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EFFECT OF GABAERGIC DRUGS ON BENZODIAZEPINE BINDING SITE SENSITIVITY IN RAT CEREBRAL CORTEX

DOROTHY W. GALLAGER,* JOHN W. THOMAS and JOHN F. TALLMAN
Section on Biochemistry and Pharmacology, Biological Psychiatry Branch, National Institute of Mental Health, Bethesda, MD 20014, U.S.A.

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Abstract—Pretreatment of rats *in vivo* with γ-aminobutyric acid (GABA) agonists and drugs which increase GABA levels in brain leads to an increased affinity of [^{3}H]diazepam binding sites in rat cerebral cortex. These drugs include amino-oxyacetic acid, muscimol, γ-butyrolactone and β-(*p*-chlorophenyl)-GABA; pentobarbital and thiosemicarbazide are without major effect. Bicuculline and picrotoxin (at high concentrations) reverse this increase in [^{3}H]diazepam binding site affinity.

An extensive literature [1–3] suggests that some of the biochemical, behavioral and electrophysiological effects of the benzodiazepines (BZ) can be attributed to their actions on γ-aminobutyric acid (GABA) mechanisms. In this laboratory [4] a synergistic effect between GABA and BZ has been observed electrophysiologically. In fact, the BZ were shown to have electrophysiological effects only in the presence of an activated GABAergic system. Thus, pretreatment with an inhibitor of GABA catabolism (amino-oxyacetic acid, AOAA) markedly increased, and a GABA antagonist (picrotoxin) reversed, the effects of BZ in this system.

Direct binding studies using [^{3}H]diazepam have indicated that a specific high-affinity binding site may be relevant to the pharmacological actions of BZ in brain [5, 6]. These results obtained in receptor binding experiments did not support a direct interaction between BZ and GABAergic systems [5, 6]. However, we have recently obtained evidence to suggest that, *in vitro*, the binding of BZ is enhanced in the presence of GABA and GABA agonists and decreased by GABA antagonists [7]. Since this effect is obtained at relatively high (5×10^{-6} M) concentrations of GABA and related GABAergic drugs, we wished to test whether this mechanism has pharmacological relevance *in vivo*. To examine this question, we pretreated animals with AOAA (previously shown to raise GABA levels [8] and to enhance the electrophysiological effects of BZ [4]) to see if this pretreatment had any effect on BZ binding.

In this paper, we demonstrate that pretreatment with AOAA does enhance BZ binding and we show evidence that this effect may be correlated directly to the *in vitro* and electrophysiological observations reported previously [4, 7]. Thus, this study supports a direct interaction between BZ binding and GABAergic mechanisms.

METHODS

Drugs and experimental animals. In these experiments, male albino Sprague–Dawley (ARS, Madison, W) rats (220–300 g) were used as experimental animals. Animals were injected with drugs intraperitoneally 1 hr prior to killing. When GABA antagonists (picrotoxin and bicuculline) were administered, an initial injection was given 15 min prior to administration of other drugs and at 15-min intervals until death 75 min after the first injection. Drugs were prepared as a solution in distilled water or saline (AOAA), titrated to pH 6.5 to 7, and injected in a volume of 0.2 ml/100 g of body weight. Sodium pentobarbital (Abbott Laboratories, N. Chicago, IL) was purchased in injectable form and diluted with saline before injection. Other chemicals used in these studies include: amino-oxyacetic acid (AOAA, Sigma Chemical Co., St. Louis, MO); picrotoxin (ICN Pharmaceuticals, Inc., Plainview, NY); bicuculline methiodide (Pierce Chemical Co., Rockford, IL); thiosemicarbazide (Sigma Chemical Co.); [^{3}H]diazepam (39.1 Ci/mmole, New England Nuclear, Boston, MA); γ-butyrolactone (GBL, Sigma Chemical Co.); γ-aminobutyric acid (GABA, Sigma Chemical Co.); muscimol and β-(*p*-chlorophenyl)GABA (Lioresal, Ciba Pharmaceutical Co., Summit, NJ). Rats were killed by decapitation, brains were removed, and the cerebral cortices were dissected rapidly and weighed.

