MOLECULAR PHARMACOLOGY, 25:352–359

Modulation by Calcium of γ-Aminobutyric Acid (GABA) Binding to $GABA_A$ and $GABA_B$ Recognition Sites in Rat Brain

Involvement of Different Mechanisms

MARIA DOROTA MAJEWSKA AND DE-MAW CHUANG

Laboratory of Preclinical Pharmacology, National Institute of Mental Health, Saint Elizabeths Hospital, Washington, D. C. 20032

Received July 29, 1983; Accepted January 19, 1984

SUMMARY

Calcium modulates sodium-independent binding of γ-aminobutyric acid (GABA) to $GABA_A$ and $GABA_B$ recognition sites located in synaptic membranes of rat brain. At 37° the binding of [^{3}H]GABA to the $GABA_B$ recognition site is dramatically stimulated by Ca^{2+} with an EC_{50} (half-saturation constant) of ≃10 μM, whereas the binding to the $GABA_A$ recognition site is only slightly, but significantly, potentiated by Ca^{2+} with an EC_{50} of ≃0.1–1.0 μM. The effect of calcium on $GABA_A$ recognition sites requires a temperature of 37° and the presence of calmodulin. Only $GABA_A$ recognition sites are linked to benzodiazepine recognition sites, and the interaction between these sites is modulated by Ca^{2+} at physiological ion concentrations. When free Ca^{2+} in the assay medium is below 10 nM, only one population of low-affinity $GABA_A$ recognition sites can be measured; however, when free Ca^{2+} is at the micromolar level, or if diazepam is present, a high affinity-binding site appears in addition to the pre-existing low-affinity component. On the basis of affinity there is a single population of $GABA_B$ bindings sites, but the number of sites is about 90% greater at 37° than at 4°. This temperature-dependent increase in the number of $GABA_B$ recognition sites is calmodulin-independent, and data with leupeptin, hemin, and antipain suggest that this temperature-dependent increase in $GABA_B$ sites might involve the activity of Ca^{2+}-dependent protease(s).

INTRODUCTION

Calcium has been shown to be required for ligand binding to a novel class of GABA[1] recognition sites discovered recently in rat brain synaptosomal membranes (1). This Ca^{2+}-dependent binding site for GABA can be labeled with (−)-baclofen and has been termed the $GABA_B$ receptor recognition site in order to distinguish it from the classical $GABA_A$ recognition site, which binds such ligands as bicuculline, THIP, muscimol, and isoguvacine (1). Little is known about this novel $GABA_B$ receptor, except that presumably it is presynaptic (2, 3) and may modulate voltage-regulated Ca^{2+} channels in neurotransmitter release (4). The mechanism whereby Ca^{2+} promotes GABA and baclofen binding to the $GABA_B$ recognition sites is also unclear. Much more is known about the classical $GABA_A$ binding site, which regulates Cl^- channel conductance and is modulated by benzodiazepines through binding to their specific recognition sites (5–8). *In vitro*, GABA enhances the affinity of recognition sites for benzodiazepines in synaptic membranes (9, 10). Conversely, the addition of benzodiazepines increases the density of high-affinity GABA recognition sites (11), and the binding of agonists to these sites is modulated *in vitro* by a heat-stable membrane protein termed GABA-modulin (11).

In view of the fundamental role of Ca^{2+} in cellular function (12) and its involvement in the processes of excitation and transmission in neurons, we have addressed the question of whether this cation may also participate in the inhibitory process mediated by GABA. It was therefore of interest for us to investigate the mechanisms whereby Ca^{2+} regulates the binding of GABA to $GABA_B$ recognition sites. We have also addressed the question of whether Ca^{2+} is involved in the regulation of $GABA_A$ recognition sites and particularly in the interaction between recognition sites of GABA and those of benzodiazepines. Data presented in this report show that Ca^{2+} modulates the binding of GABA to both $GABA_A$ and $GABA_B$ sites but by different mechanisms.

EXPERIMENTAL PROCEDURES

Materials. [^{3}H]GABA (34.7 Ci/mmole) was purchased from New England Nuclear Corporation (Boston, Mass.). (−)-Baclofen was kindly

[1] The abbreviations used are: GABA, γ-amino-*n*-butyric acid; THIP, 4,5,6,7-tetrahydroisoxazolo[4,5-*c*]pyridine-3-ol; EGTA, ethylene glycol bis(β-aminoethyl ether)-*NN,N′,N′*-tetraacetic acid; TFP, trifluoperazine.

0026-895X/84/030352-08$02.00/0

supplied by Ciba-Geigy (Summit, N. J.). Bicuculline methiodide was a product of Pierce Chemical Company (Rockford, Ill.). THIP was kindly supplied by P. Krogsgaard-Larsen (Copenhagen, Denmark). Calmodulin, leupeptin, antipain, hemin, and iodoacetamide were purchased from Sigma Chemical Company (St. Louis, Mo.).

Preparation of crude synaptosomal membranes. Cerebral cortex from male Sprague-Dawley rats (175–200 g) was homogenized at 4° in 15 volumes of medium containing 0.32 M sucrose and 50 mM Tris-citrate (pH 7.4), using a glass-Teflon homogenizer. The homogenate was centrifuged at 1000 × *g* for 10 min, and the postnuclear supernatant was further centrifuged at 48,000 × *g* for 10 min. The pellet was resuspended by Polytron (Ultra-Turrax) treatment for 10 sec and then subjected to hypoosmotic shock in 5 mM Tris-citrate (pH 7.4) for 15 min in ice. Following centrifugation at 48,000 × *g*, the membrane pellet was resuspended in 50 mM Tris-citrate (pH 7.4) and frozen at −20° for 12 hr or longer. Before use, the membrane suspension was thawed and centrifuged. The pellet was resuspended, and the suspension was incubated at 37° for 30 min to facilitate the removal of GABA and endogenous binding inhibitors. The membrane pellets were washed four additional times by centrifugation, twice in Tris-citrate buffer and twice in Tris-Cl buffer (50 mM, pH 7.4). The final membrane pellet was resuspended to give a protein concentration of about 500 μg/ml in the above Tris-Cl buffer (Method I). In some experiments (where indicated), Tris-citrate medium was replaced with Tris-Cl during the entire course of preparation (Method II).

