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Relation of the [^{3}H]γ-Hydroxybutyric Acid (GHB) Binding Site to the γ-Aminobutyric Acid$_B$ (GABA$_B$) Receptor in Rat Brain

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ABSTRACT. γ-Hydroxybutyric acid (GHB) is a naturally occurring compound that has the ability to induce generalized absence seizures when given to animals. GHB has been hypothesized to induce this effect via the postsynaptic γ-aminobutyric acid$_B$ (GABA$_B$) receptor. We sought to test this hypothesis by examining the affinity of GABA$_B$ agonists and antagonists for the [^{3}H]GHB binding site, the affinity of GHB and a GHB antagonist for the [^{3}H]GABA$_B$ binding site, and the effect of guanine nucleotides and pertussis toxin on both, using autoradiographic binding assays. GHB and its antagonist, NCS 382, did not compete for [^{3}H]GABA$_B$ binding, nor did (−)-baclofen or the [^{3}H]GABA$_B$ antagonists, CGP 35348 or SCH 50911, compete for [^{3}H]GHB binding; however, the GABA$_B$ agonist 3-amino-propylphosphinic acid (3-APPA), and the GABA$_B$ antagonists phaclofen and 2-hydroxysaclofen (2-OH saclofen) did show a weak affinity for [^{3}H]GHB binding in frontal cortex. GTP and the nonhydrolyzable GTP analogues, GTPγS and Gpp(NH)p, depressed [^{3}H]GABA$_B$ binding throughout the brain, but increased [^{3}H]GHB binding in frontal cortex and thalamus, those regions involved in GHB-induced absence seizures. Pertussis toxin significantly depressed [^{3}H]GABA$_B$ binding throughout the brain, but attenuated [^{3}H]GHB binding only in frontal cortex, and to a lesser degree than [^{3}H]GABA$_B$ binding. The guanine nucleotide-induced changes in [^{3}H]GHB and [^{3}H]GABA$_B$ binding were due to a change in K_D for both. Moreover, GTPγS reversed the ability of 3-APPA, phaclofen, and 2-OH saclofen to compete for [^{3}H]GHB binding. These data do not support the hypothesis that GHB acts through the postsynaptic GABA$_B$ receptor to produce absence seizures. Rather, they raise the possibility either that the [^{3}H]GHB binding site may be an isoform of the presynaptic GABA$_B$ receptor or that an independent GHB site is operative in the GHB model of absence seizures. BIOCHEM PHARMACOL 52;8:1235–1243, 1996.

KEY WORDS. GABA$_B$ receptor; γ-hydroxybutyrate; G protein; pertussis toxin; epilepsy; absence seizures; binding; presynaptic

GHB† is a naturally occurring short chain fatty acid that is synthesized from GABA [1]. This compound has biological significance for two reasons. First, GHB has many properties that suggest it may play a role in the brain as a neurotransmitter or neuromodulator [2]. These include a discrete, subcellular anatomical distribution for GHB and its synthesizing enzyme [3–6], the presence of specific, high affinity [^{3}H]GHB binding sites with an anatomical distribution that correlates with GHB turnover [7–9], the presence of Ca^{2+}-dependent release [10] and Na^+-dependent uptake mechanisms [11] and a distinct ontogeny [12]. Second, when given in low doses, GHB has the ability to induce absence-like seizures in a number of animal species [13, 14].

It has been proposed that GHB acts at the GABA$_B$ postsynaptic receptor to induce these experimental absence seizures [15]. There are two sets of data to support this hypothesis. First, GHB has been reported specifically to displace bound [^{3}H]baclofen and [^{3}H]CGP 27492, a potent GABA$_B$ receptor ligand, from GABA$_B$ sites in cortical and thalamic homogenates with a low affinity [15]. However, the regional distribution of [^{3}H]GHB binding differs significantly from that of [^{3}H]GABA$_B$ binding. [^{3}H]GHB binding is maximal in hippocampus and layers I–III of cerebral cortex and to a lesser extent in the ventrolateral thalamic nuclei [16], whereas [^{3}H]GABA$_B$ binding is prominent in the superficial cortical layers of frontal cortex, the granular cell layer of the cerebellum, the thalamus, and the olfactory bulb [17].

The second group of data to support the hypothesis that GHB acts at the GABA$_B$ postsynaptic receptor consists of electrophysiological studies that indicate that GHB induces long-lasting IPSPs and rebound Ca^{2+} spikes in a manner similar to (−)-baclofen [18, 19]. The experiments described below were designed to test the hypothesis that the [^{3}H]GHB and [^{3}H]GABA$_B$ binding sites in rat brain are

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† *Abbreviations:* GHB, γ-hydroxybutyric acid; GABA, γ-aminobutyric acid; IPSP, inhibitory postsynaptic potential; 2-OH saclofen, 2-hydroxy-saclofen; and 3-APPA, 3-amino-propylphosphinic acid.

