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Influences of Ions, Enzymes, and Detergents on γ-Aminobutyric Acid-Receptor Binding in Synaptic Membranes of Rat Brain

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SUMMARY

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The effects of ions, detergents, and enzymes on γ-aminobutyric acid (GABA) binding to synaptic receptor sites in membranes of rat brain in the absence of sodium is contrasted with influences on sodium-dependent GABA binding. The anions thiocyanate, iodide, and nitrate increase the potency of the GABA antagonist bicuculline 10-fold in inhibiting sodium-independent GABA-receptor binding without affecting the potency of GABA agonists. Very low concentrations of the detergent Triton X-100 increase sodium-independent GABA-receptor binding up to 5-fold. In the presence of Triton X-100, GABA-receptor binding can be detected with considerable sensitivity using fresh or previously frozen tissue in the presence of sodium, facilitating studies of GABA receptors in small tissue samples and increasing the sensitivity of radioreceptor assays for endogenous GABA.

INTRODUCTION

Sodium-independent binding of GABA[3] to synaptic membrane preparations of mammalian brain has characteristics consistent with labeling of postsynaptic GABA receptors. The relative potencies of amino acids in competing for these binding sites parallel their abilities to mimic GABA neurophysiologically (1-5). In monkey brain there is a close correlation between the regional distribution of sodium-independent GABA binding and the activity of glutamic acid decarboxylase, the GABA-synthesizing enzyme (2), although the correlation is less close in rat brain (1, 4). The (+) isomer of the GABA antagonist bicuculline is a powerful inhibitor of sodium-independent GABA binding and is active as a neurophysiological GABA antagonist, whereas (−)-bicuculline is weak both neurophysiologically and in competing for sodium-independent GABA binding. Receptor binding for other neurotransmitters is not affected in this stereospecific fashion by bicuculline isomers.[4]

Numerous investigations have reported

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[3] The abbreviations used are: GABA, γ-aminobutyric acid; APSA, 3-aminopropanesulfonic acid.

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sodium-dependent binding of GABA to brain membranes (6–12). The characteristics of this binding suggest that it represents attachment to a transport site, possibly glial, for this neurotransmitter (1). In the present study we describe the influence of ions, detergents, and enzymes on both sodium-dependent and sodium-independent GABA binding in synaptic membranes of rat brain.

MATERIALS AND METHODS

To prepare synaptic membranes, male Sprague-Dawley rats (100–200 g) were decapitated, and their brains were rapidly removed and homogenized in 15 volumes of ice-cold 0.32 M sucrose in a Potter-Elvehjem glass homogenizer fitted with a Teflon pestle. The homogenate was centrifuged at 1000 × *g* for 10 min; the pellet was discarded, and the supernatant fluid was centrifuged at 20,000 × *g* for 20 min. The crude mitochondrial pellet was resuspended in distilled water and dispersed with a Brinkmann Polytron PT-10 (setting 6) for 30 sec. The suspension was centrifuged at 8000 × *g* for 20 min. The supernatant fluid was collected, and the pellet, a bilayer with a soft, buffy coat, was rinsed carefully with the supernatant fluid to collect the upper layer. This suspension was then centrifuged at 48,000 × *g* for 20 min. The final crude synaptic membrane pellets were resuspended in H_2O and centrifuged at 48,000 × *g* for 20 min. In some cases the membranes were immediately suspended in buffer for GABA binding assay (fresh tissue), whereas in other cases the membranes were stored at −20° for at least 18 hr (frozen tissue). Frozen pellets were resuspended in H_2O, maintained at 25° for 20 min, and centrifuged at 48,000 × *g* for 10 min, and the pellets were then suspended in buffer for GABA binding assay. Sodium-independent [^{3}H]GABA binding capacity remained intact for at least 30 days under these conditions.

For the standard [^{3}H]GABA binding assay procedure, aliquots of previously frozen crude synaptic membranes (0.8–1.2 mg of protein) were incubated in triplicate at 4° for 5 min in 2 ml of 0.05 M Tris-citric acid buffer (pH 7.1) containing 8 nM [^{3}H]GABA (500,000 cpm) alone or in the presence of 1 mM GABA, 0.1 mM bicuculline, or other indicated drugs. Freshly prepared synaptic membranes were assayed in an identical manner except that the buffer normally contained 100 mM NaCl. After incubation, the reaction was terminated by centrifugation for 10 min at 48,000 × *g*. The supernatant fluid was decanted, and the pellet was rinsed superficially with 15 ml of ice-cold distilled H_2O. Bound radioactivity was extracted into 1 ml of Protosol (New England Nuclear), 10 ml of toluene phosphor were added, and radioactivity was assayed by liquid scintillation spectrometry (Packard Tri-Carb model 3385 or 3375) at a counting efficiency of 35%. Total specific [^{3}H]GABA binding was obtained by subtracting from the total bound radioactivity the amount not displaced by a high concentration of GABA (1 mM). In routine experiments, for sodium-independent [^{3}H]GABA binding, approximately 4000 cpm/mg of protein were bound in the absence of displacer, whereas only 1800 cpm/mg of protein were bound in the presence of 1 mM GABA. Specifically bound [^{3}H]GABA is defined as the amount displaced by the unlabeled GABA (2200 cpm/mg of protein). Protein was measured by the method of Lowry *et al.* (13).

In experiments using enzymes or detergents, the synaptic membranes were suspended in 0.05 M Tris-citrate buffer (pH 7.1) to a final protein concentration of 1 mg/ml, the appropriate amount of enzyme or detergent was added, and the suspension was incubated at 37° for 30 min. Following this incubation, the suspension was centrifuged at 48,000 × *g* for 10 min, and the pellet was suspended in buffer for [^{3}H]GABA binding assay.

In experiments using salts, the salt was present in the buffer only during the 5-min incubation with [^{3}H]GABA.

[^{3}H]GABA (specific activity, 36.73 Ci/mmole) was obtained from New England Nuclear. Muscimol was a gift from Dr. E. Costa, *trans*-4-aminocrotonic acid and *cis*- and *trans*-3-aminocyclopentane-1-carboxylic acids were gifts from Dr. G. A. R. Johnston, and (−)-bicuculline was a gift from Dr. J. F. Collins. All other com-

pounds and reagents were obtained from commercial suppliers.