Binding studies. The cortices were homogenized in 10 vol. (w/v) of 0.05 M Tris–HCl buffer (pH 7.5) at 4°. Aliquots of homogenate (100 μl), corresponding to 10 mg cortex (wet weight), were used for each sample. Proteins were determined according to Lowry *et al.* [9]. A typical binding assay contained, in addition to the aliquot of homogenate described above, [^{3}H]-diazepam (1 nM final concn) and 0.05 M Tris–HCl (pH 7.5) buffer to 500 μl. Assays were carried out in quadruplicate and, for each datum point a replicate sample, which included 1×10^{-5} M chlordiazepoxide in addition to the above, was incubated to assess the level of nonspecific binding. Incubations were carried out at 4° for 30 min; after incubation the samples

* Visiting Scientist (IPA- NTE 080778) on leave from Department of Psychiatry, Yale University School of Medicine, New Haven, CT 06508.

Table 1. Effects of amino-oxyacetic acid on [^{3}H]diazepam binding

Treatment	[^{3}H]diazepam bound (fmoles/10 mg tissue)
Control	107.4 ± 2.1
AQAA*	121.3 ± 2.5†
AOAA + 10^{-5} M GABA‡	122.3 ± 3.8†
AOAA + 10^{-4} M bicuculline methiodide‡	80.2 ± 4.2† §
AOAA + 10^{-4} M picrotoxin‡	106.0 ± 1.6§
AOAA + 10^{-3} M picrotoxin‡	90.7 ± 3.7†,§

* Animals (N = 5) were treated with AOAA (40 mg/kg, i.p.) 60 min prior to death, brains were removed and cortical homogenates were prepared and assayed as described in Methods.
† $P < 0.01$ vs control %
‡ Drugs were added *in vitro* to cortical homogenates from AOAA-pretreated rats in concentrations indicated.
§ $P < 0.01$ vs AOAA.

Table 2. Effects of systematically administered GABAergic drugs on [^{3}H]diazepam binding

Treatment*	[^{3}H]diazepam bound (fmoles/10 mg tissue)
Saline	123.7 ± 6.7
Muscimol (3.5 mg/kg)	143.7 ± 3.0†
Lioresal (20 mg/kg)	152.2 ± 2.6‡
AOAA (40 mg/kg)	151.7 ± 5.2†
GBL (450 mg/kg)	150.9 ± 7.9‡
Pentobarbital (25 mg/kg)	132.4 ± 7.6
Thiosemicarbazide (10 mg/kg)	122.8 ± 2.6

* Animals (N = 6) received i.p. injections 60 min prior to death.
† $P < 0.02$, compared to saline controls.
‡ $P < 0.005$, compared to saline controls.

were rapidly filtered through GF/B filters (Whatman Co., U.K.) under low vacuum. The filters were washed with two 7-ml aliquots of Tris buffer and counted in a liquid scintillation counter in 10 ml Aquasol (New England Nuclear). Results are reported as specific diazepam binding, which represents total binding minus binding obtained in the presence of 1×10^{-5} M chlordiazepoxide.

RESULTS

As seen in Table 1, [^{3}H]diazepam binding was increased by 20–25 per cent after pretreatment of the animals with AOAA. As reported previously [7], *in vitro* addition of GABA to the homogenate of control animals increases the specific [^{3}H]diazepam binding. However, the addition of GABA to brain homogenates from AOAA-pretreated animals did not further increase specific [^{3}H]diazepam binding. AOAA added *in vitro* did not alter [^{3}H]diazepam binding [7]. Additionally, when either the GABA antagonist bicuculline (1×10^{-4} M) or the GABA antagonist picrotoxin (1×10^{-3} M) was included in the binding assay of homogenates from AOAA-pretreated rats, the specific binding of [^{3}H]diazepam was decreased below control levels.