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identical. The strategy was to examine the ability of a number of $GABA_B$ receptor agonists and antagonists to displace [^{3}H]GHB binding, using autoradiographic binding techniques. Some of the antagonists used in these experiments, namely phaclofen, 2-OH saclofen, and CGP 35348 were chosen because they have been reported to differ in their ability to modulate the presynaptic release of GABA and glutamate from rat cerebral cortex synaptosomes and thus to discriminate between pre- and postsynaptic $GABA_B$ receptor subtypes [20–22]. In addition, the ability of GHB and the specific GHB antagonist NCS 382 [23] to displace [^{3}H]GABA from $GABA_B$ sites was determined. Finally, the effect of guanyl nucleotides and pertussis toxin on [^{3}H]GHB and [^{3}H]$GABA_B$ binding was ascertained. The rationale for this approach was that both guanyl nucleotides and pertussis toxin are known to decrease [^{3}H]$GABA_B$ binding because of the linkage of this receptor to a G protein [24, 25]; hence, we sought to demonstrate whether this property existed for [^{3}H]GHB binding.

[^{3}H]GHB and [^{3}H]$GABA_B$ binding were analyzed in all experiments in the thalamus, cortex, and hippocampus. The rationale for choosing these regions for analysis is that both lesioning and depth electrode electroencephalographic recordings have demonstrated that the spike wave discharges that characterize GHB-induced absence seizures originate from thalamus and cortex, but not from hippocampus [26, 27].

MATERIALS AND METHODS

Drugs

GHB, isoguvacine, pertussis toxin, GTP, GTPγS, Gpp(NH)p, phaclofen, and 2-OH saclofen were obtained from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). CGP 35348 was a gift from Raymond Bernasconi (Ciba Geigy, Basel, Switzerland). NCS 382 was a gift from J. J. Bourguignon (Centre de Neurochimie, Strasbourg, France). The baclofen isomers were a gift from Dr. John Dailey (University of Illinois College of Medicine, Peoria, IL, U.S.A.). SCH 50911 and 3-APPA were gifts from Dr. William Kreutner (Schering Plough Research Institute, Kenilworth, NJ, U.S.A.). [^{3}H]GHB or 4-hydroxy-[2,3-^{3}H]butyric acid, ammonium salt (sp. act. 44.5 Ci/mmol) was synthesized by Amersham (Arlington Heights, IL, U.S.A.) from γ-crotonolactone (Fluka Chemika-Biochemika, Buchs, Switzerland). The purity of the custom-made radioligand was determined by thin-layer chromatography [28]. [^{3}H]GABA or 4-amino-[2,3-^{3}H]butyric acid (sp. act. 91.4 Ci/mmol) was obtained from New England Nuclear (Boston, MA, U.S.A.). All other drugs and reagents were obtained from commercial sources and were of the highest possible purity.

Animals

Male Sprague–Dawley rats (Harlan, Indianapolis, IN, U.S.A.) weighing 250–300 g were used for all experiments. These animals were housed singly, with *ad lib.* access to food and water, and maintained on a 12-hr light/dark cycle. All animals were drug naive. The animals that were utilized for the pertussis toxin studies described below had a cannula implanted stereotaxically in the lateral ventricle under halothane anesthesia. The coordinates utilized for this placement were obtained from Paxinos and Watson [29]: AP: −0.92 mm; ML: 1.8 mm; DV: 3.60 mm. The animals were rested for 7 days prior to intracerebroventricular administration of pertussis toxin.

Preparation of Brain for Binding Studies

Animals were killed, and the brains were excised and chilled in isopentane at −40° for 60 sec. Brains were then mounted on a cryostat chuck, allowed to equilibrate to −15°, and cut into coronal sections of 20 μm. The sections were thaw-mounted onto gelatin-coated slides and stored at −80° until either [^{3}H]GHB or [^{3}H]$GABA_B$ binding was performed.

[^{3}H]GHB Autoradiography

[^{3}H]GHB binding was performed by a modification of the method described earlier [16]. Tissue sections were thawed at room temperature for 1 hr, preincubated in 100 mM phosphate buffer (pH 6.0) for 30 min at 4°, and dried under a stream of cold air. Tissue sections were incubated in triplicate in the same buffer containing 25 nM [^{3}H]GHB for 30 min at 4°. Because the GHB transport system is strongly Na^+ dependent [9], non-specific binding was determined in the presence of 5 mM unlabeled Na^+-free GHB. After incubation, there were three successive washes (10 sec/wash) at 4° in buffer followed by a dip in ice-cold water. The slides were then dried in a stream of cold air.

[^{3}H]$GABA_B$ Autoradiography

[^{3}H]$GABA_B$ binding was carried out by the method described by Bowery *et al.* [17]. Briefly, slides were preincubated in Tris–HCl (pH 7.4) containing 190 mM sucrose and 2.5 mM $CaCl_2$ for 40 min at room temperature. After air drying, the sections were covered for 20 min at room temperature with 100 μL of incubation buffer containing 50 nM [^{3}H]GABA and 100 μM isoguvacine. Nonspecific binding was determined in the presence of 100 μM (−)-baclofen. Following incubation, each section was rinsed rapidly in fresh buffer for 2–3 sec and then air dried.