RESULTS

Specific sodium-independent [^{3}H]GABA binding to previously frozen synaptic membranes is reduced about 50% by 0.5 μM concentrations of the potent GABA agonist 3-aminopropanesulfonic acid and by 5 μM bicuculline (1, 4). The extent to which various ionic and other treatments alter specific sodium-dependent and sodium-independent GABA binding and the influence of such treatments on the ability of 0.5 μM APSA and 5 μM bicuculline to reduce binding have been evaluated.

Influence of cations. At concentrations up to 200 mM, NaCl fails to influence total specific sodium-independent [^{3}H]GABA binding, but, at 200 mM, NaCl significantly reduces the percentage inhibition of [^{3}H]GABA binding elicited by 0.5 μM APSA. The effect of NaCl is fairly specific, since up to 200 mM lithium chloride fails to affect specific [^{3}H]GABA binding or competition by APSA and bicuculline. Similarly, potassium (1–10 mM) and the divalent cations calcium, manganese, magnesium, copper, nickel, and mercury, all tested as the chloride salts, have little influence on total specific GABA binding or the percentage inhibition by APSA and bicuculline. For all cations examined there is no change in nonspecific [^{3}H]GABA binding measured in the presence of 1 mM GABA or 0.1 mM bicuculline.

Although sodium fails to affect the GABA binding to previously frozen tissue, [^{3}H]GABA binding by fresh membranes is profoundly influenced by this cation (Fig. 1). As little as 25 mM NaCl increases specific [^{3}H]GABA binding about 2-fold, with

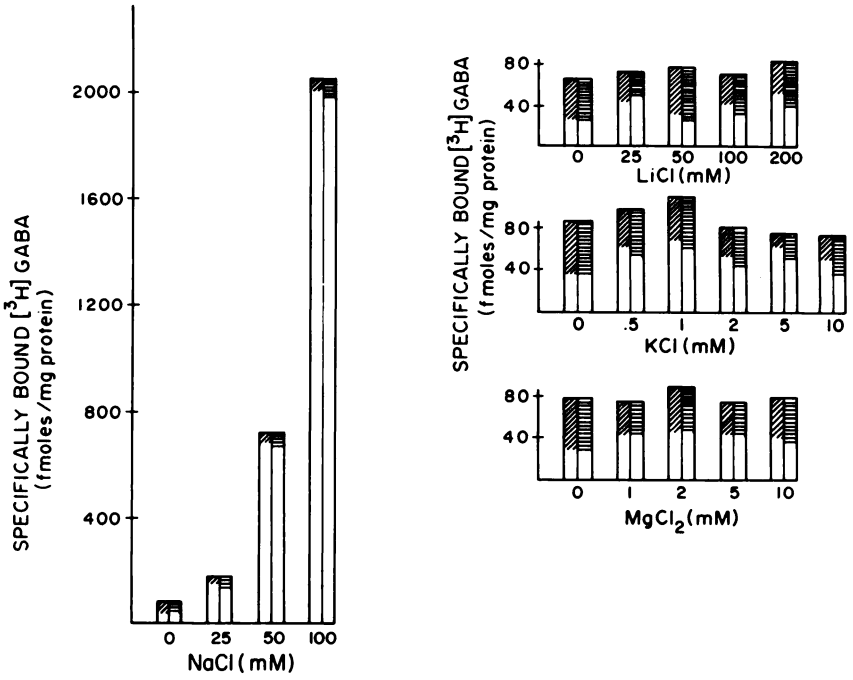

FIG. 1. *Effects of cations on [^{3}H]GABA binding to freshly prepared synaptic membranes*

Rat brain synaptic membranes were prepared and assayed immediately for receptor binding as described in MATERIALS AND METHODS. The receptor binding assay was conducted in the presence of various concentrations of the individual salts. The heights of the bars indicate total, specifically bound [^{3}H]GABA as determined by the amount of radioactive ligand displaced by 1 mM unlabeled GABA. The diagonally striped areas represent the amounts of specifically bound [^{3}H]GABA displaced by 0.5 μM APSA, and the horizontally striped areas represent the amounts displaced by 5 μM bicuculline. Each experiment was replicated three times. Data presented are the means for four separate determinations, which varied less than 10%.

a maximum augmentation of 25-fold at 100 mM NaCl. Beyond 200 mM NaCl, total [^{3}H]GABA binding in freshly prepared membranes decreases (data not shown). In the presence of NaCl, GABA binding appears to be localized to APSA- and bicuculline-resistant sites, with 0.5 μM APSA and 5 μM bicuculline, concentrations which produce about 50% inhibition of sodium-independent GABA binding, inhibiting less than 10% of sodium-dependent GABA binding.

To determine whether other cations could substitute for sodium in fresh membranes, the effects of lithium chloride (25–200 mM), potassium chloride (0.5–10 mM), and magnesium chloride (1–10 mM) were determined on [^{3}H]GABA binding to freshly prepared membranes (Fig. 1). None of these cations produces an increase in [^{3}H]GABA binding comparable to the effect of NaCl, and they do not affect the degree of inhibition by APSA or bicuculline (Fig. 1).

The influence of various cations on [^{3}H]GABA binding in freshly prepared membranes measured in the presence of 100 mM NaCl was also evaluated. In these experiments, 1 μM nonradioactive GABA was used as a displacer instead of APSA and bicuculline, since it is apparent that [^{3}H]GABA binding to freshly prepared membranes in the presence of sodium occurs at sites which are insensitive to these agents. Under these conditions, LiCl (25 and 250 mM) progressively decreases total specific [^{3}H]GABA binding, with the percentage inhibition by 1 μM nonradioactive GABA unchanged. While potassium chloride (1–10 mM) has no influence on specific [^{3}H]GABA binding or on inhibition by 1 μM nonradioactive GABA, both calcium chloride and manganese chloride inhibit total specific [^{3}H]GABA binding (Fig. 2). Neither calcium nor manganese alters the percentage inhibition by nonradioactive GABA. While magnesium chloride and nickel chloride have no significant effect on specific [^{3}H]GABA binding or inhibition by GABA, cupric chloride and mercuric chloride are potent inhibitors of total specific [^{3}H]GABA binding, although neither alters the percentage inhibition by 1 μM nonradioactive GABA. None of the cations examined affects nonspecific [^{3}H]GABA binding observed in the presence of 1 mM GABA.

