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Characterization of γ-Aminobutyric Acid Receptor Binding in Cultured Brain Cells

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SUMMARY

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The binding of the inhibitory neurotransmitter γ-[^{3}H]aminobutyric acid to its receptor sites was investigated in extensively washed membranes of primary brain cultured cells. Extensive washing of the brain cultured cells was necessary to eliminate the endogenous inhibitors of γ-aminobutyric acid receptor binding. The binding of γ-[^{3}H]aminobutyric acid to cultured brain cells was specific, rapid, and saturable. Scatchard analysis of the binding data revealed the presence of two classes of sites. The high-affinity site had a K_{D1} of 9 nM and a B_{max1} of 380 fmol/mg protein, and the low-affinity site had a K_{D2} of 250 nM and a B_{max2} of 2400 fmol/mg protein. The specific binding was displaced by muscimol (IC_{50} = 0.005 μM), γ-aminobutyric acid (IC_{50} = 0.025 μM), and (+)-bicuculline (IC_{50} = 4 μM). Specific binding was not inhibited by nipecotic acid, picrotoxin, or pentobarbital. On the basis of saturation, classes of sites, affinity constants, and ligand specificity, it appears that cultured brain cells have γ-aminobutyric acid receptors which appear identical to those found in mammalian brain.

INTRODUCTION

γ-Aminobutyric acid (GABA),[1] a major neurotransmitter in the mammalian central nervous system (CNS), produces its inhibitory responses by activating receptor-linked chloride ionophores and thereby increasing the permeability of neuronal membranes to chloride ions (1–3). Several lines of evidence suggest that a variety of centrally acting drugs, like benzodiazepines (4), barbiturates (5, 6), and convulsants (7, 8), may act via the GABA receptor–ionophore system. There is also evidence that other macromolecular structures, in addition to the receptor and ionophore components, may be involved in the regulation of GABA receptor–ionophore activity (9).

Due to the complex cellular heterogeneity of the mammalian CNS, elucidation of the molecular mechanisms involved in the physiological and pharmacological events at the GABA synapse has not been achieved. Although the binding of GABA to its receptor sites has been extensively studied in whole brain homogenates, chloride permeability changes following agonist occupancy of the GABA receptors have been demonstrated in invertebrates like crayfish (3); however, in the mammalian CNS, they have yet to be demonstrated.

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[1] Abbreviation used: GABA, γ-aminobutyric acid.

In contrast to brain homogenates, neuronal tissue cultures offer a cell population which maintains the characteristics of neuronal activity. The neuronal cell cultures are electrically excitable (10), possess mechanisms for synthesis, uptake, and release of neurotransmitters (11), and have been used for analyzing synaptic transmission and drug action (6, 7, 12). In the present manuscript, we describe the binding characteristics of GABA to receptor-like sites in brain cells grown in tissue culture. Our results indicate that the properties of the GABA binding sites in cultured brain cells are similar to those found in mammalian brain.

MATERIALS AND METHODS

[^{3}H]GABA (65–66 Ci/mmol) was purchased from Amersham (Arlington Heights, Illinois), muscimol was from Research Organics (Cleveland, Ohio), and other chemicals were from Sigma Chemicals (St. Louis, Missouri). Isoguvacine and THIP were gifts from Dr. Krogsgaard-Larsen, and (±)-nipecotic acid was a gift from Dr. G. A. R. Johnston.

Primary brain cultured cells. Cultures of dissociated mouse brain were prepared by a modification of previously published methods (10, 12). Briefly, 13- to 14-day-old embryos were removed from pregnant C57BL/6J mice which had been sacrificed by cervical dislocation. Brains were rapidly dissected from the embryos and

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minced in sterile saline (D_1; Colorado Serum) with iridectomy scissors under sterile conditions in a laminar flow hood. The fragments were further dissected by titration with a Pasteur pipet. The final suspension contained the equivalent of 0.4 brain/ml. The dissociated cells were added in 5-ml aliquots to collagen-coated sterile 60-mm Falcon tissue culture plates. The freshly plated cultures were incubated for 2 days in a nutrient medium consisting of Eagle's medium (MEM; GIBCO) modified with heat-inactivated (56°C × 30 min) horse serum (GIBCO; 10%, v/v), fetal calf serum (Colorado Serum; 10%, v/v), glucose (500 mg%), and $NaHCO_3$ (1.5 g/liter), the latter added to increase the buffering capacity and allow incubation in a humid, 90% air:10% CO_2 atmosphere. After 2 days in culture, the cells were changed to a medium containing 10% heat-inactivated horse serum–MEM, 2′-deoxy-5-fluorouridine, and uridine (final concentrations: 15 and 35 μg/ml, respectively). 2′-Deoxy-5-fluorouridine was added to control the rapid division of nonneuronal fibroplastic elements. Following 2 days of incubation in this medium, the cultures were again changed to a medium containing 10% heat-inactivated horse serum–MEM in which they remained for about 2.5 weeks prior to harvesting, with twice weekly changes of medium. Twenty-one days after initiating the cultures, the medium was removed and the cells were rinsed with ice-cold 0.05 M Tris–HCl buffer (pH 7.4). Cells were removed from the culture plates with a plastic spatula and added to a centrifuge tube containing Tris–HCl (1 ml/plate). The cells were then pelleted at 1000*g* for 5 min and frozen on dry ice.

