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Studies on the Relationship of γ-Aminobutyric Acid-Stimulated Diazepam Binding and the γ-Aminobutyric Acid Receptor

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SUMMARY

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The three binding parameters [^{3}H]muscimol binding, [^{3}H]diazepam binding, and γ-aminobutyric acid (GABA)-stimulated [^{3}H]diazepam binding are compared in various brain areas and in response to various protein and membrane modifying reagents. Comparison of rat forebrain and cerebellum revealed a much higher ratio of [^{3}H]muscimol- to [^{3}H]diazepam-binding sites in cerebellum and less GABA-stimulated [^{3}H]diazepam binding. Both sulfhydryl reducing and alkylating agents reduced [^{3}H]muscimol binding (decreased binding affinity), and increased GABA-stimulated [^{3}H]diazepam binding. Triton X-100-treated membranes display an increase in both the number and affinity of [^{3}H]muscimol-binding sites, no change in basal [^{3}H]diazepam binding, and a decrease in GABA-stimulated [^{3}H]diazepam binding. The observed relationship between [^{3}H]-muscimol-binding sites and the degree of GABA-stimulated [^{3}H]diazepam binding suggests that the high affinity muscimol-binding site is not mediating the GABA enhancement effect.

INTRODUCTION

The benzodiazepines are minor tranquilizers that have profound anxiolytic, anticonvulsant, and muscle-relaxant effects. The relatively recent discovery of a stereospecific, saturable receptor site for [^{3}H]diazepam in brain tissue membrane fractions (1–3) has shed new light on the possible molecular mechanisms underlying the action of these agents. The high correlation between pharmacological potency and receptor binding affinity for a series of benzodiazepines strongly suggests that this binding site is relevant to the mechanism of action of these drugs (2).

The availability of specific benzodiazepine receptors has prompted renewed interest in characterizing the detailed molecular mechanisms involved in the action of these drugs. Recent studies have focused on the isolation and characterization of endogenous ligands for the benzoidiazepine receptor (4). The naturally occurring purines inosine and hypoxanthine have been shown to be low-affinity competitive inhibitors of [^{3}H]diazepam binding (5–7) with "benzodiazepine-like" neurophysiological (8) and pharmacological (9) properties. These studies suggest that endogenous modulators of the benzodiazepine receptor may exist and that defined neurochemical systems may exist that mediate diffuse parameters such as anxiety and convulsant activity.

The potentiation of GABAergic[1] neuronal systems by the benzodiazepines at both the neurophysiological (10–12) and behavioral level (13) indicates that the benzodiazepines may exert their effects by potentiating the synaptic actions of GABA. At the biochemical level, GABA has been shown to stimulate the binding of diazepam to extensively washed synaptosomal membranes (14, 15). GABA agonists such as 3-guanidinopropionic acid, muscimol, and imidazoleacetic acid, also stimulate diazepam binding, whereas the GABA receptor blocker bicuculline methiodate inhibits the stimulation. These observations have suggested that the enhancement of diazepam binding caused by GABA is mediated by the GABA receptor (16).

Recent observations have suggested that GABA stimulation of diazepam binding may not be mediated by those GABA receptor sites characterized by high-affinity [^{3}H]GABA or [^{3}H]muscimol binding. The EC_{50} for GABA stimulation of diazepam binding is high (1 μM) (14, 15) relative to the reported K_D for high affinity (5–10 nM) and the lower affinity, 30–70 nM (17, 18) [^{3}H]GABA or [^{3}H]muscimol binding. This, coupled with the recent report of distinct structural requirements for the stimulation of diazepam binding compared to the inhibition of GABA binding (19), suggests that a novel GABA-binding

[1] The abbreviation used is: GABA, γ-aminobutyric acid.

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site may exist which is coupled to the benzoidazepine receptor.

To date, no studies have been reported where GABA receptors have been modified and the consequent effects on GABA-stimulated diazepam binding studied. Such studies should provide further insights concerning the presence or absence of coupling between high affinity GABA and benzodiazepine receptors. We therefore undertook the present study in which protein and membrane modifying reagents were assessed for their differential effects on each of three parameters; [^{3}H]muscimol binding, [^{3}H]diazepam binding, and GABA-stimulated diazepam binding.