Influence of anions. Specific sodium-independent GABA binding is reduced by 100 mM nitrate, iodide, sulfate, phosphate (monobasic), and sulfate, but not by fluoride, phosphate (dibasic), or formate (Fig. 3). At concentrations up to 50 mM, ammonium nitrate has no effect on specific [^{3}H]GABA binding (Fig. 3). However, concentrations as low as 5 mM markedly increase the inhibition by bicuculline without altering inhibition by APSA. A similar effect is observed with ammonium iodide and ammonium thiocyanate, whereas the ammonium salts of formate, sulfate, phosphate (monobasic), chloride, sulfite, and fluoride fail to display this effect.

Thiocyanate, nitrate, and iodide increase the potency of bicuculline in inhibiting sodium-independent GABA binding about 10-fold (Table 1). This phenomenon is not a function of the ammonium ion, since sodium nitrate and sodium iodide produce essentially the same effect. Interestingly, whereas iodide, thiocyanate, and nitrate enhance the potency of bicuculline, none of these anions alters the potencies of the agonists APSA and GABA. The displacement curve for [^{3}H]GABA binding inhibition by bicuculline in the presence of 50 mM ammonium nitrate is parallel to that obtained in the absence of the anion, but is shifted by about an order of magnitude to the left (Fig. 4). A similar parallel leftward shift in the displacement curve is obtained with either ammonium iodide or ammonium thiocyanate. As little as 5 mM ammonium thiocyanate enhances bicuculline inhibition of [^{3}H]GABA binding, and a maximal augmentation of potency of about 10–20-fold is observed at 50–100 mM ammonium thiocyanate (Table 2).

Several ammonium salts of anions decrease specific [^{3}H]GABA binding assayed in fresh tissue in the presence of 100 mM sodium (Fig. 5). As little as 10 mM ammonium nitrate reduces specific [^{3}H]GABA binding about 50%, while a similar reduction requires 50 mM ammonium formate, ammonium iodide, or ammonium thiocya-

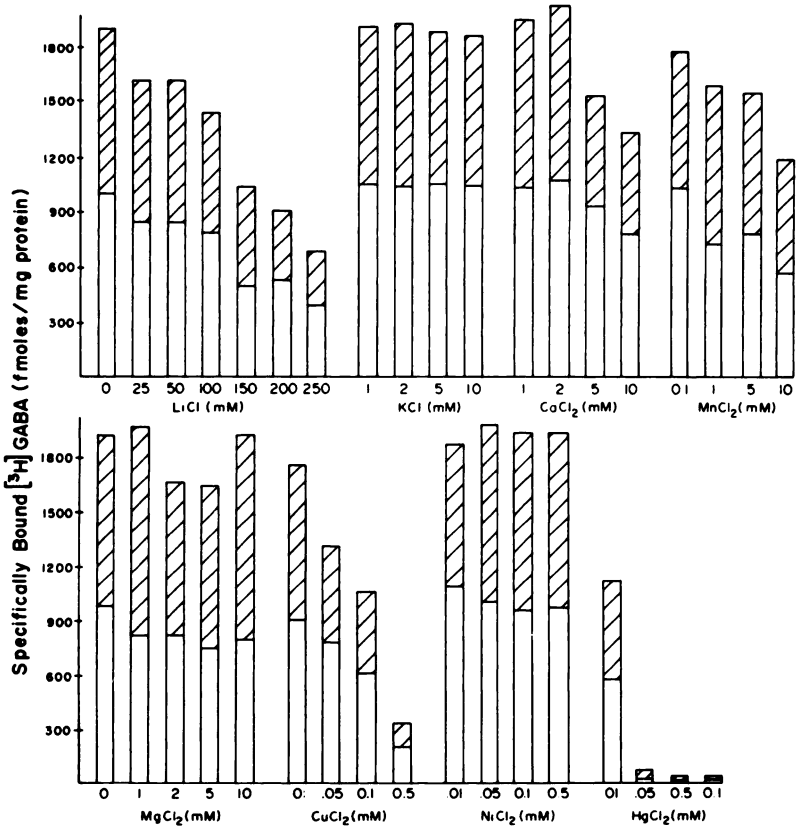

FIG. 2. *Effects of cations on sodium-dependent [³H]GABA binding to freshly prepared synaptic membranes*

Rat brain synaptic membranes were prepared and assayed immediately for receptor binding as described in MATERIALS AND METHODS. All assays were conducted in the presence of 100 mM NaCl in addition to the indicated concentrations of other salts. The heights of the bars indicate total, specifically bound [³H]GABA as determined by the amount of radioactive ligand displaced by 1 mM unlabeled GABA. The diagonally striped areas represent the amounts of specifically bound [³H]GABA displaced by 1 μM unlabeled GABA. Data presented are the means for four separate determinations, which varied less than 10%.

nate. The most potent of these ions is ammonium phosphate (monobasic), which causes a 50% reduction in binding at 5 mM. The influence of ammonium salts of sulfite, sulfate, chloride, and phosphate (dibasic) resembles that of ammonium formate. None of these treatments markedly changes the percentage inhibition of binding by 1 μM nonradioactive GABA. These effects are not due to the ammonium ion, since similar changes are observed with sodium salts of the anions, and the effects are not brought about by a change in pH, since the pH values of all incubations were adjusted to 7.1. Nonspecific [³H]GABA binding, measured in the presence of 1 mM nonradioactive GABA, is unaffected by any of these treatments.