Preparation of cell membranes. Primary brain cultured cells were thawed, homogenized in 0.32 M sucrose, and centrifuged at 480*g* for 10 min. The resulting supernatant was centrifuged at 100,000*g* for 45 min to collect the particulate fraction (high-speed pellet). The pellet was osmotically shocked by homogenizing in double-distilled iced H_2O and repelleted as before. Osmotic shock treatment was repeated, the cell homogenate was recentrifuged as before, and the pellet was resuspended in 0.05 M Tris–citrate buffer (pH 7.1) and frozen overnight. The cell homogenate was thawed, repelleted, washed once in Tris buffer, and frozen in the same buffer. On the day of the assay, the pellet was thawed, repelleted, washed once in the buffer (referred to as the last wash), and resuspended in the same buffer at a protein concentration of 0.3–0.6 mg/ml for binding studies. The freeze–thaw and extensive washing procedures are necessary to eliminate the endogenous inhibitors of GABA receptor binding (see Results; 13). These procedures also eliminate the need for using Triton X-100 during tissue preparation (13–15).[2] All tissue preparation and other assays were performed at 0–4°C and using Na^+-free buffer. Protein was estimated by the method of Lowry *et al.* (16).

GABA binding studies. [^{3}H]GABA binding was studied by a modification of the centrifugation assay of Enna and Snyder (17), as previously described for mammalian brain (13–15). Aliquots of cultured brain cell membranes (0.3–0.6 mg/ml) were incubated in triplicate with [^{3}H]-GABA (65 Ci/mmol) for 10 min at 0–4°C, in a total volume of 1 ml. For routine binding experiments, a 2 nM [^{3}H]GABA concentration was used. For saturation isotherms, the concentration of [^{3}H]GABA (65 Ci/mmol) was varied for the low concentration (0.5–4 nM) points, and for the high GABA concentration points (25–504 nM), nonradioactive GABA was varied and [^{3}H]GABA was maintained constant at 4 nM. Following incubation, the vials were centrifuged at 48,000*g* for 10 min in a JA 20.1 rotor, the supernatant was decanted, and the pellet was rapidly rinsed with iced buffer. The pellet was then solubilized overnight in 0.2 ml of Soluene-350 (Packard), and radioactivity was estimated in 3 ml of toluene containing 5 g/liter of 2,5-diphenyloxazole. The background determined in the presence of 0.1 mM nonradioactive GABA was subtracted from the total radioactivity in the pellet to obtain the specific binding. Specific binding usually represented 75 ± 6% of the total binding activity in the pellet. In displacement experiments, a concentration of 2 nM [^{3}H]GABA was used and various concentrations of the ligands were added to the vials prior to the addition of the cell homogenate. The efficiency of the counting was 40.5 ± 1%.

The binding data were plotted by Scatchard plots. The Scatchard plots were curvilinear and were subjected to the graphic analysis methods of Rosenthal (18) and Feldman (19) for one ligand and two binding sites, to obtain the values of affinities (K_D's) and the number of binding sites (B_{max}).

RESULTS

Initial binding studies revealed that [^{3}H]GABA bound specifically to crude homogenates of cultured brain cells. However, the binding was highly variable and irreproducible. When we prepared the particulate fraction of the cultured brain cells and subjected it to freeze–thaw and extensive washing procedures, as described in Materials and Methods, binding was highly reproducible. Furthermore, the supernatant (S_1) of the high-speed pellet (100,000*g*) of cultured brain cells contained an inhibitory substance(s) which potently inhibited the binding of [^{3}H]GABA to extensively washed brain culture cells and rat brain membranes (Table 1). The supernatant from rat brain membranes also inhibited [^{3}H]GABA binding to cultured brain cells and rat brain membranes (Table 1). The GABA binding inhibitory activity of these supernatants was not destroyed by heating at 95°C for 15 min (Table 1). These findings demonstrate the presence of endogenous inhibitors of GABA receptor binding in brain cells grown in tissue culture and are consistent with similar observations in rat brain (13). The supernatant derived from the last washing of the pellet (see Materials and Methods) had no GABA receptor binding inhibitory activity.

The binding of [^{3}H]GABA to extensively washed brain cultured cells reached an equilibrium within 5–10 min (Fig. 1). Specifically bound [^{3}H]GABA was linear with protein concentrations in the range of 0.04–0.6 mg, and usually represented 76 ± 6% of the total binding. A typical binding experiment, using 0.2 mg protein and 2 nM [^{3}H]GABA per assay, gave 13,037 ± 780 dpm/mg bound in the absence and 3172 ± 383 dpm/mg in the presence of 0.1 mM nonradioactive GABA (i.e., back-

[2] Ticku, M. K., unpublished observations.