Analysis of Binding

Dried tissue sections were apposed to tritium-sensitive film (Amersham) with [^{3}H] microscale standards (Amersham) for 3 weeks at room temperature. Following exposure to tritium-sensitive film, the tissue sections were stained with cresyl violet. The films were developed in D-19 (Kodak), fixed, and dried. Quantitative analysis of the resulting au-

toradiograms was performed densitometrically using a microcomputer-based densitometer system (MCID; Imaging Research, Ontario, Canada). Briefly, a standard curve between the O.D. of [^{3}H] standards and tissue radioactivity equivalents was constructed using a non-linear regression analysis. Tissue O.D. values were measured and the O.D. values converted to femtomoles bound per milligram of protein using the standard curve. Five to eight readings were determined and averaged for each anatomic area analyzed. Cresyl violet stained tissue sections were overlayed upon the corresponding audioradiogram and anatomic areas identified with the assistance of the atlas of Paxinos and Watson [29]. Because the spike wave discharges in the GHB model emanate from the thalamus and cortex [26, 27], [^{3}H]GHB and [^{3}H]$GABA_B$ binding density was analyzed in these structures. In addition, particular attention was paid to the CA1 region of the hippocampus because this is the site of the highest density of [^{3}H]GHB binding [16], yet this region is not involved in the genesis of GHB-induced absence seizures [26, 27].

Experimental Design

The regional affinity of GHB and the specific GHB antagonist, NCS 382 [23], for the [^{3}H]$GABA_B$ binding site was determined. Also, the effects of the specific $GABA_B$ receptor agonists 3-APPA [30], (−)-baclofen, the inactive isomer (+)-baclofen, and the specific $GABA_B$ receptor antagonists, CGP 35348, phaclofen, 2-OH saclofen [31], and SCH 50911 [32, 33], in the [^{3}H]GHB autoradiographic binding assay were ascertained. In addition, because the $GABA_B$ receptor is known to be coupled to G proteins [25] and, therefore, guanine nucleotides modulate binding to the $GABA_B$ receptor [24], the effects of GTP and of the non-hydrolyzable GTP analogues GTPγS and Gpp(NH)p on regional [^{3}H]GHB and [^{3}H]$GABA_B$ binding in rat brain were determined. The concentration of all drugs except GHB in these experiments was 100 μM. The concentration of GHB in all competition experiments was 250 μM because this is the threshold concentration in brain associated with the onset of absence seizures in the GHB model of generalized absence seizures [34]. The concentration of drugs used in concentration–response experiments to examine potency ranged from 10^{-9} to 10^{-3} M. Finally, in another series of experiments rats were implanted with intraventricular cannulae and 24 hr later were administered either 1.5 μg pertussis toxin or BSA intracerebroventricularly [25]. Three days later, the animals were killed, the brains were removed, and regional [^{3}H]GHB and [^{3}H]$GABA_B$ binding was measured.

Data Analysis

All data were expressed as the arithmetic mean ± SEM. In all autoradiographic binding studies done in slide-mounted tissue sections, nonspecific binding images were subtracted from adjacent total binding images to determine specific binding, and 5–8 O.D. readings were taken for each anatomic area analyzed. For the kinetic analysis of the effect of GTPγS on [^{3}H]$GABA_B$ binding in frontal cortex, the method of Chu *et al.* [35] was utilized. More specifically, the affinity (K_D) and density (B_{max}) of [^{3}H]$GABA_B$ binding was determined using the method of isotopic dilution of [^{3}H]GABA (1–30 nM) with non-radioactive GABA. The range of free [^{3}H]GABA concentrations spanned from 1 nM to 1 μM. Nonspecific binding was determined in serially adjacent sections for each concentration of free radioactive [^{3}H]GABA. Values of bound [^{3}H]GABA were quantified in the frontal cortex. These were used to construct Scatchard plots that were analyzed by the computer curve-fitting program LIGAND [36].

For the kinetic analysis of the effect of GTPγS on [^{3}H]GHB binding in frontal cortex, the method of Banerjee *et al.* [26] was employed. The K_D and B_{max} of [^{3}H]GHB binding were determined using a concentration of [^{3}H]GHB that ranged from 2 to 500 nM. As in the [^{3}H]GABA binding experiments, nonspecific binding was determined in serially adjacent sections for each concentration of free radioactive [^{3}H]GHB. Values of bound [^{3}H]GHB were quantified in the frontal cortex, and Scatchard analysis was performed using the curve-fitting program EBDA [37]. The N for each group of kinetic experiments was six. Levels of significance were determined by Dunnett's two-tailed test for multiple comparisons [38].

RESULTS

There were no visible images in the nonspecific autoradiograms of [^{3}H]GHB or [^{3}H]$GABA_B$ binding observed. The pattern of [^{3}H]GHB binding was charcterized by a high density of binding in the CA1 region of hippocampus, septum, and superficial (I–III) laminae of frontal, parietal, and temporal cortex (Fig. 1A). The distribution of [^{3}H]$GABA_B$ binding (Fig. 1B) differed markedly from that of [^{3}H]GHB binding. There was a greater intensity of [^{3}H]GHB binding