METHODS

Membrane preparation. Fresh rat (male Sprague-Dawley, 100–125 g) forebrain or cerebellum was homogenized in 10 volumes of 0.32 M sucrose by using a Teflon-glass apparatus (eight passes). The homogenate was centrifuged at 1000 × *g* for 10 min and the resulting supernatant centrifuged at 30,000 × *g* for 30 min. The pellet was resuspended in 50 original tissue volumes of 50 mM Tris-chloride buffer (assay buffer), pH 7.3, by using a Brinkman polytron (setting 5, 10 sec) and centrifuged at 30,000 × *g* for 20 min. This procedure was repeated two additional times with the final membrane suspension being frozen at −20°. Prior to use, the membranes were thawed and washed two additional times in 50 volumes of assay buffer. Membranes prepared in this manner were used for both the basal and GABA-stimulated [^{3}H]diazepam-binding assay and the [^{3}H]muscimol-binding assay. Protein determinations were performed according to the procedure of Lowry *et al.* (20).

Membranes utilized in the Triton X-100 experiments were prepared in a similar manner except that after the first three washes the membranes were split into two aliquots. Each aliquot was incubated at 37° for 30 min, one with 0.1% Triton and one without Triton. Each membrane suspension was then washed twice in 100 volumes of assay buffer and used immediately.

In experiments where sulfhydryl reagents were employed, the membranes were incubated at 0° with the indicated concentration of each reagent for 20 min prior to initiation of the receptor-binding assay.

Receptor-binding assays. The [^{3}H]diazepam-binding assay was performed as previously described with slight modifications (2, 6). Each assay contained between 0.15 and 0.25 mg of membrane protein, [^{3}H]diazepam (80 Ci/mM, New England Nuclear Corporation, Boston, Mass.) in assay buffer at a final volume of 0.5 ml. The assay was initiated by the addition of membranes and incubated for 15 minutes in an ice-water bath (0°). Assays were terminated by vacuum filtration using Whatman GF-B filters and four 3-ml washes. The filters were then air dried, mixed thoroughly in 10 ml of Aquasol (New England Nuclear Corporation) and counted by liquid scintillation counting. Single-point binding analysis was performed by using 1.25 nM [^{3}H]diazepam and Scatchard analysis with 1.25–12.5 nM [^{3}H]diazepam. Nonspecific binding was determined by incorporation of 3 μM unlabeled diazepam (courtesy of Dr. William Scott; Roche, Nutley, New Jersey) into the binding assay and routinely represented less than 5% of total binding at 1.25 nM [^{3}H]diazepam.

GABA-stimulated [^{3}H]diazepam-binding assays were performed as described above with the incorporation of 0.5–200 μM GABA (Sigma Chemical Company, St. Louis, Mo.). All of these assays were performed by using 1.25 nM [^{3}H]diazepam. Nonspecific binding was determined by the addition of 3 μM unlabeled diazepam and routinely represented less than 5% of total binding. In most experiments, 0.5, 10, and 200 μM GABA was used, although results for only the 10 μM point are usually presented.

The [^{3}H]muscimol-binding assays contained between 0.2 and 0.3 mg of membrane protein and 4-[^{3}H]muscimol (New England Nuclear Corporation; 17.3 Ci/mM) in assay buffer and a total volume of 0.5 ml. Assays were incubated at 0° for 20 min and terminated by filtration through Whatman GF-B filters. The filters were washed four times with 3 ml of ice-cold 50 mM Tris-chloride buffer, dried, and counted in 10 ml of Aquasol. Single-point binding analysis was performed at 5.84 nM [^{3}H]-muscimol and Scatchard analysis at concentrations ranging from 1.46–14.6 nM or 5.84–146 nM [^{3}H]muscimol. Nonspecific binding was determined by incorporating 10 μM unlabeled GABA in the assay and routinely represented between 3 and 6% of total binding at 5.84 nM [^{3}H]muscimol.

Statistical analysis. Both K_D and B_{max} values were obtained by Scatchard analysis. Statistical evaluation of the data was performed by paired *t*-test analysis (two-tailed). The coefficient of intra-assay variance was 2.4% for all the binding assays. All direct comparisons were made from data generated on a given day with identical membrane preparations. All experiments where statistically significant differences are presented were repeated at least four times, on separate days with the data from each experiment treated as one pair.