Influence of enzymes. Sodium-independent GABA receptor binding to previously frozen synaptic membranes is resistant to enzyme treatment. No significant influence on specific [³H]GABA binding or percentage inhibition by APSA and bicuculline is detected at up to 3 μg/ml of trypsin, 50 μg/ml of neuraminidase, 200 μg/ml of phospholipase D, or 25 μg/ml of chymotrypsin. Some reduction in specific [³H]-GABA binding is observed at 50 μg/ml of chymotrypsin. Low concentrations of phospholipase A tend to increase the inhibition of specific [³H]GABA binding by both

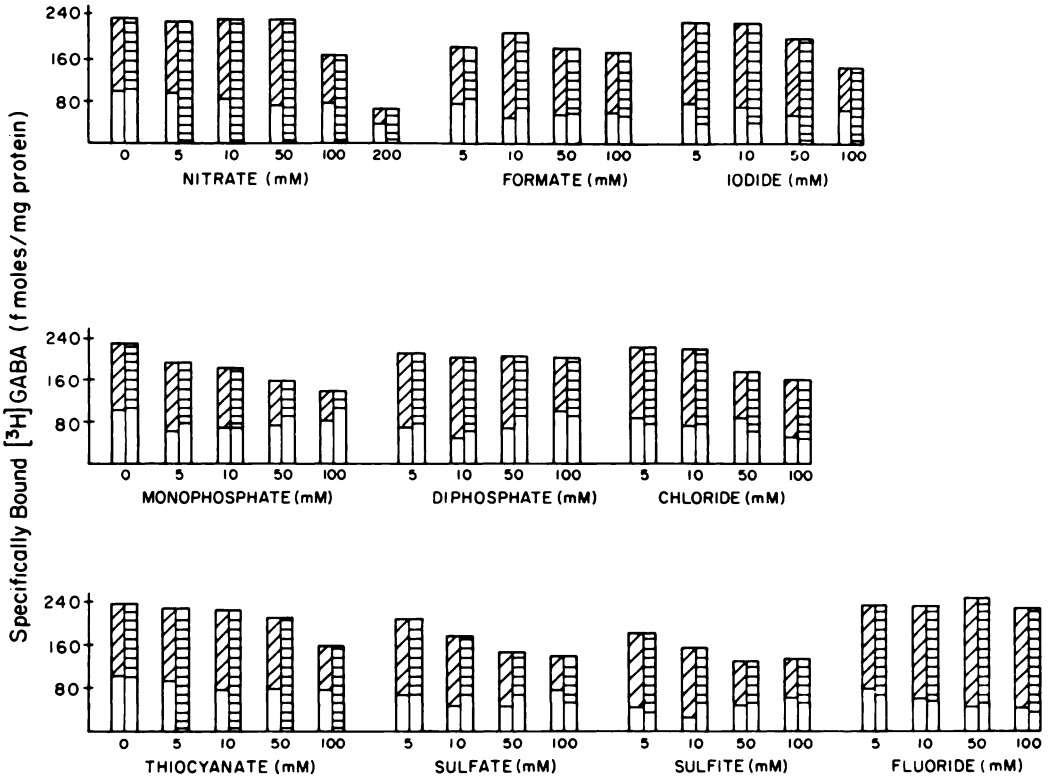

FIG. 3. *Effects of anions on sodium-independent [3H]GABA binding to previously frozen synaptic membranes*

See Fig. 1 for explanation.

APSA and bicuculline, although this increase is not statistically significant. No effect on total specific [3H]GABA binding is observed with phospholipase A.

Sodium-dependent GABA binding to freshly prepared synaptic membranes in the presence of 100 mM NaCl is more sensitive to enzymatic treatment than is sodium-independent GABA binding (Fig. 6). At 1–3 μg/ml, trypsin reduces specific [3H]GABA binding by about 50%, with no change in the percentage inhibition by nonradioactive GABA, and as little as 5 ng/ml of phospholipase A reduce sodium-dependent GABA binding by 80%. The very small amount of GABA binding remaining after treatment with 10–50 ng/ml of phospholipase A is inhibited to a much greater extent by GABA than are control levels of sodium-dependent GABA binding. Apparently, these concentrations of phospholipase A eliminate the sodium-dependent GABA binding, leaving only sodium-independent sites, for which GABA has a substantially higher affinity. This conclusion is reinforced by the observation that in freshly prepared membranes treated with 50 ng/ml of phospholipase A and incubated in the presence of 100 mM NaCl, the IC_{50} for nonradioactive GABA is 0.2 μM, similar to values obtained under sodium-independent conditions. No decrease in GABA binding is obtained with phospholipase A preparations without added calcium, indicating that the degradation of binding is due to phospholipase A enzymatic activity.

Between 10 and 50 μg/ml of chymotrypsin and neuraminidase produce a 25–40% decrease in total specific sodium-dependent [3H]GABA binding, with no change in percentage inhibition by nonradioactive GABA. Phospholipase D does not alter sodium-dependent specific [3H]GABA binding but, at all concentrations studied, reduces the percentage inhibition by nonra-

dioactive GABA. None of these enzymes alters the level of nonspecific [^{3}H]GABA binding.

Influence of detergents. Treatment with Triton X-100 markedly augments specific sodium-independent [^{3}H]GABA binding (Fig. 7). At 0.05% Triton X-100, specific [^{3}H]GABA binding increases about 5-fold, and the potency of APSA, but not bicuculline, is also increased. In contrast, as little as 0.03% deoxycholate reduces total [^{3}H]GABA binding to negligible levels. Unlike the increase in sodium-independent binding observed after treatment with Triton, the detergent markedly reduces sodium-dependent GABA binding in fresh tissue, with a 50% reduction at 0.01–0.02% (Fig. 8). At concentrations above 0.02%, the proportion of sodium-dependent binding inhibited by nonradioactive GABA is increased, indicating that at these levels of detergent, even though sodium is present, binding probably involves the same sodium-independent GABA receptor sites that were found in membranes which had previously been frozen. Furthermore, sodium-dependent GABA binding is more sensitive to deoxycholate than is sodium-independent binding.