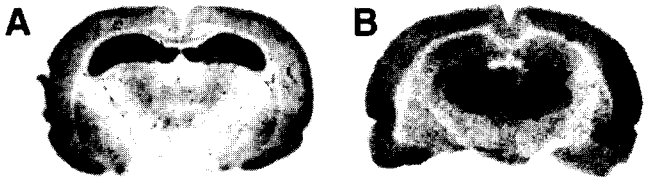

FIG. 1. Autoradiograms of [^{3}H]GHB (A) and [^{3}H]$GABA_B$ (B) binding in a rat brain coronal section −2.5 mm from the bregma [29]. These were 20-μm sections incubated with either 25 nM [^{3}H]GHB or 50 nM [^{3}H]GABA as described in Materials and Methods. The pattern of [^{3}H]GHB binding was characterized by a high density of binding in the hippocampus and superficial (I–III) laminae of cerebral cortex, with less intense binding seen in the ventrobasal nuclei of the thalamus. [^{3}H]$GABA_B$ binding was characterized by a high density of binding in thalamus and the superficial laminae of cerebral cortex. There was colocalization of [^{3}H]GHB and [^{3}H]GABA binding in superficial laminae of cortex and ventrobasal thalamus.

in the hippocampus (Fig. 1A) vs a greater intensity of [^{3}H]GABA$_B$ binding in the thalamus (Fig. 1B); however, there was high intensity and colocalization of both [^{3}H]GABA$_B$ and [^{3}H]GHB binding in laminae I–III of cortex and in ventrobasal thalamus (Fig. 1, A and B), those regions from which the spike wave discharges emanate in the GHB model of absence seizures [26, 27].

As shown in Table 1, there was no significant affinity detected of GHB or its specific antagonist for the [^{3}H]GABA$_B$ binding site. The results of the converse experiments in which the ability of GABA$_B$ agonists and antagonists to compete for [^{3}H]GHB binding was determined are shown in Table 2 and Fig. 2. While there was no significant competition for [^{3}H]GHB binding by (−)-baclofen, nor by the specific antagonists CGP 35348 or SCH 50911, phaclofen, 2-OH saclofen, and 3-APPA showed a weak affinity for the [^{3}H]GHB binding site (Table 2; Fig. 2). The inhibition of [^{3}H]GHB binding by phaclofen and 2-OH saclofen was most significant in the superficial laminae of frontal cortex (Figs. 3 and 4).

GTP and its analogues resulted in a significant decrease in [^{3}H]GABA$_B$ binding in all areas of brain examined. However, incubation with GTPγS resulted in a significant increase in [^{3}H]GHB binding localized to the superficial layers of frontal cortex (Table 3). Pertussis toxin treatment resulted in a marked and significant decrease in [^{3}H]GABA$_B$ binding in all brain areas examined, and a less pronounced decrease in [^{3}H]GHB binding, which achieved statistical significance only in the frontal cortex (Table 3). Kinetic studies of [^{3}H]GHB and [^{3}H]GABA$_B$ binding showed that the observed change in binding in the presence of guanine nucleotides was secondary to a decreased affinity of the [^{3}H]GABA$_B$ binding site for GABA and an increased affinity of the [^{3}H]GHB binding site for GHB (Table 4). The nonhydrolyzable GTP analogue GTPγS reversed the ability of 3-APPA, phaclofen, phaclofen, and 2-OH saclofen to compete for [^{3}H]GHB binding (Table 2).

DISCUSSION

GHB induces absence seizures that are blocked by GHB antagonists and GABA$_B$ antagonists and exacerbated by GABA$_B$ agonists; however, GABA$_B$ agonists alone do not induce absence seizures [39–43]. Although GHB is metabolically derived from GABA and is structurally similar to that compound, GHB does not seem to be a GABA agonist. GHB fails to compete for binding at the GABA$_A$ site [44, 45], nor do GABA$_A$ agonists or antagonists compete for binding at the GHB site [8, 28]. Although GHB has been reported specifically to displace bound [^{3}H]baclofen and [^{3}H]CGP 27492, a potent GABA$_B$ receptor ligand, from GABA$_B$ sites in cortical and thalamic rat brain homogenates with a low affinity [15], there are a number of lines of evidence, several generated in the current group of experiments, which mitigate against the hypothesis that GHB is a simple GABA$_B$ agonist that induces absence seizures by acting directly upon the postsynaptic GABA$_B$ receptor.

TABLE 1. [^{3}H]GABA$_B$ binding in laminae I–III of frontal cortex

Treatment	Concentrations	[^{3}H]GABA$_B$ binding (fmol/mg protein)
Control		338 ± 31
GHB	250 μM	302 ± 27
	500 μM	349 ± 32
	1 mM	328 ± 29
NCS 382	100 μM	288 ± 29
(−)-Baclofen	100 μM	81 ± 9*

Values are means ± SEM; N = 6, each performed in triplicate. Compounds were incubated in the concentrations shown with tissue sections in the presence of 50 nM [^{3}H]GABA as described in Materials and Methods.

Nonspecific binding was ≤ 10%.

* Significantly ($P < 0.05$) decreased from control (no drug) binding.

First, the ontogeny of [^{3}H]GHB binding sites is distinctly different from that of [^{3}H]GABA$_B$ sites. [^{3}H]GHB binding in rat brain does not appear until the third postnatal week of life, whereas [^{3}H]GABA$_B$ binding is present at birth [12]. More importantly, GHB-induced absence seizures emerge only with the appearance of GHB receptors, not with the appearance of GABA$_B$ receptors [12].