RESULTS

The newly available 4-[^{3}H]muscimol was utilized in all the muscimol-binding experiments, since this ligand has a higher specific activity than the methylene-labeled compound and gave higher specific binding. Nonspecific binding was less than 5% with 4-[^{3}H]muscimol at 5.8 nM, whereas it ranged from 10–20% with the methylene-labeled compound. Scatchard analysis results obtained with this ligand indicate that two binding sites are observed in forebrain with affinity constants of 6.5 and 24 nM. Approximately two-thirds of the sites (1060 fmoles/mg of protein) are high-affinity sites and one-third (500 fmoles/mg of protein) are lower affinity sites. These binding parameters are in good agreement with previously published data using the methylene-labeled [^{3}H]-muscimol (17, 18).

The distribution of [^{3}H]muscimol and [^{3}H]diazepam-binding sites in rat forebrain and cerebellum and the degree of GABA-stimulated [^{3}H]diazepam binding is shown in Table 1. The forebrain contains approximately 2-fold more [^{3}H]diazepam-binding sites than cerebellum, whereas the reverse is true for [^{3}H]muscimol-binding sites. These observations are consistent with a previous report (15). GABA-stimulated [^{3}H]diazepam binding is,

TABLE 1

Distribution of [^{3}H]muscimol, [^{3}H]diazepam, and GABA-stimulated [^{3}H]diazepam binding in forebrain and cerebellum

The total number of [^{3}H]diazepam and high-affinity [^{3}H]muscimol-binding sites were determined by Scatchard analysis. GABA-stimulated [^{3}H]diazepam was determined by using 1.25 nM [^{3}H]diazepam and 10 μM GABA. The values presented are the means of four determinations for the B_{max} data (femtomoles/milligram of protein) and eight experiments for the GABA-stimulated [^{3}H]diazepam binding data ± standard deviation. Forebrain and cerebellum GABA-stimulated [^{3}H]diazepam binding were significantly different at $p < 0.005$.

Binding parameter	Forebrain	Cerebellum
[^{3}H]Diazepam (B_{max})	1200 ± 58	570 ± 41
[^{3}H]Muscimol (B_{max})	1060 ± 64	1880 ± 108
[^{3}H]Muscimol/[^{3}H]diazepam	0.88	3.29
% GABA enhancement	61 ± 4	39 ± 3

however, significantly higher in forebrain (61%) compared to the cerebellum (39%), $p < 0.01$.

The effect of the sulfhydryl reducing agent β-mercaptoethanol on the three binding parameters under study is shown in Fig. 1. Both diazepam and muscimol binding were significantly reduced in a concentration-dependent manner by β-mercaptoethanol. The stimulation of diazepam binding by GABA was, however, increased from 58–90% at 90 mM β-mercaptoethanol ($p < 0.005$). Ethanol at concentrations up to 100 mM had no effect on any of the binding parameters (data not shown), indicating that the effects observed were not due to the ethanol moiety.

Other sulfhydryl reducing agents such as glutathione (0–100 mM) and dithiothreitol (0–50 mM) were also tested with very similar results, i.e., a decrease in muscimol and diazepam binding with a significant increase in GABA stimulation of diazepam binding (data not shown). Scatchard analysis of both [^{3}H]diazepam and [^{3}H]muscimol binding in the presence and absence of 90 mM β-mercaptoethanol revealed that the affinity of each receptor was

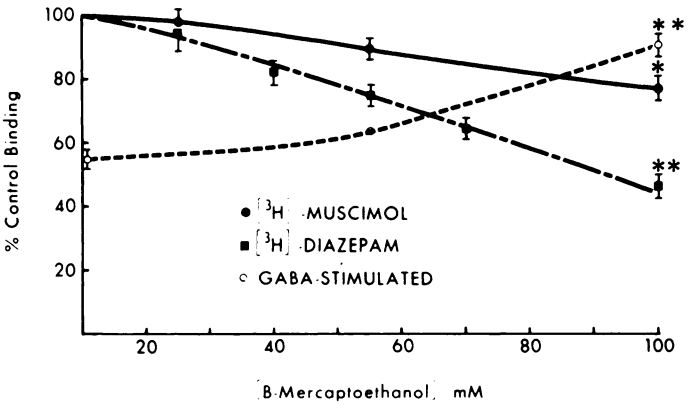

FIG. 1. *Effect of β-mercaptoethanol on [^{3}H]diazepam, [^{3}H]muscimol, and GABA-stimulated diazepam binding*