To ensure that specific GABA binding in the presence of Triton involves the same receptor sites labeled in the absence of so-

TABLE 1

Effects of iodide, nitrate, and thiocyanate on IC_{50} of GABA, 3-aminopropanesulfonic acid, and bicuculline for displacing specifically bound, sodium-independent [^{3}H]GABA from previously frozen synaptic membranes

The displacement of sodium-independent, specifically bound [^{3}H]GABA by various concentrations of GABA, APSA, or bicuculline in the presence of a 50 mM concentration of the various salts was assayed using the standard procedure for previously frozen membranes as described in MATERIALS AND METHODS. The concentration of the compound which inhibited specific binding of [^{3}H]GABA by 50%, IC_{50}, was determined by log probit analysis. Each value is the mean of four separate determinations, which varied less than 10%.

Salt (50 mM)	IC_{50}		
	GABA	APSA	Bicuculline
	μM	μM	μM
None	0.4	0.3	7.0
Ammonium iodide	0.2	0.4	0.5
Ammonium thiocyanate	0.4	0.3	0.5
Ammonium nitrate	0.5	0.4	0.4
Sodium nitrate	0.4	0.5	0.5
Sodium iodide	0.4	0.4	0.5

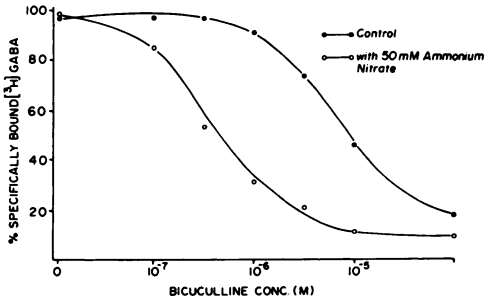

FIG. 4. *Effect of 50 mM ammonium nitrate on bicuculline displacement of sodium-independent [^{3}H]GABA binding from previously frozen synaptic membranes*

Rat brain synaptic membranes were prepared, frozen, and assayed as described in MATERIALS AND METHODS. The displacement of specifically bound [^{3}H]GABA was determined in the presence and absence of 50 mM ammonium nitrate, and the results were plotted as percentage of maximal binding in the absence of bicuculline. Each point represents the mean of four separate determinations, each analyzed in triplicate. The experiment was replicated twice.

TABLE 2

Effect of ammonium thiocyanate on IC_{50} of bicuculline for displacing sodium-independent, specifically bound [^{3}H]GABA from previously frozen synaptic membranes

The displacement of sodium-independent, specifically bound [^{3}H]GABA by various concentrations of bicuculline was assayed in the presence of three different concentrations of ammonium thiocyanate, using the standard procedure for previously frozen membranes as described in MATERIALS AND METHODS. The concentration of bicuculline which inhibited specifically bound [^{3}H]GABA, IC_{50}, was determined by log probit analysis. Each value is the mean ± standard error of four separate determinations, each done in triplicate.

Ammonium thiocyanate	Bicuculline IC_{50}
mM	μM
0	5 ± 0.3
5	3 ± 0.6
50	0.5 ± 0.02
100	0.3 ± 0.05

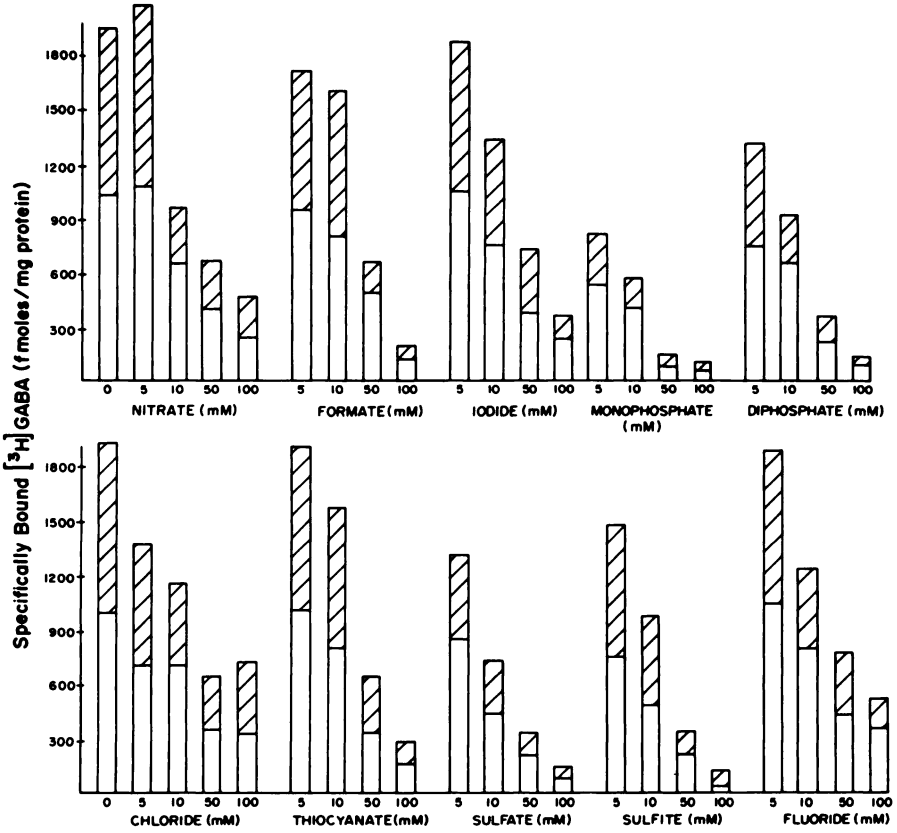

FIG. 5. *Effects of anions on sodium-dependent [³H]GABA binding to freshly prepared synaptic membranes*

See Fig. 2 for explanation.