Sodium-independent binding of [^{3}H]GABA to crude synaptic membranes. In order to mimic *in vivo* conditions, all of the binding assays were performed at 37° in 50 mM Tris-Cl (pH 7.4), which was found to be more favorable for the binding of GABA than the Tris-citrate buffer (pH 7.1). Unless otherwise indicated, the binding mixtures in a final volume of 0.5 ml contained 50 mM Tris-Cl (pH 7.4), 20 nM [^{3}H]GABA, about 200 μg of membrane protein, and 0.5 mM aminooxyacetic acid, which was found to be optimal for inhibiting the activity of GABA transaminase. All of the binding ingredients were added on ice. The membrane suspension was incubated in the absence of labeled GABA for 5 min at 37°, and the binding was initiated by the addition of [^{3}H]GABA and was further incubated for 5–10 min at 37°. This period of incubation allowed the binding to both $GABA_A$ and $GABA_B$ recognition sites to reach steady state. The binding reaction was terminated by centrifugation at 48,000 × *g* for 15 min at 4°. The pellet was washed once in 3 ml of ice-cold binding buffer by gentle aspiration and then dispersed in 3 ml of Atomlight (New England Nuclear Corporation) for counting radioactivity. Specific binding was determined by subtracting nonspecific binding, measured in the presence of 1 mM unlabeled GABA, from total [^{3}H]GABA binding. (The IC_{50} value for GABA competing for [^{3}H]GABA binding is about 0.7 μM, and full competition is attained by 0.1 mM GABA.) All assays were performed in triplicate or quadruplicate. To differentiate the binding of [^{3}H]GABA to $GABA_A$ and $GABA_B$ recognition sites, 100 μM (−)-baclofen (to block "B" receptor sites) or 100 μM bicuculline or THIP (to block "A" receptor sites) was included during the preincubation of the membrane preparation as reported previously (1). At the concentrations used, these drugs are selective ligands for their respective receptor sites.

Calculation of free Ca^{2+} content. Free ionized Ca^{2+} in the system was varied by the addition of exogenous EGTA or $CaCl_2$ (or both) to the binding mixtures. Concentration of free Ca^{2+} depends on the dissociation constant of $(EGTA\text{-}Ca)^{2-}$ complex and on the pH of the medium. Complex formation between $(Ca)^{2+}$ and $(EGTA)^{4-}$ may be presented by the following equations:

$$(EGTA)^{4-}(Ca)^{2+} = K_I(CaEGTA)^{2-} \quad (1)$$

$$(CaEGTA)^{2-} = \sum Ca - (Ca)^{2+} \quad (2)$$

$$(EGTA)^{4-} = \sum EGTA - (CaEGTA)^{2-} \quad (3)$$

where K_I is the apparent dissociation constant of $(CaEGTA)^{2-}$ and ∑Ca and ∑EGTA represent the total amounts of these chemicals. The solution for $(Ca)^{2+}$ is:

$$(Ca)^{2+} = \tfrac{1}{2}\left[\sqrt{(\sum EGTA - \sum Ca + K_I)^2 + 4K_I\sum Ca} - (\sum EGTA - \sum Ca + K_I)\right]$$

K_I takes into account the true dissociation constant of this complex ($K = 10^{-11}$ M) as well as formation of the $HEGTA^{3-}$ and H_2EGTA^{2-} in addition to $CaEGTA^{2-}$, which depends on the pH of the system (13). (The formation of $CaHEGTA^-$ is much lower and can be neglected.)

There is the following relationship between K_I and pH of the system (13);

$$pK_I = 2pH - 7.28$$

The chelating capacity of the system decreases exponentially as the ratio of Ca^{2+}/EGTA approaches unity. When Ca/EGTA ≥1, the chelating capacity of EGTA is exceeded and the concentration of Ca^{2+} (endogenously present or added to the system) has to be considered.

The approximate concentration of Ca^{2+} endogenously present in our membrane preparation was assumed to be 5 μM, based on the values measured by absorption spectrometry in synaptosomal membrane suspensions (14) and adipocyte plasma membranes (15).

Protein determination. Protein content was determined according to the method of Lowry *et al.* (16).

RESULTS

Effect of Ca^{2+} on the binding of [^{3}H]GABA to synaptic membrane preparations. The results in Fig. 1 show that at 37° the specific binding of [^{3}H]GABA to crude synaptic membranes of rat brain was increased when a concentration of more than 0.1 mM of exogenous $CaCl_2$ was added to the binding mixture containing 0.25 mM EGTA or when the concentration of free Ca^{2+} was above 22 nM. This increase in the specific binding of [^{3}H]GABA

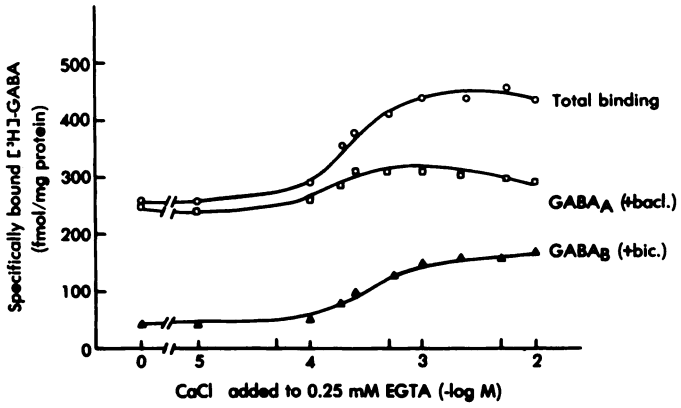

FIG. 1. *Effects of Ca^{2+} on the binding of [^{3}H]GABA to $GABA_A$ and $GABA_B$ recognition sites in brain synaptic membranes*

Membranes were prepared according to Method I, and binding was performed as described under Experimental Procedures except that the binding mixtures contained 0.25 mM EGTA and the indicated concentrations of added $CaCl_2$. Determinations of the binding to $GABA_A$ and $GABA_B$ sites are also described under Experimental Procedures. Results represent means of triplicate determinations from a typical experiment. Free Ca^{2+} concentrations were calculated with the assumption that endogenous Ca^{2+} was 5 μM. When only 0.25 mM EGTA was added, free Ca^{2+} was $\simeq 10^{-9}$ M. With 10 μM exogenous Ca^{2+}, free Ca^{2+} was 2 nM. At 100 μM exogenous Ca^{2+}, free Ca^{2+} was 22 nM. At 200 μM exogenous Ca^{2+}, free Ca^{2+} was 0.14 μM. At 250 μM exogenous Ca^{2+}, free Ca^{2+} was 6.2 μM. When exogenous was more than 250 μM, free Ca^{2+} was the net value of exogenous Ca^{2+} + endogenous Ca^{2+} − EGTA present in the medium. *bacl.*, (−)-Baclofen; *bic.*, bicuculline.

reached a plateau when 1 mM exogenous Ca^{2+} was added (or when the free Ca^{2+} concentration was about 0.76 mM).