Second, concentration–response data in electrophysiologic experiments suggest that the concentration of GHB in brain required to mimic the postsynaptic effects of baclofen is in the millimolar range [18, 19]. However, the brain concentrations of GHB associated with the onset of GHB-induced generalized absence seizures in the GHB model of this disorder is in the micromolar range [34]. A similar concentration–response relationship has been shown in regard to putative presynaptic effects of GHB. The concentration of GHB associated with significant alterations in basal GABA release and K^+-stimulated GABA and glutamate release in *in vivo* microdialysis experiments was 250 μM [46].

Third, pharmacological and quantitative differences have been demonstrated between GHB and (−)-baclofen in their ability to reduce basal and K^+-stimulated GABA and glutamate release [46]. Perfusion of GHB into the thalamus inhibited the basal release of GABA in a concentration-dependent fashion with a significant decrease in basal GABA release at the lowest concentration of GHB tested, 250 μM, the same concentration associated with GHB-induced absence seizures. However, while the basal release of glutamate was not altered significantly by GHB perfusion, K^+-evoked release of both GABA and glutamate was decreased significantly by all concentrations of GHB tested. (−)-Baclofen perfusion into the thalamus produced a significant concentration-dependent decrease in both basal and K^+-evoked release of GABA and glutamate. The postsynaptic GABA$_B$ receptor antagonist CGP 35348 antagonized the effect of (−)-baclofen and GHB on K^+-evoked glutamate release, but had no apparent effect on either GHB- or (−)-baclofen-induced changes in basal or K^+-evoked GABA release. Perfusion of the presynaptic

TABLE 2. Effects of $GABA_B$ agonists and antagonists on [^{3}H]GHB binding in frontal cortex

Treatment	Concentrations	[^{3}H]GHB binding (fmol/mg protein)	% Total binding
Control		422 ± 28	
(−)-Baclofen	100 μM	399 ± 31	97 ± 0.2
CGP 35348	100 μM	409 ± 60	95 ± 0.6
2-OH Saclofen	100 μM	243 ± 15*	69 ± 6.6*
Phaclofen	100 μM	233 ± 17*	58 ± 4.9*
3-APPA	100 μM	269 ± 12*	66 ± 4.6*
GTPγS + phaclofen	100 μM (of each)	374 ± 16	87 ± 4.1
GTPγS + 2-OH saclofen	100 μM (of each)	385 ± 24	91 ± 3.2
GTPγS + 3-APPA	100 μM (of each)	393 ± 16	93 ± 3.4

Each value represents the mean ± SEM of six animals, each performed in triplicate. Compounds were incubated in a concentration of 100 μM with tissue sections in the presence of 25 nM [^{3}H]GHB as described in Materials and Methods. Nonspecific binding was <10% in all experiments.

* Significantly lower than control at $P < 0.01$.

$GABA_B$ receptor antagonist phaclofen into thalamus produced a blockade of GHB- and (−)-baclofen-induced changes in basal and K^+-induced GABA extracellular levels, but had no effect on either GHB- or (−)-baclofen-induced alterations in glutamate release [46].

The differences between CGP 35348, phaclofen, and 2-OH saclofen in regard to their ability to compete for [^{3}H]GHB binding in the current experiments are relevant to the *in vivo* microdialysis data described above, particularly in view of recent electrophysiological and biochemical studies that show a differing sensitivity of presynaptic $GABA_B$ receptor function to (−)-baclofen, 3-APPA, phaclofen, and CGP 35348 [20–22, 47]. Phaclofen, an antagonist at presynaptic $GABA_B$ receptors [20], antagonizes (−)-baclofen-induced inhibition of GABA release without influencing (−)-baclofen-induced inhibition of glutamate release. Alternatively, the postsynaptic $GABA_B$ receptor antagonist CGP 35348 [20] blocks the effect of (−)-baclofen on glutamate release, but has little effect

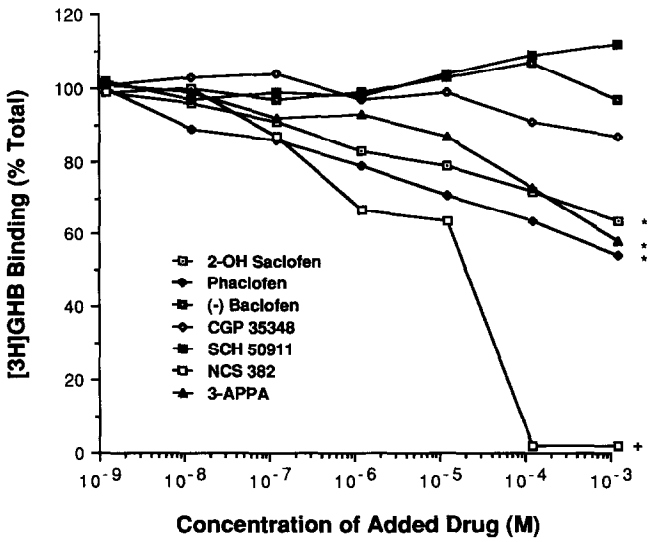

FIG. 2. Concentration–response curves for the effect of $GABA_B$ agonists and antagonists and a GHB antagonist on [^{3}H]GHB binding in frontal cortex. Each point represents the mean of six animals, each of which was determined in triplicate. Compounds were incubated (1 nM to 1 mM) with tissue sections in the presence of 25 nM [^{3}H]GHB as described in Materials and Methods. Standard error was ≤10% for each point and is not shown. [^{3}H]GHB binding in frontal cortex was inhibited significantly by NCS 382, 3-APPA, phaclofen, and 2-OH-saclofen. Key: (*) $P < 0.05$; and (+) $P < 0.001$.