dium, the influence of a wide range of drugs with varying affinities for GABA receptors was evaluated, using membranes treated with the detergent (Table 3). The neurophysiological effects of GABA are antagonized stereospecifically by bicuculline, with essentially all activity residing in the (+) isomer. (+)-Bicuculline is about 70 times more potent in inhibiting sodium-independent [³H]GABA binding in frozen tissues than the (−) isomer, whereas no stereospecificity is detectable in bicuculline effects on receptor binding for other neurotransmitters.[4] There is a similar degree of stereospecificity in the inhibition of [³H]GABA binding in fresh tissue treated with Triton. The geometrical isomers of GABA agonists also provide a means of assessing the specificity of GABA-receptor binding. *trans*-3-Aminocyclopentane-1-carboxylic acid is a more potent GABA agonist in neurophysiological studies than the *cis* isomer (14). Similarly, differences are detected in the influence of these agents on sodium-independent [³H]GABA receptor binding, with the *trans* isomer being 28 and 600 times as potent as the *cis* isomer, respectively, in frozen tissue and in fresh tissue treated with Triton. Muscimol is one of the more potent GABA agonists examined in neurophysiological studies, being more active than GABA itself (14, 15). In parallel with neurophysiological results, muscimol is the most potent agent so far examined in competing for sodium-independent [³H]-GABA receptor binding sites, being about 4 times as active as GABA in frozen preparations and 6 times as active in fresh preparations treated with Triton. Triton also

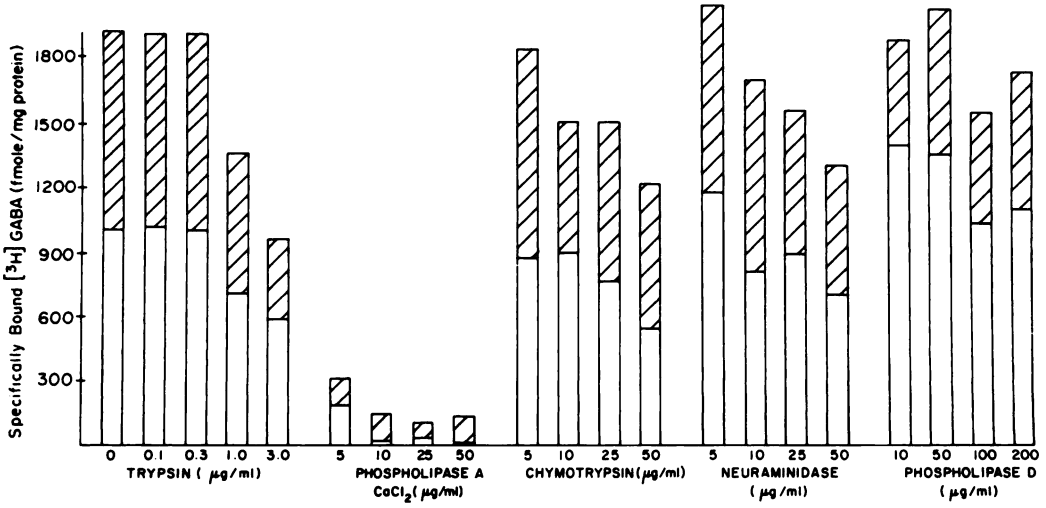

FIG. 6. *Effects of enzymes on sodium-dependent [³H]GABA binding to freshly prepared synaptic membranes*

Immediately after preparation, rat brain synaptic membranes were incubated in buffer containing sufficient enzyme to make the indicated concentrations, and the suspension was incubated at 37° for 30 min, followed by centrifugation at 48,000 × *g* for 10 min. The pellet was then suspended in buffer, and [³H]GABA binding was assayed. Phospholipase A was incubated with buffer containing 5 mM $CaCl_2$. Data are the means of five separate determinations, which varied less than 15%. [³H]GABA binding was assayed in the presence of 100 mM NaCl in all cases. See Fig. 2 for explanation of bars.

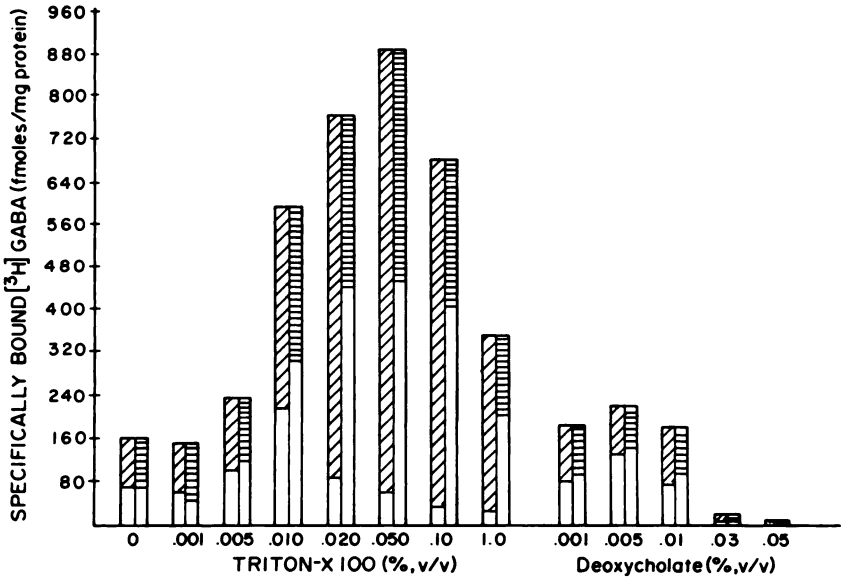

FIG. 7. *Effects of detergents on sodium-independent [³H]GABA binding to previously frozen synaptic membranes*

Frozen rat brain synaptic membranes, prepared as described in MATERIALS AND METHODS, were suspended in 0.05 M Tris-citrate buffer (pH 7.1) (1 mg of protein per milliliter), with sufficient detergent added to make the indicated concentrations, and the suspensions were incubated at 37° for 30 min, followed by centrifugation at 48,000 × *g* for 10 min. The pellet was then suspended in buffer and [³H]GABA binding was assayed. See Fig. 1 for explanation of bars. Data are the means of four separate determinations, which varied less than 15%.