The binding of [^{3}H]GABA to $GABA_A$ and $GABA_B$ receptor recognition sites, respectively, was determined by the presence of excess (−)-baclofen (to block $GABA_B$ sites) or bicuculline (to block $GABA_A$ sites). The binding of GABA to $GABA_B$ sites was enhanced in a concentration-related manner up to a maximum of about 400% when the free Ca^{2+} concentrations were increased from 22 nM to 0.76 mM, with an EC_{50} for the Ca^{2+} effect of 10 μM. Under the optimal conditions, the binding to $GABA_B$ recognition sites constituted about 30–40% of the total binding. When the free Ca^{2+} concentration was below 22 nM, almost no binding of [^{3}H]GABA to $GABA_B$ sites was detected.

In contrast, at nanomolar concentrations of free Ca^{2+}, the binding of $GABA_A$ recognition sites was substantial but could be increased by 20–30% when free Ca^{2+} was increased from 22 nM to 10 μM. The concentration of Ca^{2+} required to give half-maximal enhancement of GABA binding to $GABA_A$ receptors was in the 0.1–1.0 μM range. At Ca^{2+} concentrations higher than 1 mM, the binding capacity of $GABA_A$ sites was gradually decreased to the EGTA control levels. It should be stressed that the increase in binding to $GABA_A$ receptors caused by Ca^{2+}, although small, is consistently reproducible.

The effect of diazepam on the binding of [^{3}H]GABA also depends on the concentration of free Ca^{2+} in the binding medium (Fig. 2). When the binding was assayed in the presence of 0.25 mM EGTA, which lowered free Ca^{2+} to nanomolar concentrations, diazepam increased GABA binding by more than 50%. With an increase of free Ca^{2+} in the medium, the over-all specific binding of [^{3}H]GABA was increased, but the enhancement of this binding by diazepam was reduced. When free Ca^{2+} was between 20 nM and 14 μM (or when exogenous Ca^{2+} was between 100 and 200 μM), a concentration which approximates the concentration of this cation during the resting state of a neuron, diazepam was still able to enhance GABA binding by 25–35% of the control values. At levels of free Ca^{2+} higher than 10 μM (or at an exogenous Ca^{2+} concentration of 0.25 mM), the effect of diazepam was lower than 20%; it practically disappeared at 1 mM Ca^{2+}.

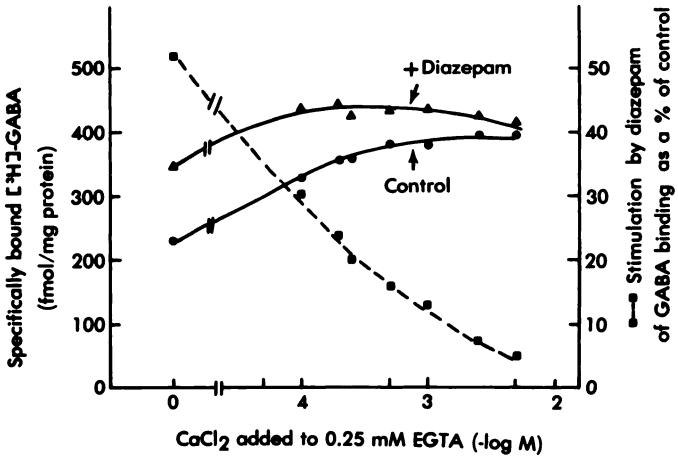

FIG. 2. *Effect of Ca^{2+} on the enhancement of [^{3}H]GABA binding to synaptic membranes by diazepam*

Membrane preparation and binding conditions are as described in the legend to Fig. 1. When used, diazepam was added to the membrane preparation to give a final concentration of 5 μM and was preincubated for 5 min in ice followed by an additional 5 min at 37° prior to the addition of [^{3}H]GABA. Results are means of triplicate determinations from a typical experiment.

Our finding that at 37° a maximal stimulation by diazepam of [^{3}H]GABA binding occurs in the presence of EGTA suggests that most likely $GABA_A$ sites but not $GABA_B$ sites are coupled to the benzodiazepine binding site and that all of the modulation by Ca^{2+} of the diazepam effect is related to this receptor recognition site. This view is further supported by the experimental results shown in Table 1.

When the effect of diazepam on GABA binding was studied in three types of media, the maximal stimulatory effect was seen in the presence of EGTA. The stimulation was less than 20% in the normal medium [which could contain 5–10 μM Ca^{2+} (14, 15)], and the effect was insignificant in the presence of 2.5 mM exogenous Ca^{2+}. When $GABA_A$ sites were selectively blocked by excess bicuculline (or THIP), diazepam failed to affect GABA binding. However, when $GABA_B$ sites were selectively blocked with baclofen, diazepam stimulated GABA binding maximally in the presence of EGTA, and this stimulation was abolished in the presence of 2.5 mM Ca^{2+}. It is of interest to note that, although in the absence of diazepam the GABA binding to the $GABA_A$ receptor was about 30% higher in the normal medium than in the medium containing EGTA, the binding measured in the presence of diazepam was the same in both types of media (Table 1).

Receptor site saturation analysis of the GABA binding to $GABA_A$ recognition sites. To obtain a better understanding of the stimulatory effect of micromolar Ca^{2+} and diazepam on GABA binding to $GABA_A$ recognition sites, the study of equilibrium saturation binding was performed. Scatchard analysis of [^{3}H]GABA binding measured in the presence of EGTA revealed a single class of binding sites with a K_D of 237 nM and a B_{max} of 4.4 pmoles/mg of protein (Fig. 3). The addition of diazepam resulted in the appearance of a high-affinity binding component with an apparent K_D of 33 nM and a B_{max} of 0.45 pmole/mg of protein. The K_D and B_{max} of the low-affinity binding component were similar to those found in the presence of EGTA alone. The appearance of the high-affinity $GABA_A$ binding component upon the addition of diazepam was highly reproducible, with nearly identical results obtained in four separate experiments.