Synaptosomal membranes incubated with the indicated concentrations of β-mercaptoethanol were assayed for [^{3}H]diazepam binding (1.25 nM [^{3}H]diazepam), [^{3}H]muscimol binding (5.8 nM), and GABA-stimulated [^{3}H]diazepam binding by using 10 μM GABA. The 0 and 100 mM β-mercaptoethanol points are the averages of six separate experiments ± standard deviation. ** Significantly different from control in the absence of β-mercaptoethanol, $p < 0.005$, * $p < 0.05$ paired t-test. One hundred per cent control binding is equal to control binding.

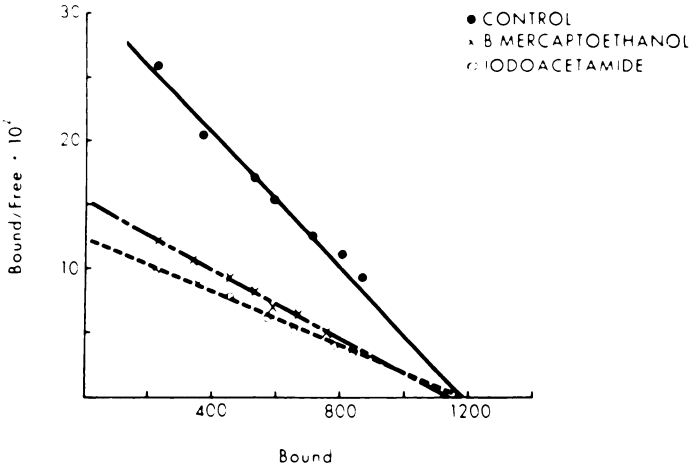

FIG. 2. *Effect of β-mercaptoethanol and iodoacetamide on [^{3}H]diazepam binding (Scatchard analysis)*

Control membranes and membranes incubated with 90 mM β-mercaptoethanol or 100 mM iodoacetamide were titrated for [^{3}H]diazepam binding. Calculated K_D values were 4.0 nM for control membranes, 9.6 nM for β-mercaptoethanol-treated membranes, and 11 nM for iodoacetamide-treated membranes. B_{max} values were 1190, 1150, and 1175 fmoles/mg of protein for control, β-mercaptoethanol-treated membranes, and iodoacetamide-treated membranes. The results represent a typical analysis which was done on four separate occasions with nearly identical results. The standard deviations of the K_D and B_{max} data were in all cases less than 10%.

decreased, whereas the maximum number of binding sites remained unchanged (Figs. 2 and 3).

Similar results to those obtained with β-mercaptoethanol were also generated with membranes that were treated with the sulfhydryl alkylating agent iodoaceta-

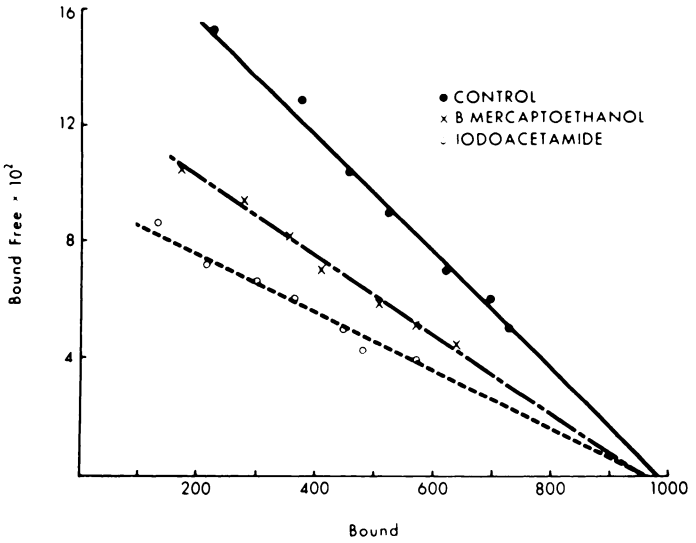

FIG. 3. *Effect of β-mercaptoethanol and iodoacetamide on [^{3}H]muscimol binding (Scatchard analysis)*

Control membranes and membranes incubated with 90 mM β-mercaptoethanol and 100 mM iodoacetamide were titrated for [^{3}H]muscimol binding. A typical plot is shown and the experiment was repeated on three separate occasions with similar results. Calculated K_D values were 6.1 nM for control membranes, 9.1 nM for β-mercaptoethanol-treated membranes, and 11.0 nM for iodoacetamide-treated membranes. The respective B_{max} values were 980, 960, and 965 fmoles/mg of protein. Standard deviations for both K_D and B_{max} data were less than 10%.