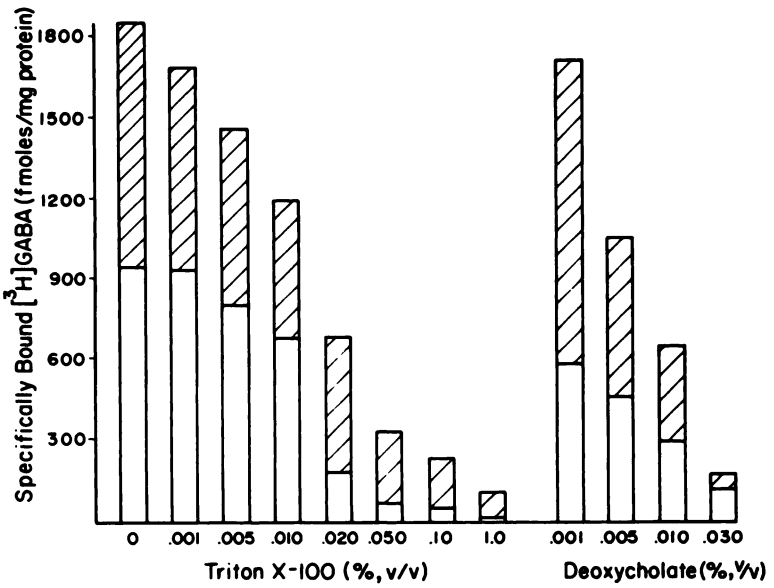

FIG. 8. *Effects of detergents on sodium-dependent [³H]GABA binding to freshly prepared synaptic membranes*

Immediately after preparation, rat brain synaptic membranes were incubated in buffer containing one of the detergents as described in Fig. 7. [³H]GABA binding was assayed in the presence of 100 mM NaCl in all cases. See Fig. 2 for explanation of bars. Data are the means of five separate determinations, which varied less than 15%.

increases the inhibitory effect of GABA agonists on [³H]GABA binding in frozen membrane preparations.[5]

These experiments demonstrate that the sites labeled in fresh or frozen tissue treated with Triton are pharmacologically similar to the GABA receptor binding sites observed in frozen tissue in the absence of Triton and sodium. Triton treatment appears to increase the inhibitory potencies of GABA agonists but not antagonists. Thus GABA, 3-aminopropanesulfonic acid, imidazoleacetic acid, and muscimol become 6–17 times more potent in Triton-treated membranes, and *trans*-4-aminocrotonic acid and (±)-*trans*-3-aminocyclopentane-1-carboxylic acid, respectively, become 200 and 140 times as potent. The potency of bicuculline is unaltered by Triton treatment.

Scatchard analysis of GABA displacement of [³H]GABA, using Triton-treated synaptic membranes which have previously been frozen, reveals two distinct components in GABA-receptor binding. A lower-affinity component has a dissociation constant of approximately 130 nM, and a higher-affinity component has a dissociation constant of about 16 nM, with approximately 1/10 the number of binding sites as the lower-affinity site (Fig. 9). The same two components of binding are detected in fresh tissue treated with Triton. The apparent K_D of the lower-affinity binding site is similar to that reported earlier for the sodium-independent GABA receptor site (1).

[5] Unpublished observations.

DISCUSSION

Sodium-dependent binding of GABA has been reported by several investigators (6–12). The exact identity of the sodium-dependent binding sites is not clear, although their pharmacological characteristics suggest they may be GABA uptake sites, possibly localized on glia (1). Because of the high levels of sodium-dependent GABA binding, detection of GABA binding sites with characteristics of GABA synaptic receptors was facilitated by freezing and thawing of tissue to destroy the

sodium-dependent GABA binding (1, 4). The enhancement, up to 5-fold, of synaptic GABA receptor binding after treatment with Triton X-100 greatly facilitates binding sites. Using Triton, fresh or frozen synaptic membranes can be employed to study sodium-independent receptor binding in the presence of high concentrations of sodium without interference by sodium-dependent GABA binding. This enhanced binding affords greater sensitivity in assays of GABA-receptor binding and should facilitate studies in small tissue samples. This procedure can also be used to increase the sensitivity of a recently described radioreceptor assay for GABA (16).

TABLE 3

Effect of Triton X-100 on substrate specificity of [³H]GABA binding to rat brain synaptic membranes

Inhibition of sodium-independent specific [³H]-GABA binding by various concentrations of the different compounds was determined using the standard assay procedure. Prior to assay, freshly prepared membranes were incubated with 0.05% Triton X-100 (v/v) for 30 min at 37°; membranes which had previously been frozen were not exposed to the detergent. IC_{50} values, the concentrations of agent which inhibit binding of [³H]GABA 50%, were calculated by log probit analysis. Values are the means of three determinations, which varied less than 20%.

Compound	IC_{50}	
	Frozen	Fresh tissue treated with triton
	μM	μM
γ-Aminobutyric acid	0.2	0.02
Muscimol	0.05	0.003
trans-4-Aminocrotonic acid	0.2	0.001
(±)-*trans*-3-Aminocyclopentane-1-carboxylic acid	0.7	0.005
(±)-*cis*-3-Aminocyclopentane-1-carboxylic acid	20.0	3.0
3-Aminopropanesulfonic acid	0.3	0.04
Imidazoleacetic acid	0.3	0.05
(+)-Bicuculline	4.0	5.0
(−)-Bicuculline	300	200
1-Methylimidazoleacetic acid	200	100
Diaminobutyric acid	>1000	>1000
Glycine	>1000	>1000
Aminooxyacetic acid	>1000	>1000

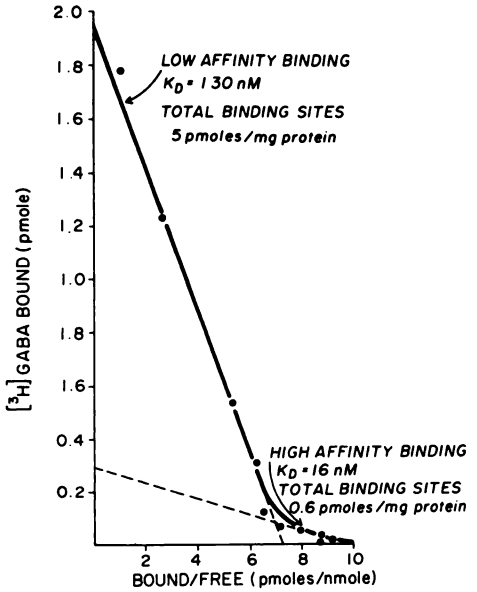

FIG. 9. *Scatchard analysis of sodium-independent [³H]GABA binding to previously frozen rat brain synaptic membranes treated with 0.05% Triton X-100*

Frozen rat brain synaptic membranes were incubated with 0.05% Triton X-100 as described in Fig. 7, and specific binding of [³H]GABA was determined in the presence of various concentrations of unlabeled GABA as described in MATERIALS AND METHODS. The experiment was repeated four times.