Scatchard analysis of the binding to $GABA_A$ sites in the absence of EGTA (i.e., in the presence of endogenous amounts of Ca^{2+}) (Fig. 4) revealed the existence of two classes of binding sites, one with a K_D of 25 nM and a B_{max} of 0.36 pmole/mg of protein and the other with a K_D of 290 nM and a B_{max} of 4.8 pmoles/mg of protein. In general, the kinetic parameters of GABA binding to $GABA_A$ sites measured in the presence of endogenous Ca^{2+} resembled the values obtained when diazepam was added to the EGTA-containing system. Our binding parameters of B_{max} and K_D values for both high- and low-

TABLE 1

Stimulation of [^{3}H]GABA binding by diazepam: involvement of $GABA_A$ but not $GABA_B$ receptor sites

Membranes were prepared by Method II. Binding conditions are described in the legend to Fig. 1 except that, when used, the concentrations of EGTA and $CaCl_2$ were 1 mM and 2.5 mM, respectively. The concentration of diazepam was 5 μM. Data represent means ± standard error of the mean of three separate experiments.

Experimental condition	Specifically bound [^{3}H]GABA					
	Total	Increase by diazepam[a]	$GABA_A$	Increase by diazepam[a]	$GABA_B$	Increase by diazepam
	fmoles/mg protein	%	*fmoles/mg protein*	%	*fmoles/mg protein*	%
EGTA						
−Diazepam	240 ± 18		235 ± 15		40 ± 5	
+Diazepam	380 ± 21	54**	385 ± 25	63**	45 ± 4	11
Normal medium						
−Diazepam	390 ± 28		305 ± 21		85 ± 10	
+Diazepam	465 ± 30	19*	380 ± 20	24*	84 ± 7	0
Ca^{2+} (2.5 mM)						
−Diazepam	460 ± 27		275 ± 18		190 ± 14	
+Diazepam	475 ± 31	3	285 ± 15	4	175 ± 12	0

[a] * $p < 0.05$ and ** $p < 0.01$ when compared with their controls.

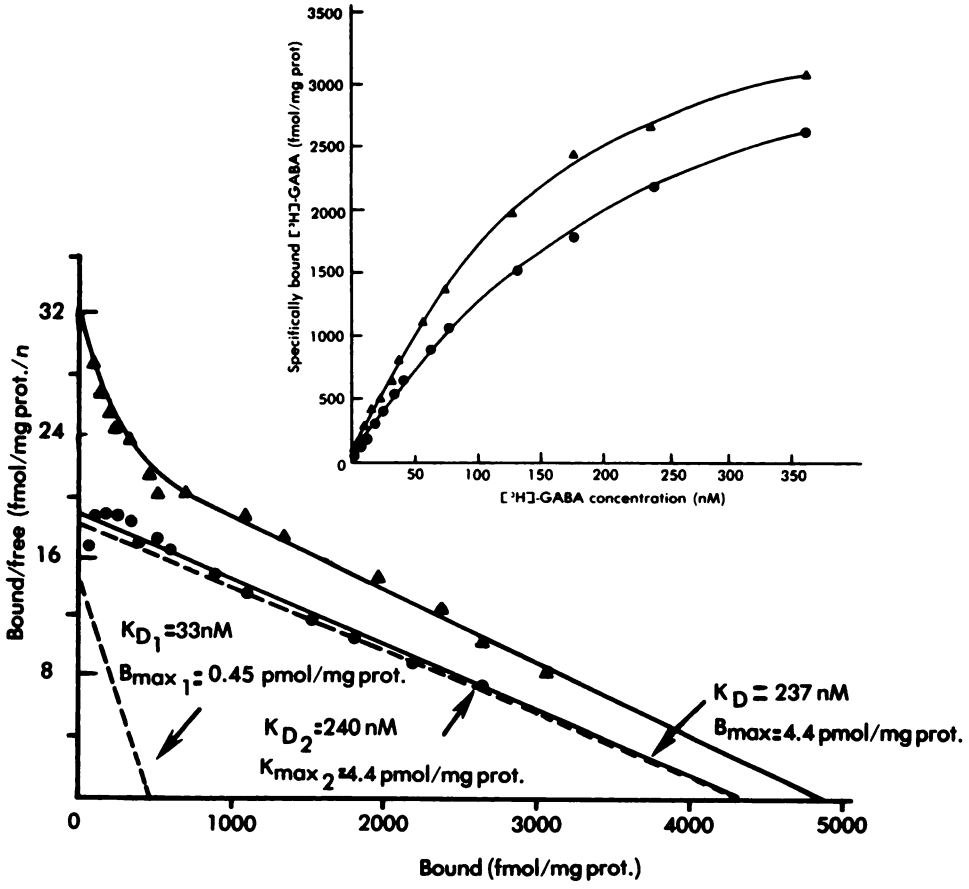

FIG. 3. *Effect of diazepam on [^{3}H]GABA binding in the presence of EGTA: saturation curve and Scatchard analysis*

Membranes were prepared according to Method II, and binding of [^{3}H]GABA was performed as described under Experimental Procedures except that the binding mixtures contained 1 mM EGTA and the concentrations of [^{3}H]GABA were varied from 3 to 360 nM. Each point represents the mean of triplicate determinations. The curve in the Scatchard analysis obtained in the presence of diazepam was best fitted to a two-site model by computer analysis (with $r_1 = 0.98$; $r_2 = 0.99$) and was resolved into two linear components (*broken lines*) using the method of Rosenthal (17). In the presence of EGTA, the data were best fitted to a one-site model with ($r = 0.99$). Results are representative of one of four such experiments.

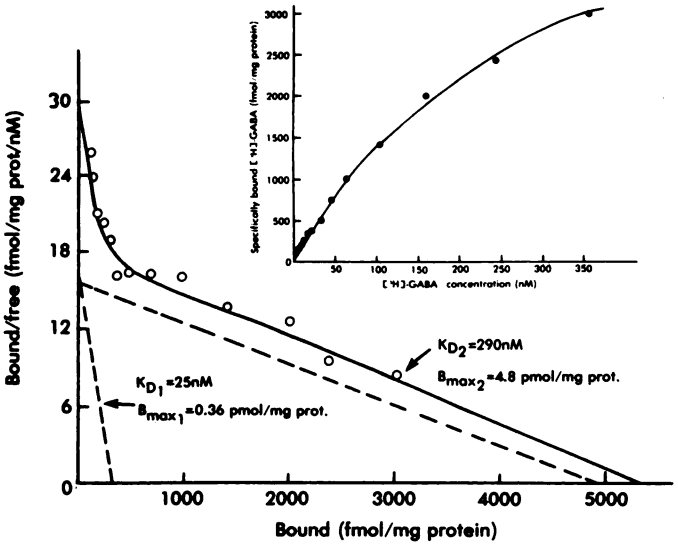

FIG. 4. *Saturation curve and Scatchard analysis of [³H]GABA binding "A" receptor recognition sites in the normal medium*

Membranes were prepared according to Method II, and binding was performed in 50 mM Tris-Cl medium in the presence of 100 μM (−)-baclofen to eliminate the binding of [³H]GABA to $GABA_B$ recognition sites. The Scatchard plot of these binding data fits best a two-site model based on computer analysis (with $r_1 = 0.99$; $r_2 = 0.99$). The curved Scatchard plot was resolved into two linear components as described in the legend to Fig. 3. Each point represents the mean of quadruplicate values. Results are from a typical experiment of four such experiments.

affinity sites are comparable to those reported by others measured at 4° (18, 19). Small differences in the values may reflect differences in experimental conditions such as temperature, membrane preparation, and assay medium.