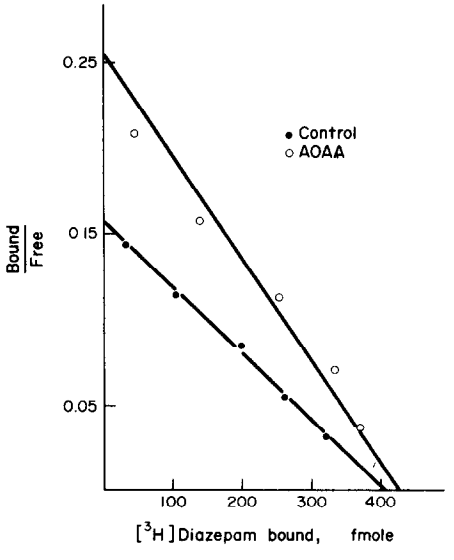

Fig. 1. Scatchard analysis of [^{3}H]diazepam in rats pretreated for 60 min with a single i.p. injection of saline (control) or AOAA (40 mg/kg). Assays were carried out on cortical homogenates as described in Methods except that the concentration of [^{3}H]diazepam was varied as indicated. (Bound/free equals [^{3}H]diazepam specifically bound/total [^{3}H]diazepam added to incubation mixture minus that bound to filters.)

The increase in specific [^{3}H]diazepam binding observed after pretreatment of animals with AOAA can be attributed to a change in the affinity of the BZ binding site for its ligand (Fig. 1). The observed K_D in control animals is 2.7 nM and the total concentration of binding sites was 405 fmoles/incubation. After AOAA pretreatment the observed K_D is 1.7 nM and the total binding was 420 fmoles/incubation. Thus, it appears that the increase in binding is due almost entirely to a change in the apparent affinity of the binding site for its ligand.

The change in the apparent K_D for [^{3}H]diazepam is due to a change in the association rate [k_1 (control) $= 4.7 \times 10^7$ M^{-1} min^{-1}; k_1 (AOAA pretreatment) $= 7.8 \times 10^7$ M^{-1} min^{-1}; Fig. 2b]; the dissociation rate constant was unaltered ($k_2 = -0.133$ min^{-1}, Fig. 2a).

A curvilinear decrease in specific [^{3}H]diazepam binding was observed at increasing temperatures between 4 and 34° (Fig. 3). No evidence for a single inflection point was obtained in this temperature range. In spite of the changes in affinity seen after pretreatment with AOAA *in vivo*, no major differences in the temperature curve could be observed which might reflect a radically altered conformation of the BZ binding site.

Various drugs were administered *in vivo* to examine the specificity of the increased diazepam binding (Table 2). Increases in specific [^{3}H]diazepam binding were observed with the rigid GABA analog, muscimol, and with β-(*p*-chlorophenyl)GABA (Lioresal) and GBL. No significant changes were observed with either thiosemicarbazide (a GABA synthesis in-