The effects of freezing, ions, enzymes, and detergents on [³H]GABA binding to synaptic membranes suggest a heterogeneity of sites. The pharmacological characteristics and regional distribution of the sodium-dependent binding to freshly prepared membranes is not consistent with the synaptic receptor sites for GABA, but seems to suggest that under these conditions binding occurs primarily to a transport site, possibly glial (1). In contrast, both the high (16 nM)- and low (130 nM)-affinity sodium-independent [³H]GABA binding to membranes which have been incubated in Triton have a sensitivity to amino acids and drugs which suggests that both these sites are biologically relevant synaptic GABA receptors. It is unlikely that the stereospecificity and pharmacological sensitivity of these two sites are merely coincidental, since it has been reported that destruction of cell types which are thought to possess synaptic receptors

for GABA causes a reduction in the sodium-independent binding (17, 18) whereas binding is unchanged after the loss of other cell types (3). Furthermore, an increase in sodium-independent [^{3}H]GABA binding has been noted after destruction of certain GABA pathways, which may suggest a "denervation supersensitivity" (19), and there is a distinct regional distribution of these binding sites in human, rat, and monkey (1, 2, 20). All these reports lend support to the notion that sodium-independent [^{3}H]GABA binding occurs at a pharmacologically and biologically important synaptic GABA receptor site.

Several anions, specifically thiocyanate, iodide, and nitrate, increase the potency of bicuculline in inhibiting sodium-independent GABA receptor binding about 10-fold. Although these anions enhance the potency of the antagonist bicuculline, they do not alter the potencies of agonists such as GABA or APSA. A variety of evidence suggests that neurotransmitter receptors in the brain may exist in interconvertible conformations with selective high affinities for agonists and antagonists, respectively (21). Conceivably, the influence of these anions is exerted selectively upon the "antagonist" state of the receptor. In the absence of binding studies with a labeled GABA antagonist, it is not feasible to determine whether the anions increase the number of "antagonist" receptor sites. Since there is no increase in [^{3}H]GABA binding itself or in the IC_{50} for GABA, the anions do not seem to alter the number of [^{3}H]GABA binding sites in concentrations which markedly increase bicuculline potency. The available data do not reveal whether the increased potency of bicuculline induced by the anions involves a specific alteration of the GABA receptor. If the anion effects are pharmacologically meaningful, the application of these anions should increase the neurophysiological potency of bicuculline as a GABA antagonist.

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REFERENCES

1. Enna, S. J. & Snyder, S. H. (1975) *Brain Res.*, **100**, 81–97.
2. Enna, S. J., Kuhar, M. J. & Snyder, S. H. (1975) *Brain Res.*, **93**, 168–174.
3. Enna, S. J., Bird, E. D., Bennett, J. P., Bylund, D. B., Yamamura, H. I., Iversen, L. L. & Snyder, S. H. (1976) *N. Engl. J. Med.*, **294**, 1305–1309.
4. Zukin, S. R., Young, A. B. & Snyder, S. H. (1974) *Proc. Natl. Acad. Sci. U. S. A.*, **71**, 4802–4807.
5. Coyle, J. T. & Enna, S. J. (1977) *Brain Res.*, in press.
6. DeFeudis, F. V. (1973) *Exp. Neurol.*, **41**, 54–62.
7. DeFeudis, F. V. (1974) *Can. J. Physiol. Pharmacol.*, **52**, 138–147.
8. DeFeudis, F. V. & Black, W. C. (1973) *Brain Res.*, **49**, 218–222.
9. Gottesfeld, Z. & Elliott, K. A. C. (1971) *J. Neurochem.*, **18**, 683–690.
10. Roberts, E. & Kuriyama, I. (1968) *Brain Res.*, **8**, 1–35.
11. Sano, K. & Roberts, E. (1961) *Biochem. Biophys. Res. Commun.*, **4**, 358–361.
12. Varon, S., Weinstein, H., Kakefuda, T. & Roberts, E. (1965) *Biochem. Pharmacol.*, **14**, 1213–1224.
13. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.*, **193**, 265–275.
14. Johnston, G. A. R. (1976) in *GABA in Nervous System Function* (Roberts, E., Chase, T. & Tower, D. B., eds.), pp. 395–411, Raven Press, New York.
15. Krogsgaard-Larsen, P., Johnston, G. A. R., Curtis, D. R., Game, C. J. A. & McCulloch, R. M. (1975) *J. Neurochem.*, **25**, 803–809.
16. Enna, S. J. & Snyder, S. H. (1976) *J. Neurochem.*, **26**, 221–224.
17. Simantov, R., Oster-Granite, M., Herndon, R. & Snyder, S. H. (1976) *Brain Res.*, **105**, 365–371.
18. Lloyd, G. K. & Hornykiewicz, O. (1977) *Brain Res.*, in press.
19. Enna, S. J., Bennett, J. P., Bylund, D. B., Bird, E. D., Iversen, L. L. & Snyder, S. H. (1976) *Brain Res.*, **116**, 531–537.
20. Enna, S. J., Bennett, J. P., Bylund, D., Creese, I., Burt, D. R., Charness, M. E., Yamamura, H. I., Simantov, R. & Snyder, S. H. (1976) *J. Neurochem.*, **28**, 233–236.
21. Snyder, S. H. & Bennett, J. P., Jr. (1976) *Annu. Rev. Physiol.*, **38**, 153–175.