What is the biochemical basis for the Ca^{2+} effect on [³H] GABA binding to $GABA_A$ recognition sites? The qualitative and quantitative changes in GABA binding to the "A" receptor site elicited by micromolar Ca^{2+} suggest that perhaps calmodulin is involved in the regulation of the high-affinity binding sites, in particular because the EC_{50} value of Ca^{2+} for this effect (0.1–1.0 μM) is similar to the values reported for a number of calmodulin-mediated processes (12). To examine the possibility of calmodulin involvement, the following experiment was performed. Membranes were depleted of endogenous Ca^{2+} and calmodulin by washing the thawed membranes three times with Tris-Cl medium containing 1 mM EGTA in ice followed by two additional washes without the chelator. The effect of micromolar Ca^{2+} on GABA binding was studied using this membrane preparation and compared with the results in the system in which exogenous calmodulin was supplemented. Data from these experiments are shown in Table 2.

The binding of [³H]GABA to $GABA_A$ recognition sites in the presence of 1 mM EGTA was similar to that measured in the presence of 20 μM Ca^{2+} in membranes depleted of calmodulin. The addition of exogenous calmodulin to these calmodulin-deficient membranes increased the binding by about 30% in the presence of 20 μM Ca^{2+} but was without the effect in the presence of EGTA. The calmodulin inhibitor, TFP, at 50 μM abolished the effect elicited by calmodulin and Ca^{2+}, although this drug alone also inhibited nonspecifically the binding to $GABA_A$ sites by about 50% in the absence or presence of Ca^{2+}. Similar effects on binding to $GABA_A$ receptors were observed when pimozid, another calmodulin antagonist, was used; however, various Ca^{2+}-protease inhibitors were without effect (data not shown). Neither calmodulin nor TFP changed the effect of Ca^{2+} on GABA binding to $GABA_B$ recognition sites under these experimental conditions (Table 2).

TABLE 2

Effect of calmodulin on [³H]GABA binding to $GABA_A$ and $GABA_B$ recognition sites in calmodulin-depleted membranes

Membranes were depleted of calmodulin as described in the text. When used, the concentration of calmodulin was 1 μM and that of TFP was 50 μM. Data represent means ± standard error of the mean of three separate determinations.

Experimental condition	[³H]GABA bound		$GABA_A$/$GABA_B$
	$GABA_A$ sites	$GABA_B$ sites	
	fmoles/mg protein		
EGTA (1 mM)			
−calmodulin	214 ± 14	45 ± 4	4.8
+calmodulin	222 ± 12	52 ± 3	4.3
EGTA + TFP			
−calmodulin	135 ± 7	50 ± 4	2.7
+calmodulin	—	—	—
$CaCl_2$ (20 μM)			
−calmodulin	228 ± 8	116 ± 6	1.9
+calmodulin	284 ± 14*	96 ± 8	3.0
$CaCl_2$ + TFP			
−calmodulin	125 ± 6	106 ± 8	1.2
+calmodulin	131 ± 8	101 ± 7	1.3

[a] * $p < 0.01$ as compared with control (20 μM Ca^{2+} in the absence of calmodulin).

The effect of Ca^{2+} on [³H]GABA binding to $GABA_A$ recognition sites was temperature-dependent. The Ca^{2+}-elicited increase in GABA binding was detected only when the binding was performed at 37° but not at 4° (data not shown). Moreover, Scatchard analysis of [³H] GABA done at 4° in the presence of EGTA revealed the presence of both high- and low-affinity binding sites with K_D = 20 nM, B_{max} = 0.38 pmole/mg of protein and K_D = 205 nM, B_{max} = 4.5 pmoles/mg of protein, respectively (data not shown), in contrast to only one low-affinity binding site measured at 37°. These parameters were similar to those found at 37° in the presence of diazepam or endogenous Ca^{2+}. The enhancement of GABA binding by diazepam at 4° was not dependent on Ca^{2+} and was usually no more than 20%. These results indicate the presence of both high- and low-affinity GABA binding sites. In the absence of Ca^{2+} (in the presence of EGTA) at 37°, the high affinity binding sites are eliminated but they are preserved in the medium containing micromolar Ca^{2+} or diazepam.

What is the biochemical basis of the Ca^{2+} effect on [³H] GABA binding to $GABA_B$ recognition sites? In the experiment shown in Fig. 5 we examined the specificity of cations for the activation of GABA binding to $GABA_B$ recognition sites. Among six cations tested at 2.5 mM, only Ca^{2+} was able to increase greatly the binding to

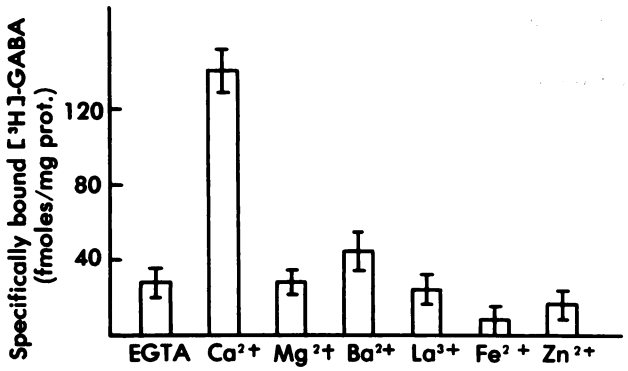

FIG. 5. *Effect of divalent cations on [^{3}H]GABA binding to $GABA_B$ recognition sites*

Binding conditions are described under Experimental Procedures. The concentration of each cation used was 2.5 mM in their chloride forms. Data represent means ± standard error of the mean from three separate experiments.

$GABA_B$ sites. The effect of Ca^{2+} was antagonized by $LaCl_3$ at concentrations higher than 1 mM, whereas verapamil, an organic calcium antagonist, at 0.5 mM had no effect (data not shown).