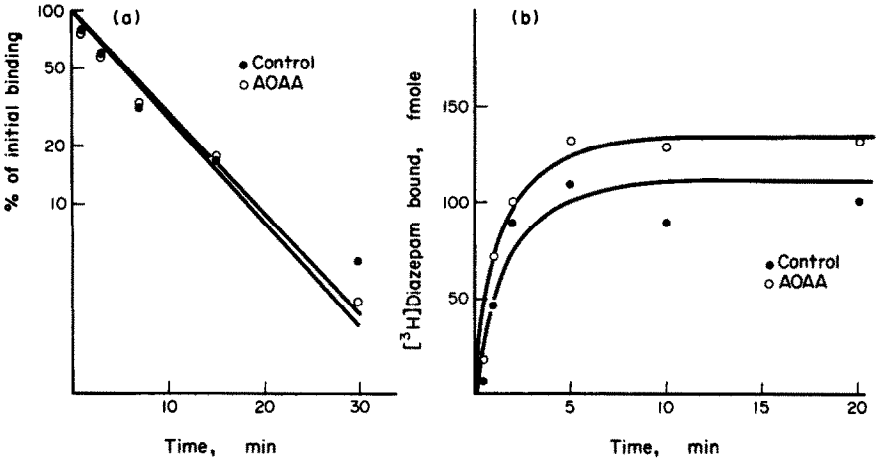

Fig. 2. (a) Dissociation of binding of [^{3}H]diazepam in control and AOAA (40 mg/kg, i.p. 60 min prior to sacrifice)-treated rats. After a 30-min preincubation period of cortical homogenates with [^{3}H]diazepam, the homogenate from either AOAA-treated or control rats was diluted 1:50 and filtered at the times indicated. (Rate of dissociation, $k_2 = -0.133$ min^{-1}.) (b) Association curve for [^{3}H]diazepam binding in cortical homogenates from control and AOAA-pretreated (40 mg/kg, i.p. 60 min prior to death) rats. [^{3}H]diazepam (1 nM) was added and the mixtures were incubated at various intervals. Samples were then filtered and assayed as described. An increase in the rate of association was seen after AOAA pretreatment ($k_1 = 7.8 \times 10^7$ M^{-1} min^{-1} vs control $k_1 = 4.7 \times 10^7$ M^{-1} min^{-1}).

hibitor) or pentobarbitol treatment. In addition, no change in binding was observed after pretreatment of the animals with the GABA antagonists picrotoxin and bicuculline (Table 3).

DISCUSSION

The results reported in these studies suggest that the interaction between GABA and BZ observed *in vitro* [7] and electrophysiologically [4] is relevant to a pharmacological action *in vivo*. Pretreatment of animals with AOAA, an inhibitor of GABA catabolism [8], results in an increase in specific [^{3}H]-diazepam binding. Since the addition of GABA *in vitro* does not further increase the binding in AOAA-pretreated animals and since bicuculline inhibits the increase in binding observed after AOAA treatment, we conclude that the increase in specific [^{3}H]-diazepam binding is mediated by GABA. It also appears from these data that the treatment with

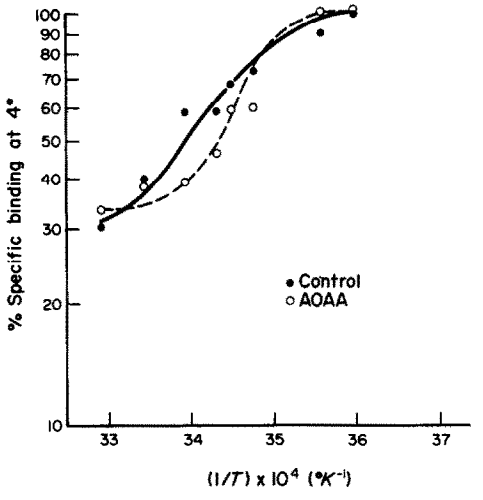

Fig. 3. Temperature dependence for binding of [^{3}H]diazepam was compared in cortical homogenates from control rats and rats pretreated with AOAA (40 mg/kg, i.p. given 60 min prior to death). Homogenates were incubated in a temperature-independent pH 7.4 buffer (0.05 M HEPES) with 1 nM [^{3}H]diazepam for 30 min at various temperatures and specific binding was measured as described in Methods.