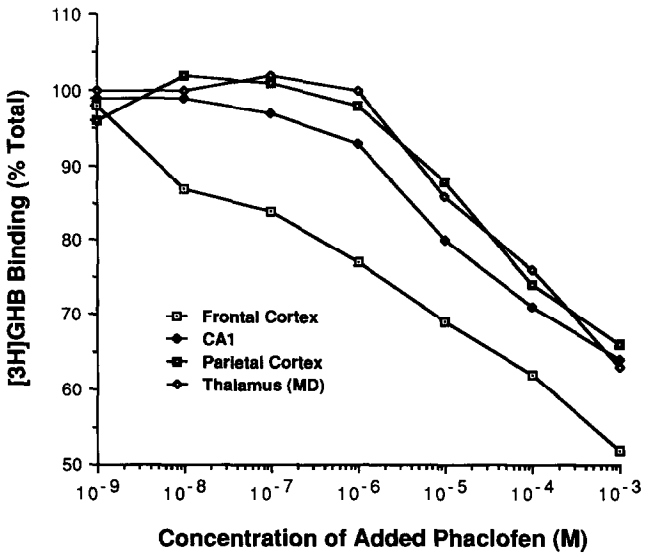

FIG. 3. Concentration–response curves for the regional effect of phaclofen on [^{3}H]GHB binding in rat brain. Each point represents the mean of six animals, each of which was determined in triplicate. Phaclofen was incubated (1 nM to 1 mM; see Fig. 2) with tissue sections in the presence of 25 nM [^{3}H]GHB as described in Materials and Methods. Standard error was ≤10% for each point and is not shown. The inhibition of [^{3}H]GHB binding was significantly greater in frontal cortex than other brain regions at all concentrations of phaclofen > 10^{-8} M ($P < 0.05$). CA1 refers to the region of hippocampus analyzed; MD refers to the mediodorsal nucleus of the thalamus.

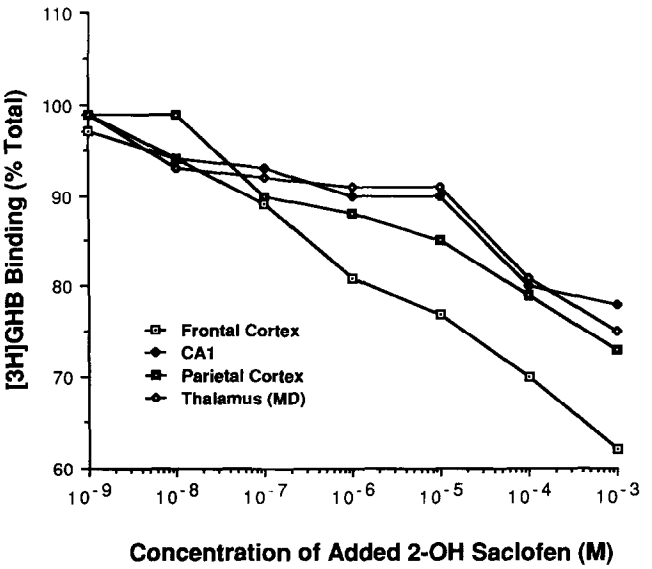

FIG. 4. Concentration–response curves for the regional effect of 2-OH saclofen on [^{3}H]GHB binding in rat brain. Each point represents the mean of six animals, each of which was determined in triplicate. 2-OH saclofen was incubated (1 nM to 1 mM; see Fig. 2) with tissue sections in the presence of 25 nM [^{3}H]GHB as described in Materials and Methods. Standard error was ≤10% for each point and is not shown. The inhibition of [^{3}H]GHB binding was significantly greater in frontal cortex than other brain regions at all concentrations of 2-OH saclofen >10^{-7} M ($P < 0.05$). CA1 refers to the region of hippocampus analyzed; MD refers to the mediodorsal nucleus of the thalamus.

on (−)-baclofen-induced changes in GABA release [18–20].

Fourth, the results of the present experiments also fail to support the hypothesis that GHB exerts its epileptogenic effect by virtue of being a direct agonist at the postsynaptic $GABA_B$ receptor. The current data (see Fig. 1) are in agreement with other published experiments [12] showing that [^{3}H]GHB and [^{3}H]$GABA_B$ binding sites have a different regional anatomic distribution in rat brain with the only brain regions common to both being the superficial laminae of cerebral cortex and ventrobasal thalamus. This may represent a coincidental finding if GHB induces absence seizures by an action at its own specific sites while still binding as an agonist to $GABA_B$ receptors; however, the importance of the colocalization of [^{3}H]GHB and [^{3}H]$GABA_B$ binding to superficial laminae of cerebral cortex and ventrobasal thalamus relates to the fact that these are the brain regions where spike wave discharges originate in the GHB model of generalized absence seizures [26, 27]. Moreover, in addition to showing that the $GABA_B$ agonist (−) baclofen has no affinity for the [^{3}H]GHB binding site, the present experiments also demonstrated that neither GHB nor its antagonist, NCS 382, displace [^{3}H]GABA in the [^{3}H]$GABA_B$ autoradiographic binding assay.