To examine the possibility that some enzymatic process might be involved in the regulation of $GABA_B$ receptor binding, we compared this binding performed at 4° and 37°. Scatchard analysis of these data shown in Fig. 6 indicated that there was only one class of $GABA_B$ recognition sites on the basis of affinity of GABA for the sites, and there were 90% more recognition sites for $GABA_B$ receptors measured at 37° than at 4°; however, the binding affinities for $GABA_B$ recognition sites were similar at these two temperatures. To study whether this temperature-dependent effect is related to the action of Ca^{2+}-activated enzymes such as phospholipase A_2 or to a Ca^{2+}-dependent protease, inhibitors of these enzymes were studied for their effect on $GABA_B$ receptor binding. Quinacrine and tetracaine, both inhibitors of phospholipase A_2 (20), did not affect the calcium-elicited stimulation of the binding to $GABA_B$ sites (data not shown). Moreover, calmodulin, which is believed to be responsible for the Ca^{2+} activation of phospholipase A_2 (21), is not involved in the $GABA_B$ receptor binding (Table 2). In contrast, inhibitors of Ca^{2+}-dependent proteases such as hemin, iodoacetamide, and leupeptin (22) were potent in the inhibition of Ca^{2+}-dependent GABA binding to "B" sites (Table 3), although antipain was relatively inactive. Scatchard analysis of the binding performed in the presence of leupeptin indicates that its effect was associated with a reduction in the number of $GABA_B$ recognition sites with no change in the receptor binding affinity (Fig. 6). The number of $GABA_B$ recognition sites measured in the presence of Ca^{2+} and leupeptin at 37° was comparable with that measured at 4° without leupeptin. Leupeptin did not affect significantly $GABA_B$ receptor binding at 4°. Similar results of Scatchard analysis were obtained with iodoacetamide at 37° and 4° (data not shown). These results suggest, but do not prove, that a membrane-bound Ca^{2+}-dependent protease may be involved in the regulation of the number of $GABA_B$ receptor sites.

DISCUSSION

In the present study we have provided evidence that Ca^{2+} plays an important role in the *in vitro* binding of

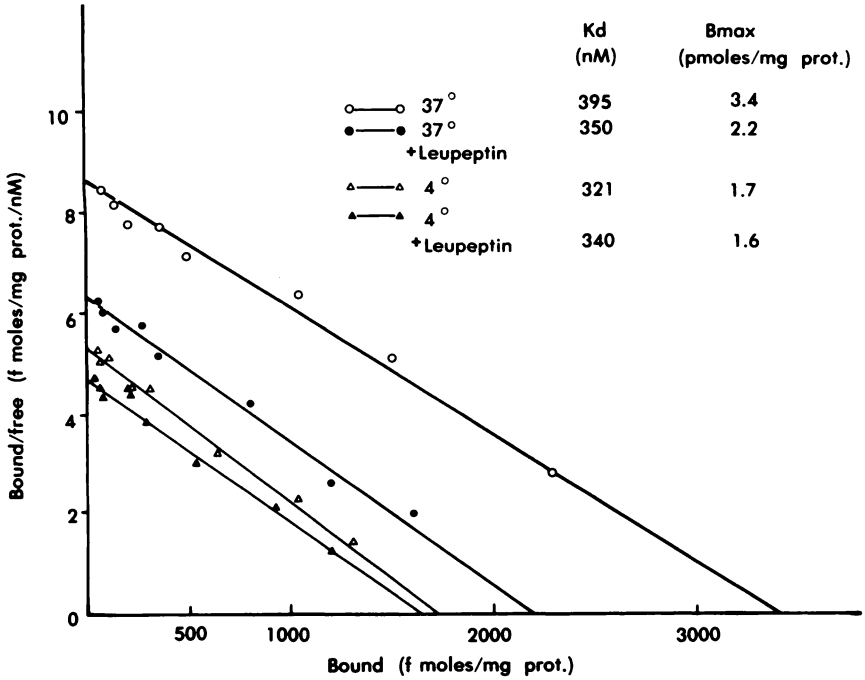

FIG. 6. *Scatchard analysis of [^{3}H]GABA binding to $GABA_B$ recognition sites: effect of temperature and iodoacetamide*

Binding was performed in the presence of 2.5 mM $CaCl_2$ and 100 μM bicuculline (to block $GABA_A$ sites) either at 4° or 37° for 10 min. When used, leupeptin was added to give a final concentration of 25 μM and preincubated for 5 min in ice followed by another 5 min at 4° or 37° prior to the addition of [^{3}H]GABA, which was varied from 5 to 800 nM in its final concentration. Data are from a typical experiment of three such experiments.

TABLE 3

Effect of Ca^{2+}-protease inhibitors on [^{3}H]GABA binding to $GABA_B$ recognition sites

Membranes were prepared according to Method II as described under Experimental Procedures. [^{3}H]GABA binding to $GABA_B$ sites was performed in the presence of 2.5 mM $CaCl_2$ and 100 μM bicuculline. Membranes were preincubated with inhibitors for 5 min in ice followed by an additional 5 min at 37° prior to the addition of [^{3}H]GABA. Data are means ± standard error of the mean of three determinations. Hemin at 100 μM decreased the binding to a level below the value measured in the presence of 0.25 mM EGTA; this effect we consider as unrelated to the protease inhibiton.

Addition	Specifically bound [^{3}H]GABA	% of control
	fmoles/mg protein	
None	148 ± 9	100
Leupeptin (μM)		
5	133 ± 5	90
10	95 ± 6	64
25	74 ± 4	50
50	75 ± 5	50
Antipain (μM)		
10	122 ± 7	83
50	118 ± 7	80
Hemin (μM)		
20	93 ± 6	63
100	9 ± 4	6
Iodoacetamide (μM)		
100	74 ± 4	50
500	81 ± 4	55

[^{3}H]GABA to both $GABA_A$ and $GABA_B$ recognition sites at physiological temperature; however, the mechanisms of Ca^{2+} modulation involved in these two systems are different. We have also provided further evidence that $GABA_A$ but not $GABA_B$ binding sites are coupled to benzodiazepine recognition sites, whereas $GABA_B$ recognition sites are not. This conclusion derives from the following observations: (a) At 37° the stimulation by diazepam of [^{3}H]GABA binding to synaptic membranes is greatest when the concentration of free Ca^{2+} is lowered below nanomolar and when the binding to $GABA_B$ recognition sites is practically nonexistent; (b) diazepam enhances [^{3}H]GABA binding only when $GABA_A$ recognition sites are free; and (c) [^{3}H]flunitrazepam binding is enhanced by GABA bound to $GABA_A$ but not $GABA_B$ receptors.[2]