Table 3. Effect of systematically administered GABA antagonists on [^{3}H]diazepam binding

Treatment*	[^{3}H]diazepam bound (fmoles/10 mg tissue)
Expt. I	
Saline	130.6 ± 3.7
AOAA	157.9 ± 4.6†
Bicuculline (0.75 mg/kg)	138.0 ± 3.2
Bicuculline + AOAA	154.2 ± 4.5†
Expt. II	
Saline	116.0 ± 3.2
AOAA	136.9 ± 3.2†
Picrotoxin (5 mg/kg)	125.2 ± 2.2
Picrotoxin + AOAA	132.6 ± 3.9†

* Animals (N = 5) received five sequential i.p. injections of the antagonist (0.15 mg/kg of bicuculline methodide, cumulative dose = 0.75 mg/kg; 1.0 mg/kg of picrotoxin, cummulative dose = 5 mg/kg; or saline) 15 min prior to the administration of AOAA (40 mg/kg, i.p.). Rats were killed 75 min after the initial injection (60 min after AOAA) and assayed as described in Methods.

† $P < 0.02$, compared to saline; results of the antagonist + AOAA pretreatment were not significantly different from AOAA treatment.

AOAA elicits a maximal increase in binding since the addition of GABA *in vitro*, or treatment with other GABAergic drugs, does not further increase binding.

As demonstrated by an *in vitro* binding study, pretreatment with AOAA (Fig. 2) alters the affinity of the BZ binding site and this may be able to modulate its response to systemic BZ. However, this is not a direct effect of AOAA since *in vitro* addition of AOAA does not alter [^{3}H]diazepam binding [7]. The increase in affinity of the BZ binding site after AOAA pretreatment is consistent with direct GABAergic modulation of BZ binding affinity observed *in vitro* using washed cortical membranes [7] and in electrophysiological studies in the dorsal raphe nucleus [4]. It is important to note that in this case a change in affinity, rather than a significant change in the number of binding sites, mediates the alteration in pharmacological responsiveness [10–12]. This change in affinity is due to a change in the association, not dissociation, rate for BZ binding. Such a change in affinity might be expected if the GABA binding site were directly coupled to a BZ binding site, or if GABA were an allosteric activator of a BZ receptor, or if the BZ binding site is part of a GABA/BZ/ionophore complex. The results reported here indicate that *in vivo* alterations of GABA levels affect this binding site, suggesting a change in its conformation. If alterations in conformation are occurring, preliminary results reported here do not indicate a major change in the binding sites' temperature profile, which might have reflected major changes in the thermostability of the protein or alterations of its hydrophobic domain. Further investigations are anticipated to demonstrate the molecular basis for this alteration of BZ binding affinity.

To further investigate GABAergic mediation of alterations in BZ binding *in vivo*, several GABA analogues and antagonists, as well as unrelated drugs, were administered to animals. Since GABA itself poorly penetrates the blood-brain barrier [13], drugs reported to alter brain GABAergic mechanisms after their systemic administration were tested in our system.

The most pronounced increases in BZ binding were observed after administration of the GABA analogs: muscimol, β-(*p*-chlorophenyl)GABA (Lioresal) and γ-butyrolactone (GBL). Muscimol, a rigid structural analogue of GABA [14], has potent GABA agonistic properties [15, 16] which are assumed to be a consequence of the structural similarity of this compound to GABA. Electrophysiological responses to muscimol are blocked by bicuculline, suggesting that this compound has a GABA-like post-synaptic action [17]. When given systematically to animals and tested in our system, muscimol was found to increase BZ binding. This effect was similar to the addition of GABA and muscimol to cortical homogenates *in vitro* [7]. Another compound which is structurally related to GABA and able to penetrate the blood–brain barrier [18] when administered systemically is β-(*p*-chlorophenyl)GABA (Lioresal). Electrophysiological studies have suggested that although this compound produces a response similar to GABA, it is not antagonized by bicuculline; thus, it does not appear to interact directly with GABA receptors [19, 20]. However, GABA transaminase activity has been shown to be inhibited by the addition of this compound *in vitro* [19]. Since our data indicate that BZ binding is increased by pretreatment of animals with β-(*p*-chlorophenyl)GABA, the results could indicate that the BZ binding site is affected by β-(*p*-chlorophenyl)GABA, which may reflect its conformational similarity to GABA or the ability to the compound to inhibit GABA transaminase, which may be sufficient to raise endogenous GABA levels and to affect BZ binding in a manner analogous to AOAA treatment. However, since both diazepam [21] and β-(*p*-chlorophenyl)GABA [22, 23] have been shown to have therapeutic efficacy in treating certain types of spasticity, one may speculate that the effects of these compounds on the binding of BZ indicate a single site of action for these therapeutic effects. Gamma-butyrolactone is a readily absorbable substance which is rapidly metabolized to γ-hydroxybutyrate, a drug considered to be a possible GABA agonist [24, 25]. In the present studies, an anesthetic dose of GBL was also found to enhance BZ binding. Since recent reports have suggested that there is negligible *in vivo* conversion of γ-hydroxybutyrate to GABA in brain [26, 27], it appears that the enhanced binding may be due to a direct action of this compound rather than by elevating brain GABA levels.

