}H]flunitrazepam to benzodiazepine receptors in brains of rats pretreated for 6 days with diazepam (12 mg/kg twice daily), tofizopam (50 mg/kg twice daily), or vehicle (1% Tween 80/water 12.5 ml/kg twice daily). Statistical significance of the differences as compared to controls, Student's t-test: *$p<0.05$, †$p<0.01$; n=number of experiments.

DISCUSSION

In vitro tofizopam slightly enhances the binding of [^{3}H]flunitrazepam to benzodiazepine receptors in the rat brain, but *in vivo* binding is more markedly stimulated after acute treatment [14]. In this study binding was also enhanced slightly; this may, however, reflect significant pharmacological effects. Occupation of only 25–30% of benzodiazepine receptors by diazepam is sufficient to protect from pentylenetetrazol-induced seizures [11], and occupation of 10–20% elicits an anxiolytic effect in the conflict test [6].

Chronic administration of tofizopam seems to cause similar changes in the binding of benzodiazepine receptors as those brought about by acute treatment [14]. Prolonged treatment with tofizopam caused no alterations in the number of benzodiazepine receptors; in fact the residual tofizopam present in the tissues at the time of the analysis (2 hr after the last dose of drug) is probably responsible for the increase in the affinity of receptors which was observed in this study.

The binding of [^{3}H]flunitrazepam in the diazepam-treated rats was markedly lower than the binding in the tofizopam group and in the control group. Consequently the level of radioactivity was low, especially in the hindbrain. This decreased the accuracy of the analysis, resulting in a poor fit for the data points (shown as non-linearity in the Scatchard plot in Fig. 1B) with a tendency of the B_{max} intercept to shift to the right. The Scatchard plots in the control group and in the tofizopam group were always linear, suggesting that radioligand was bound to a single population of benzodiazepine receptors. Recently several reports have been published about the existence of multiple subclasses of benzodiazepine receptors [3, 9, 16]. β-Carboline-3-carboxylic acid ethyl ester has been shown to bind preferentially to a subclass of benzodiazepine receptors that is most abundant or perhaps present exclusively in the cerebellum [9]. In this study we used the hindbrain tissue blocks so that each rat could be analyzed individually with-

out pooling tissues from several animals; use of the cerebellum would have caused difficulties because of the small amount of homogenate available. Therefore on the basis of these results it is not possible to conclude whether tofizopam acts similarly on all putative subclasses of benzodiazepine receptors. When using tritiated β-carboline-3-carboxylic acid ethyl ester as a ligand, we found that *in vitro* and *in vivo* tofizopam failed to enhance or to inhibit its binding to benzodiazepine receptors in rats and mice. Under similar conditions, diazepam was effective in reducing binding (Saano, manuscript in preparation).

Gamma-aminobutyric acid (GABA) receptors and benzodiazepine receptors are closely linked to each other. Endogenous inhibitory protein modulates the sensitivity of the GABA/benzodiazepine receptor complex; 1,4-benzodiazepines compete with this neuromodulator, increasing GABA receptor binding [6]. On the other hand, GABA and GABA-agonist muscimol stimulate the binding of benzodiazepine receptors by increasing their affinity for 1,4-benzodiazepines [6]. The enhancement of binding by tofizopam also seems to result from the increased affinity of the receptors; in this study the number of binding sites was not increased. Similar effects have been reported for pyrazolopyridines, which have anti-anxiety effect, but do not cause sedation or muscle relaxation or protect from convulsions induced by electroshock [1]. Pyrazolopyridines also stimulate the binding of tritiated GABA and muscimol to GABA receptors [13]; pyrazolopyridines may inhibit the action of the inhibitory neuromodulator in the GABA/benzodiazepine receptor complex [1]. Tofizopam enhanced the binding of [^{3}H]muscimol to GABA receptors in the brains of rats and mice to the same extent as diazepam did; enhancement was abolished when frozen, thawed and Tritonized tissue was used (Saano, manuscript in preparation). This treatment has been shown to inactivate the inhibitory protein that regulates the sensitivity of the receptor complex [5].

The results of this study support our earlier view [14] that the effects of tofizopam on benzodiazepine receptors are mediated indirectly, possibly through competition with endogenous modulatory proteins of the GABA/benzodiazepine receptor complex.

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