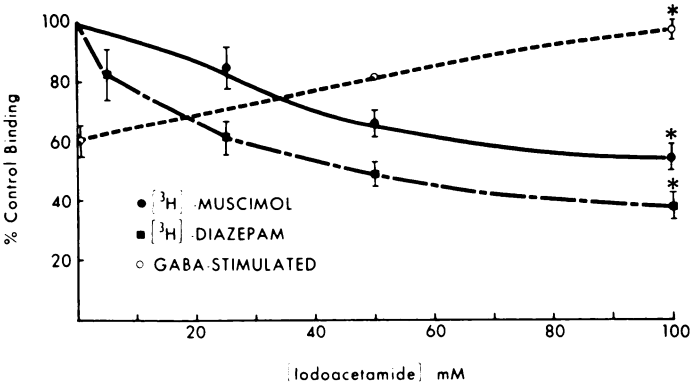

FIG. 4. *Effect of iodoacetamide on [^{3}H]diazepam, [^{3}H]muscimol, and GABA-stimulated [^{3}H]diazepam binding*

Conditions are identical to those described in the legend to Figure 1. The 0 and 100 mM iodoacetamide points are the averages of five separate experiments ± standard deviation. * Significantly different from control, $p < 0.01$. One hundred per cent control binding is equal to control binding.

mide. As shown in Fig. 4, both [^{3}H]diazepam and [^{3}H]-muscimol binding were decreased to 38 and 55% of control, respectively, at 100 mM iodoacetamide, whereas GABA-stimulated [^{3}H]diazepam binding was increased from 60% in the absence of iodoacetamide to 96% in the presence of 100 mM iodoacetamide, $p < 0.01$. Iodoacetamide reduced the affinity of both [^{3}H]diazepam and [^{3}H]muscimol for their respective receptors (Figs. 2 and 3) while having no effect on the total number of available binding sites. It therefore appears that both sulfhydryl reducing and alkylating reagents have similar effects on the three binding parameters.

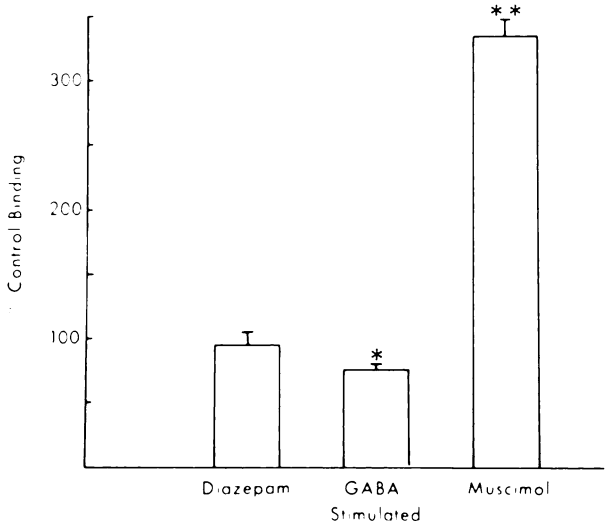

FIG. 5. *Effect of Triton X-100 treatment on [^{3}H]diazepam, [^{3}H]-muscimol, and GABA-stimulated [^{3}H]diazepam binding*

The percentage of control binding of 1.25 nM [^{3}H]diazepam, 4.8 nM [^{3}H]muscimol, and 1.25 nM [^{3}H]diazepam + 10 μM GABA is shown. For Triton X-100-treated membranes compared to control membranes, the actual values were as follows: for control membrane, [^{3}H]diazepam binding = 212 ± 11; GABA-stimulated binding = 339 ± 10; [^{3}H]muscimol = 338 ± 21. For Triton X-100-treated membranes, the respective values are 201 ± 17, 294 ± 8, and 1198 ± 84. All values are fmoles/mg of protein ± standard deviation. The experiment was repeated four times (* $p < 0.05$, ** $p < 0.005$ paired t-test).