Another anesthetic agent, pentobarbital, showed only a slight tendency to increase BZ binding in our system. It has been reported [28] that barbiturates produce small increases in central nervous system GABA levels, and recent electrophysiological experiments have suggested that barbiturates can potentiate GABA-mediated transmission [29–31]. However, since the barbiturates have also been reported to decrease the post-synaptic effects of glutamic acid and to directly alter membrane potentials in some systems [32], the small enhancement of BZ binding seen in our system may not be related specifically to GABAergic mechanisms.

When thiosemicarbazide, a GABA synthesis inhibitor, was given to rats, no alteration in BZ binding was observed. Soubrie *et al.* [3] have also reported that thiosemicarbazide failed to modify diazepam-induced behavior. However, since GABA synthesis blocking agents have been reported to have only weak effects on GABA levels [33], the decrease in GABA produced by thiosemicarbazide may not have been sufficient to affect BZ binding significantly.

Finally, *in vivo* administration of subconvulsant doses of the GABA antagonists, bicuculline methiodide and picrotoxin, failed to alter BZ binding. Our data suggest that, if either antagonist is present in sufficient concentrations (in our studies, by addition *in vitro*), both bicuculline and picrotoxin can counteract the AOAA-induced increase in BZ binding. Because of alterations in brain GABA levels induced by convulsions (including asphyxia [34] and seizure generation [35]), *in vivo* doses of these convulsants, sufficient to achieve antagonistic levels in brain homogenates (1×10^{-4} M bicuculline, 1×10^{-3} M picrotoxin), could not be tested. However, since the electrophysiological effects of BZ in the dorsal raphe nucleus have been reversed by subconvulsant doses of these antagonists [4], it is possible that subcortical BZ binding is affected at lower doses. Regional

differences in GABA-receptor sensitivities to both bicuculline and picrotoxin have been pointed out in both electrophysiological [35, 36] and GABA-receptor binding [37] studies. As observed previously in our *in vitro* studies [7], the antagonists appear to decrease the affinity of the BZ binding site for diazepam below untreated control levels. This suggests that in the control animal the BZ binding site may already be partially activated. Whether this apparent partial activation reflects the true *in vivo* condition, a post-decapitation change [38] or some other effect is not understood at this time.

In summary, our results demonstrate that many drugs which have been previously shown to significantly alter GABAergic mechanisms *in vivo* affect the binding of [^{3}H]diazepam to a high-affinity binding site in brain. When administered *in vivo*, drugs which increase GABA or behave as GABA agonists increase the specific binding of diazepam. This increase in binding is almost entirely due to a change in the apparent affinity of the binding site (increase in association rate) for its ligand. Thus, our study indicates that the interaction between BZ binding and GABA, observed *in vitro*, has pharmacological relevance *in vivo*. Since many of the drugs shown to be effective in our study have been shown, or have been theorized to be clinically effective in treating certain types of spasticity, it is tempting to speculate that the effects of these compounds on the binding of BZ may indicate a common site of action for these therapeutic effects.

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