The current study, utilizing autoradiographic binding techniques and more specific $GABA_B$ agonists and antagonists, both confirms previous studies showing that (−)-baclofen has no demonstrable affinity for [^{3}H]GHB binding [8, 28] and suggests that there is heterogeneity in regard to the ability of 3-APPA and some $GABA_B$ antagonists to compete for [^{3}H]GHB binding. CGP 35348 and SCH 50911 were inactive while phaclofen and 2-OH saclofen appeared to displace [^{3}H]GHB from its binding site, albeit with a low affinity (Table 2). Moreover, this displacement of [^{3}H]GHB binding was most significant in the frontal

TABLE 3. Effects of GTP, its nonhydrolyzable analogues, and pertussis toxin on [^{3}H]GHB and [^{3}H]$GABA_B$ binding

Treatment	Regions	Binding (fmol/mg protein) [^{3}H]$GABA_B$	[^{3}H]GHB
Control	Frontal cortex	271 ± 22	284 ± 7.9
	Parietal cortex	323 ± 8	304 ± 15
	Thalamus (MD)	297 ± 11	137 ± 7.2
	CA1	115 ± 3	536 ± 32
GTP	Frontal cortex	69 ± 6.1*	261 ± 18
	Parietal cortex	99 ± 7.8*	325 ± 13
	Thalamus (MD)	98 ± 8.2*	145 ± 7.3
	CA1	32 ± 2.1	545 ± 45
GTPγS	Frontal cortex	37 ± 7.0*	347 ± 15†
	Parietal cortex	78 ± 5.7*	334 ± 19
	Thalamus (MD)	45 ± 3.1*	166 ± 23
	CA1	17 ± 2.7*	591 ± 51
Gpp(NH)p	Frontal cortex	49 ± 5.6*	329 ± 18
	Parietal cortex	86 ± 6.9*	359 ± 22
	Thalamus (MD)	60 ± 5.8*	155 ± 26
	CA1	31 ± 3.6*	576 ± 63
Pertussis toxin	Frontal cortex	46 ± 8.7*	244 ± 11*
	Parietal cortex	93 ± 26*	270 ± 19
	Thalamus (MD)	144 ± 34*	119 ± 12
	CA1	58 ± 13*	492 ± 35

Each value represents the mean ± SEM of six experiments, each performed in triplicate. GTP and its nonhydrolyzable analogues were incubated with tissue sections in a concentration of 100 μM as described in Materials and Methods. Animals were treated with pertussis toxin intracerebroventricularly as described in Materials and Methods. The concentrations of [^{3}H]GABA and [^{3}H]GHB were 50 and 25 nM, respectively.

MD = mediodorsal nucleus of the thalamus.

* Significantly lower than control at $P < 0.001$ for the [^{3}H]$GABA_B$ binding experiments and $P < 0.03$ for the [^{3}H]GHB binding experiments.

† Significantly higher than control at $P < 0.03$.

TABLE 4. Effect of GTPγS on K_d and B_{max} of [^{3}H]$GABA_B$ and [^{3}H]GHB binding in frontal cortex

	K_D (nM)	B_{max} (pmol/mg protein)
[^{3}H]$GABA_B$, control	354 ± 55	3.22 ± 0.28
[^{3}H]$GABA_B$, GTPγS	615 ± 86*	3.25 ± 0.35
[^{3}H]GHB, control	81.6 ± 7.2	0.190 ± 0.015
[^{3}H]GHB, GTPγS	67.8 ± 5.9†	0.185 ± 0.019

GTPγS was incubated with tissue sections in a concentration of 100 μM as described in Materials and Methods. Kinetic analysis of [^{3}H]$GABA_B$ binding was done by the method of Chu *et al.* [35] and that of [^{3}H]GHB binding by the method of Banerjee *et al.* [26] (see Materials and Methods). Values are means ± SEM, N = 6.

* Significantly increased from control ($P < 0.05$).

† Significantly decreased from control ($P < 0.05$).

cortex (Figs. 3 and 4), a region intimately involved in the generation of GHB-induced absence seizures [26, 27].

The $GABA_B$ receptor is bound to G protein [24, 25, 48] and is thought to gate K^+ and Ca^{2+} channels through a G-protein-mediated mechanism either directly or via second messenger systems [49]. The rationale for the use of guanine nucleotides in the current binding studies relates to this finding and to the fact that a G-protein coupled receptor agonist such as GABA has a higher affinity for that conformation of the $GABA_B$ receptor which binds most tightly to a $G_{\alpha\beta\gamma}$ with no bound nucleotide. When added, a guanine nucleotide such as GTP, GDP, or a hydrolysis-stable guanine nucleotide analogue such as Gpp(NH)p or GTPγS, binds to the vacant site on the G-protein α subunit. The transition state of the $G_{\alpha\beta\gamma}$ is thus lost, and a weakened association between the receptor and the $G_{\alpha\beta\gamma}$ occurs. The end result is decreased binding of agonist to the receptor because of a decreased affinity [50]. In this way, guanine nucleotides may decrease the affinity of the $GABA_B$ receptor for GABA [24].