Membranes treated with 0.1% Triton X-100 were assayed for each of the three binding parameters. Figure 5 shows that [^{3}H]diazepam binding is not significantly changed, whereas [^{3}H]muscimol binding was increased to 340% of control values at 4.8 mM. GABA-stimulated [^{3}H]diazepam binding was decreased to 70% of control values ($p < 0.01$). The decrease in GABA-stimulated [^{3}H]diazepam binding was also observed at 200 μM and 1 mM GABA concentrations, indicating that the decrease in GABA stimulation observed in Triton-treated membranes is not simply due to a shift in the GABA EC_{50} that has been observed previously (21). A recent report (21) has shown a decrease in EC_{50} for GABA enhancement of diazepam binding with Triton-treated membranes and no apparent decrease in total stimulation. It is, however, difficult to directly compare our results with this study since the membrane preparations are quite different. Scatchard analysis of [^{3}H]muscimol binding to control and Triton X-100-treated membranes revealed that the total number of available binding sites increases from 1050 ± 71 fmoles/mg of protein with the control membranes to 2100 ± 94 fmoles/mg of protein with the Triton X-100-treated membranes. The affinity of the receptor for [^{3}H]muscimol is also increased by Triton X-100 treatment from 7.1 ± 0.6 to 2.1 ± 0.2 nM. Therefore, treatment of the membranes with Triton X-100 apparently exposes a class of high-affinity GABA receptors, a finding consistent with previous reports (22, 17).

Table 2 summarizes the differential relationships observed between the three binding parameters in various brain regions and in response to the protein and membrane modifying treatments employed. Circumstances in which high-affinity muscimol binding was low, i.e., forebrain versus cerebellum, or where the membrane treatments decreased muscimol binding, without exception displayed a higher degree of GABA stimulation of diazepam binding. Conversely, Triton X-100 treatment, which greatly increased muscimol binding, significantly decreased GABA-stimulated diazepam binding.

DISCUSSION

The intimate association of the major inhibitory neurotransmitter GABA with benzodiazepine action has become increasingly clear (19, 23). The benzodiazepines enhance GABA-mediated inhibition in both brain and spinal cord and inhibit convulsions associated with reduced GABAergic function (10–13). The stimulation of

TABLE 2

Relationship of [^{3}H]muscimol, [^{3}H]diazepam, and GABA-stimulated [^{3}H]diazepam binding

Results obtained in the various brain areas and in response to the indicated treatments are qualitatively summarized.

Binding parameter	Effect observed		
	[^{3}H] Diazepam	[^{3}H] Muscimol	GABA enhancement
Forebrain B_{max}	High	Low	High
Cerebellum B_{max}	Low	High	Low
β-Mercaptoethanol treatment	Decrease	Decrease	Increase
Iodoacetamide treatment	Decrease	Decrease	Increase
Triton X-100 treatment	No change	Increase	Decrease

diazepam binding by GABA and its inhibition by GABA receptor antagonists (14, 15) provides biochemical evidence that the GABA and benzodiazepine receptor are functionally associated. In fact, it has been postulated that the GABA and benzodiazepine receptor together with the chloride ionophore exist as a macromolecular complex (24). The recent demonstration that benzodiazepine receptor ligands are more potent as inhibitors of GABA-stimulated diazepam binding compared to basal binding (25) provides additional evidence concerning the importance of GABA in determining benzodiazepine receptor status.

It was initially suggested that the stimulation of diazepam binding observed in response to GABA was mediated by the same GABA receptor studied by [^{3}H]-GABA or [^{3}H]muscimol binding. Recent evidence has, however, raised serious questions concerning the coupling of the classically studied high-affinity GABA receptor with the benzodiazepine receptor. The evidence consists of several observations which include the fact that the EC_{50} for GABA-stimulated diazepam binding is approximately 1 μM (14–16), whereas the reported affinity constants for high and low affinity [^{3}H]GABA or [^{3}H]-muscimol-binding sites range from 5–10 and 20–70 nM, respectively (17, 18, 14, 15). Also, distinct structural requirements for the stimulation of diazepam binding and the inhibition of GABA binding have been reported (19). These reports suggest that a novel form of the GABA receptor which is coupled to the benzodiazepine receptor may exist.