At 37°, Ca^{2+} enhances [^{3}H]GABA binding to $GABA_A$ recognition sites in a calmodulin-dependent manner (with an EC_{50} for Ca^{2+} of ≃0.5 μM). When the concentration of free Ca^{2+} is lowered by EGTA below 10 nM, only one low-affinity binding component of $GABA_A$ recognition site can be detected (K_D = 237 nM and B_{max} = 4.4 pmoles/mg of protein). However, when diazepam or Ca^{2+} is present in the binding system, a high-affinity binding component of the "A" binding site can be measured (Figs. 3 and 4). The diazepam- and Ca^{2+}-mediated effects on GABA binding are not additive (Table 1), suggesting that a common mechanism may be involved in these two events. Since the effect of Ca^{2+} depends on the presence of calmodulin in the synaptic membranes and is detected at physiological temperature but not at 4°, one may surmise that some calmodulin-dependent enzymatic reaction is involved in this Ca^{2+}-mediated process. Potential candidates for this could be membrane-bound phospholipases which may modify the phospholipid composition in the receptor vicinity, thereby affecting the $GABA_A$ receptor binding (23). Unfortunately, using inhibitors of phospholipase A_2, quinacrine and tetracaine, we were unable to obtain a conclusive answer with regard to the role of this enzyme in mediating the effect dependent on Ca^{2+} and calmodulin, because these drugs inhibit nonspecifically the $GABA_A$ binding even in the presence of EGTA (data not shown). Another possible explanation of our results would be the participation of GABA-modulin. This protein inhibitor has been shown recently to lose its inhibitory effect on GABA binding when it is phosphorylated by a cyclic AMP-dependent protein kinase (24). Assuming that some GABA-modulin is present in a phosphorylated form in our membrane preparation, one may speculate that Ca^{2+}-calmodulin inhibits the dephosphorylation of GABA-modulin by membrane-bound phosphatase in a manner analogous to that reported for phosphorylated histone (25).

Our observation of the Ca^{2+} participation in the regulation of $GABA_A$ receptors is in agreement with the results of others (26–28) demonstrating the existence of cation recognition sites in $GABA_A$/benzodiazepine/Cl^- channel receptor complexes. Although the high-affinity GABA binding sites which are exposed by diazepam or Ca^{2+} represent only about 10% of the total $GABA_A$ recognition sites, they may play important physiological roles. Because their affinity for the agonist is about 10 times higher than that of the low-affinity site, they may provide a more efficient control of neuronal firing by potentiating Cl^- influx through the receptor-associated channel. When the exogenous Ca^{2+} concentration is 2.5 mM or higher, GABA binding to "A" receptors is reduced by 10–20% as compared with values measured in the normal medium[2] (Table 1, Fig. 1). This effect is similar to the results of Corda and Guidotti (29), who reported a temperature-dependent inhibition of [^{3}H]GABA and [^{3}H]muscimol binding to $GABA_A$ receptor sites by Ca^{2+} at millimolar concentrations. However, it is doubtful that this Ca^{2+} effect is of physiological relevance, because intracellular Ca^{2+} usually does not reach this high level and extracellular Ca^{2+} remains rather constant in millimolar ranges.

The enhancement by Ca^{2+} of the GABA binding to $GABA_B$ recognition sites involves a different mechanism. This effect requires higher concentrations of Ca^{2+} and is not mimicked by other divalent cations such as Mg^{2+} at equimolar concentrations (Fig. 5). This observation is in contrast to the report of Hill and Bowery (1) that both Mg^{2+} and Ca^{2+} can increase the binding of (−)-baclofen to $GABA_B$ recognition sites with equal potency. It is possible, however, that the binding of the natural ligand (GABA) has more stringent ion requirements than the binding of baclofen. We have also found the La^{3+}, a Ca^{2+} antagonist, inhibits this effect of Ca^{2+} whereas verapamil does not.

[2] M. D. Majewska and D.-M. Chuang, unpublished observations.

The binding of [^{3}H]GABA to $GABA_B$ receptor sites is also temperature-dependent, as reflected by more than a 90% increase in the B_{max} of $GABA_B$ recognition sites when the binding temperature was raised from 4° to 37° (Fig. 6). This temperature-dependent increase in the number of $GABA_B$ recognition sites may be mediated by Ca^{2+}-dependent proteases, since inhibitors of this enzyme, such as leupeptin, iodoacetamide, and hemin, reduce GABA binding at 37° by decreasing the number of $GABA_B$ sites (Fig. 6, Table 3). However, noncompetitive inhibition of $GABA_B$ sites by these drugs cannot be totally eliminated. Investigation for the participation of phospholipase A_2, another Ca^{2+}-dependent enzyme, has yielded negative results. In our experimental system, Ca^{2+} appears to play dual roles in the GABA binding to $GABA_B$ recognition sites. This cation is directly involved in the ligand binding to the receptor; it also increases the receptor number, presumably by the activation of membrane-bound proteases. We do not know at present whether this latter process is physiologically significant, since our synaptosomal membrane preparation is inherently heterogeneous and contains different types of proteases, which can be activated *in vitro*. However, one may postulate the involvement of a receptor-coupled, specific enzyme which plays a physiological role in exposing a "latent" pool of $GABA_B$ receptors, in a way similar to that described for glutamate receptors (30).

Most of the experimental data in the literature suggest that the baclofen-sensitive $GABA_B$ recognition sites are presynaptic, based on the baclofen inhibition of K^+-evoked release of catecholamine and 5-hydroxytryptamine (2) and excitatory neurotransmitters such as aspartate and glutamate from brain slices (2, 3). The $GABA_B$ receptor has also been suggested to participate selectively in the decrease of the voltage-sensitive Ca^{2+} channel conductance (4). In our *in vitro* study, GABA binding to "B" receptors is regulated by concentrations of Ca^{2+} corresponding to the levels of this cation in the cytoplasm of nerve ending during excitation ($EC_{50} \simeq 10$ μM). It is therefore logical to suggest that perhaps GABA binding to this receptor is regulated intracellularly by an increase during depolarization of free Ca^{2+} in the synaptoplasm.

Our data indicate that *in vitro* binding of the principal inhibitory neurotransmitter, GABA, to its "A" and "B" recognition sites in the central nervous system is regulated or modulated by Ca^{2+} at the concentrations corresponding to the intracellular level during excitation. This may represent an "emergency" mechanism of turning on an inhibitory process to prevent neurons from being overstimulated. This hypothesis and its physiological significance remain to be tested.