A similar rationale was employed for the pertussis toxin studies since this compound inhibits $G_{i\alpha}$ and $G_{o\alpha}$ via ADP-ribosylation [51]. A response to pertussis toxin, therefore, is considered presumptive evidence of G-protein modulation of the activity being studied. Since the GABA receptor is known to be coupled to a G protein, [^{3}H]$GABA_B$ binding is reduced in the presence of pertussis toxin [24]. The hypothesis of presynaptic $GABA_B$ receptor heterogeneity described above is relevant to these G-protein–$GABA_B$ binding data because it is supported by experimental evidence regarding pertussis toxin sensitivity of $GABA_B$ receptors. The $GABA_B$ receptor located on presynaptic GABAergic inhibitory nerve terminals may be linked to a pertussis toxin-sensitive G protein, whereas those $GABA_B$ receptors located on presynaptic excitatory terminals seem to be both pertussis toxin sensitive and insensitive [52].

We reasoned that if GHB is an agonist that binds to the $GABA_B$ receptor, [^{3}H]GHB binding would be expected to respond to GTP, its nonhydrolyzable analogues, and pertussis toxin in a manner similar to that observed with [^{3}H]$GABA_B$ binding. However, although [^{3}H]$GABA_B$ binding decreased in a global and predictable fashion in the presence of GTP, its nonhydrolyzable analogues, and pertussis toxin, [^{3}H]GHB binding behaved very differently. GTP had no effect, but the non-hydrolyzable analogues of this guanine nucleotide produced a significant increase in [^{3}H]GHB binding due to an increased affinity of the site for GHB. The kinetic studies confirmed the hypothesis detailed above, i.e. that the observed changes in both [^{3}H]GHB and [^{3}H]$GABA_B$ binding in response to GTPγS were due to an alteration of the affinity of the respective binding sites for GHB and GABA.

The explanation for the increased [^{3}H]GHB binding observed in the presence of GTP and its nonhydrolyzable analogues is not clear. Several reports have indicated that binding of antagonists to G-protein coupled receptors may be increased upon addition of guanine nucleotides [50]; however, it is difficult to reconcile the hypothesis that GHB causes absence seizures by acting as an antagonist at the $GABA_B$ receptor with the findings that GHB agonists induce and $GABA_B$ agonists exacerbate generalized absence seizures and both GHB and $GABA_B$ antagonists block or attenuate this phenomenon [39–43].

Although pertussis toxin exposure resulted in a decrease of both [^{3}H]GHB and [^{3}H]$GABA_B$ binding in frontal cortex, there was a great difference in terms of the magnitude of the effect. Pertussis toxin-treated animals showed an 83% decrease in [^{3}H]$GABA_B$ binding in frontal cortex compared with only a 14% decrease in [^{3}H]GHB binding in the same region. This pertussis toxin effect on [^{3}H]GHB binding in frontal cortex achieved statistical significance ($P < 0.05$), but was not different in magnitude from [^{3}H]GHB binding in other parts of the brain in pertussis toxin-treated animals. Contrary to [^{3}H]GHB binding in pertussis toxin-treated animals which was decreased significantly only in frontal cortex, there was a marked and global decrease of [^{3}H]$GABA_B$ binding throughout the brains of animals exposed to pertussis toxin.

In summary, these data do not support the hypothesis that the [^{3}H]GHB binding site and the [^{3}H]$GABA_B$ receptor are identical, nor do they give credence to the theory that GHB induces absence seizures solely by acting at the postsynaptic $GABA_B$ receptor. However, even though the GHB site and the $GABA_B$ receptor appear to be separate from one another in many respects, both seem to be involved in the modulation of neurotransmitter release [46] and in the pathogenesis of experimental absence seizures. Evidence for the latter may be found in experiments which show that GHB induces and $GABA_B$ agonists exacerbate absence seizures, while both specific GHB and $GABA_B$ receptor antagonists block the occurrence of spike wave discharges in experimental absence seizure models [39–43]. An hypothesis that may reconcile these disparate binding, microdialysis, and electrophysiologic data is that the GHB site operative in the GHB model of generalized absence seizures is an isoform of the presynaptic $GABA_B$ receptor. Alternatively, the GHB binding site, or a subgroup of this binding site, may be independent of the $GABA_B$ receptor, i.e. GHB may be an agonist at some $GABA_B$ receptors, but induce absence seizures by acting at independent GHB sites. This hypothesis is supported by the divergent binding results and the differential effects of manipulating G proteins shown in these experiments as well as by electrophysiological studies that demonstrate differences between GHB and baclofen. In this scenario, the potentiating effects of $GABA_B$ agonists and GHB on absence seizure models, as well as the similar effects of these compounds on neurotransmitter release, could be effected through a common downstream pathway.

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