In the present study, the question of whether GABA stimulation of the benzodiazepine receptor is mediated by the [^{3}H]muscimol binding site (GABA receptor) was approached in a different manner. Paradigms were devised in which muscimol receptor sites could be altered and the consequent effect on GABA enhancement of diazepam binding could be studied. Initial studies comparing the ratio of GABA and benzodiazepine receptors in the forebrain and cerebellum with the degree of GABA-stimulated diazepam binding observed in the respective brain areas (Table 1) suggested that the observed stimulation was not mediated by the muscimol-binding site. The ratio of high-affinity muscimol-binding sites to diazepam-binding sites was 0.88 in forebrain and 3.29 in cerebellum, whereas the stimulation of diazepam binding by GABA was significantly greater in forebrain. The reverse situation would be more likely if the muscimol site was in fact mediating the GABA-induced stimulation of diazepam binding. However, this would only be true if a 1:1 relationship existed between these two receptor systems throughout the brain and if the same type of linkage existed at all sites.

Treatment of membranes with either sulfhydryl reducing or alkylating agents (Figs. 1 and 4) produced similar differential effects in the binding parameters, i.e., a decrease in [^{3}H]muscimol binding and an increase in GABA-stimulated diazepam binding. Again, the reverse situation would be expected if the muscimol site was modulating the stimulation effect. The most striking differential effect is that obtained when Triton X-100-treated membranes were compared to control membranes. In this case, [^{3}H]muscimol binding increases while GABA-stimulated [^{3}H]diazepam binding is significantly decreased (Fig. 5). Alterations in both the affinity (sulfhydryl reagents) and the total number (Triton X-100 treatment) of [^{3}H]muscimol-binding sites therefore both failed to produce the changes in GABA-enhanced [^{3}H]diazepam binding that would be expected if the GABA receptor was mediating the phenomenon.

The functional relationship between the benzodiazepine and GABA systems has been amply displayed at both the pharmacological neurophysiological and biochemical levels. However, the evidence presented in this report taken in conjunction with other recent findings indicates that the high-affinity GABA receptor sites characterized by [^{3}H]muscimol binding do not appear to be mediating the GABA enhancement of diazepam binding. The differential effects of various treatments on [^{3}H]muscimol binding and GABA-stimulated [^{3}H]diazepam binding reported here compliment the previous report concerning the differential potency of various GABA receptor ligands in these two paradigms (19). It therefore appears that enhanced diazepam binding elicited by GABA may be mediated by a novel heretofore undescribed GABA recognition site. It is possible that a population of low-affinity GABA sites not readily quantifiable by using presently available techniques exist that modulate the affinity of the benzodiazepine receptor. It should be stressed that the treatments employed in this study are indirect and nonspecific in nature. Sulfhydryl reducing or alkylating agents and Triton X-100 can therefore be expected to effect many different systems. It is therefore difficult to conclude from any of these studies that GABA-enhanced [^{3}H]diazepam binding is not mediated by high-affinity GABA receptor, but only to say that the data are consistent with such an interpretation.

It is also possible that the treatments utilized in this report are effecting a third factor which may serve as a membrane coupling factor between GABA and benzodiazepine receptors. This would, however, fail to explain the different concentrations of GABA required for Na^+ independent binding and the stimulation of [^{3}H]diazepam binding.

The recent preliminary report that solubilized benzodiazepine receptors retain their responsiveness to GABA (26) suggest that the benzodiazepine receptor contains a GABA recognition site. Such a stiuation would be consistent with the data presented in this report especially if this proposed site has a low affinity for GABA. The recent finding that kainic acid treatment has a differential effect on the GABA and benzodiazepine receptor in rat cerebellum (27) also indicates that these two recognition sites, at least in the cerebellum, are not located on the same cells. Further study of the GABA effect on benzodiazepine binding and of the solubilized benzodiazepine receptor should provide information useful in the development of more specific GABAergic ligands of potential therapeutic value. More detailed characterization of the GABA agonists 4,5,6,7-tetrahydroisoxazola(4,5-C)-pyridin-3-OL and isoguvacine which have differential effects at the GABA receptor and as stimulators of [^{3}H] diazepam binding (28) should also prove useful.

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