ACKNOWLEDGMENT

We are indebted to Dr. E. Costa for his continuous guidance and support throughout the course of this study.

REFERENCES

1. Hill, D. R., and N. G. Bowery. ^{3}H-Baclofen and ^{3}H-GABA bind to bicuculline insensitive $GABA_B$ sites in rat brain. *Nature* (*Lond.*) **290**:149–152 (1981).
2. Bowery, N. G. Baclofen: 10 years on. *Trends Pharmacol. Sci.* October, 400–403 (1982).
3. Collins, G. G. S., J. Anson, and E. P. Kelly. Baclofen: effects on evoked field potentials and amino acid neutrotransmitter release in rat olfactory cortex slice. *Brain Res.* **238**:371–383 (1982).
4. Dunlap, K., and G. D. Fischbach. Neurotransmitters decrease the calcium conductance activated by depolarization of embrionic chick sensory neurons. *J. Physiol.* (*Lond.*) **317**:519–535 (1981).
5. Costa, E., A. Guidotti, C. C. Mao, and A. Suria. New concepts on the mechanism of action of benzodiazepines. *Life Sci.* **17**:167–186 (1975).
6. Gallager, D. W., P. Mallorga, J. Thomas, and J. F. Tallman. GABA-benzodiazepine interactions: physiological, pharmacological and developmental aspects. *Fed. Proc.* **39**: 3043–3049 (1980).
7. Geller, H. M., B. J. Hoffer, and D. A Taylor. Electrophysiological actions of benzodiazepines. *Fed. Proc.* **39**:3016–3023 (1980).
8. Olsen, R. W. GABA-benzodiazepine-barbiturate receptor interactions. *J. Neurochem.* **37**:1–13 (1981).
9. Tallman, J. F., J. W. Thomas, and D. W. Gallager. GABA-ergic modulation of benzodiazepine binding sites sensitivity. *Nature* (*Lond.*) **274**:383–385 (1978).
10. Wastek, G. J., R. C. Speth, T. D. Reisine, and H. I. Yamamura. The effect of gamma-aminobutyric acid on ^{3}H-flunitrazepam binding in the rat brain. *Eur. J. Pharmacol.* **50**:445–447 (1978).
11. Guidotti, A., G. Toffano, and E. Costa. An endogenous protein modulates the affinity of GABA and benzodiazepine receptors in rat brain. *Nature* (*Lond.*) **275**:553–555 (1978).
12. Rasmussen, H., and D. M. Waisman. Modulation of the cell function in the calcium messenger system. *Rev. Physiol. Biochem. Pharmacol.* **95**:111–148 (1983).
13. Nanninga, L. B., and R. Kempen. Role of magnesium and calcium in the first and second contraction of glycerin-extracted muscle fibers. *Biochemistry* **10**:2449–2454 (1971).
14. Schmidt, W. K., and E. L. Way. Direct assay for calcium in brain homogenates and synaptosomal pellets. *J. Neurochem.* **32**:1095–1098 (1979).
15. Pershadsingh, H. A., and J. M. McDonald. A high affinity calcium-stimulated magnesium-dependent adenosine triphosphatase in rat adipocyte plasma membranes. *J. Biol. Chem.* **255**:4087–4093 (1980).
16. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
17. Rosenthal, H. E. A graphic method for the determination and presentation of binding parameters in a complex system. *Anal. Biochem.* **20**:525–532 (1967).
18. Enna, S. J., and S. H. Snyder. Influences of ions, enzymes and detergents on gamma-aminobutyric acid receptor binding in synaptic membranes of rat brain. *Mol. Pharmacol.* **13**:442–453 (1977).
19. Olsen, R. W., M. O. Bergman, P. C. Van Ness, S. C. Lummis, A. E. Watkins, C. Napias, and D. V. Greenlee. γ-Aminobutyric acid receptor binding in mammalian brain: heterogeneity of binding sites. *Mol. Pharmacol.* **19**:217–227 (1981).
20. Volpi, M., R. I. Shaafi, P. M. Epstein, D. M. Andrenyak, and M. B. Feinstein. Local anesthetics, mepacrine and propranolol are antagonist of calmodulin. *Proc. Natl. Acad. Sci. U. S. A.* **78**:795–799 (1981).
21. Cheung, W. Y. Calmodulin plays a pivotal role in cellular regulation. *Science* (*Wash. D. C.*) **207**:19–27 (1980).
22. Waxman, L. Calcium activated proteases in mammalian tissues. *Methods Enzymol.* **80**:664–681 (1982).
23. Toffano, G., C. Aldinio, M. Balzano, A. Leon, and G. Savoni. Regulation of GABA receptor binding to synaptic plasma membrane of rat cerebral cortex: the role of endogenous phospholipids. *Brain Res.* **222**:95–102 (1981).
24. Wise, B. C., A. Guidotti, and E. Costa. Phosphorylation induces a decrease in the biological activity of the protein inhibitor (GABA-modulin) of gamma-aminobutyric acid binding sites. *Proc. Natl. Acad. Sci. U. S. A.* **80**:886–890 (1983).
25. Wolff, D. J., J. M. Ross, P. N. Thompson, M. A. Brostrom, and C. O. Brostrom. Interaction of calmodulin with histones: alteration of histone dephosphorylation. *J. Biol. Chem.* **256**:1846–1860 (1981).
26. Squires, R. F., and E. Saederup. γ-Aminobutyric acid receptors modulate cation binding sites coupled to independent benzodiazepine, picrotoxin, and anion binding sites. *Mol. Pharmacol.* **22**:327–334 (1982).
27. Hirch, J. D., and R. L. Kochman. Coupling of putative calcium channels with brain benzodiazepine receptors. *Abstr. Soc. Neurosci.* **8**:574 (1982).
28. Supavilai, P., A. Mannonen, and M. Karobath. Modulation of GABA binding sites by CNS depressants and CNS convulsants. *Neurochem. Int.* **4**:259–268 (1982).
29. Corda, M. G., and A. Guidotti. Modulation of GABA receptor binding by Ca^{2+}. *J. Neurochem.* **41**:277–280 (1983).
30. Baudry, M., M. C. Bundman, E. K. Smith, and G. S. Lynch. Micromolar calcium stimulates proteolysis of glutamate binding in rat brain synaptic membranes. *Science* (*Wash. D. C.*) **212**:937–938 (1981).

Send reprint requests to: Dr. De-Maw Chuang, Laboratory of Preclinical Pharmacology, National Institute of Mental Health, Saint Elizabeths Hospital, Washington, D. C. 20032.