TABLE 1

Effect of the supernatant containing an inhibitory substance(s) on [^{3}H]GABA binding in cultured brain cells and rat brain

Cultured brain cells were prepared as described in Materials and Methods for binding studies. [^{3}H]GABA binding was studied by centrifugation assay using 0.2 mg protein and 2 nM [^{3}H]GABA (65 Ci/mmol). The supernatant (S_1) was obtained by homogenizing cultured brain cells or rat brain with 10 vol of 0.05 mM Tris–citrate (pH 7.1) and centrifuging at 100,000*g* for 45 min. Supernatant (S_1) was added to the incubation vial as such or as a cooled solution after heating at 95°C for 15 min. Binding assays were performed as described in the text. Rat brain membranes were prepared as described elsewhere (13, 15). The values are the mean ± SD or two experiments, each done in triplicate.

Treatment	Specific [^{3}H]GABA binding	
	dpm/mg protein	*% of control*
[^{3}H]GABA binding in cultured brain cells		
Control (0.05 M Tris–citrate, pH 7.1)	9,850 ± 620	100.0
20 μl S_1 cultured brain cells	4,432 ± 640	45.0
50 μl S_1 cultured brain cells	1,182 ± 175	12.0
50 μl S_1 cultured brain cells, 95°C–15 min	1,084 ± 232	11.0
50 μl S_1 rat brain	985 ± 210	10.0
50 μl S_1 rat brain, 95°C–15 min	939 ± 180	9.5
[^{3}H]GABA binding in rat brain membranes		
Control (0.05 M Tris–citrate, pH 7.1)	8,854 ± 407	100.0
50 μl S_1 rat brain	499 ± 110	6.0
50 μl S_1 rat brain, 95°C–15 min	428 ± 90	5.0
50 μl S_1 cultured brain cells	534 ± 78	6.0
50 μl S_1 cultured brain cells, 95°C–15 min	299 ± 112	3.0

ground). The difference of 9865 dpm/mg represents the specific binding.

With increasing concentrations of [^{3}H]GABA, specific binding was saturable (Fig. 2A). A Scatchard plot of the [^{3}H]GABA binding in cultured brain cells was curvilinear, which suggested the presence of at least two classes of binding sites (Fig. 2B). The binding constants were calculated by subjecting the Scatchard data to a one ligand and two binding sites model (assuming no interaction between the sites) by the graphic analyses of

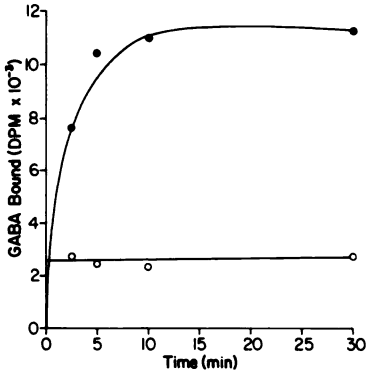

FIG. 1. *Time course of [^{3}H]GABA binding to cultured brain cells*
Cultured brain membranes (0.2–0.3 mg protein) were incubated with 2 nM [^{3}H]GABA (65 Ci/mmol) in the presence (○——○) and absence (●——●) of 0.1 mM nonradioactive GABA. The vials were incubated for various times prior to centrifugation at 48,000*g* for 10 min. Background (○——○) represented approximately 20% of the total binding and remained constant, while specific binding (●——●) reached an equilibrium within 10 min.

Rosenthal (18) and Feldman (19). The high-affinity site had a K_{D1} of 9 ± 3 nM and a B_{max1} of 380 ± 35 fmol/mg protein, and the low-affinity site had a K_{D2} value of 250 ± 30 nM and a B_{max2} of 2400 ± 125 fmol/mg protein (values are means ± SD of three experiments).

Figure 3 shows the displacement of specifically bound [^{3}H]GABA by agonists like muscimol and GABA and by the antagonist (+)-bicuculline. Muscimol was the most potent inhibitor of [^{3}H]GABA binding, displacing 50% of the binding at 0.005 μM, and was fivefold more potent than GABA (Table 2). Other GABA mimetics, like isoguvacine (IC_{50} = 0.11 μM) and THIP (IC_{50} = 0.23 μM), were potent inhibitors of [^{3}H]GABA binding. The potency and the rank order of these ligands in inhibiting [^{3}H]GABA binding in cultured brain cells are in agreement with their reported neurophysiological activity (22) and biochemical potency in displacing GABA binding in mammalian brain (13–15, 17).

Specifically bound [^{3}H]GABA was not displaced by GABA neuronal uptake blockers like nipecotic acid. Similarly, binding was not inhibited by picrotoxinin or pentobarbital. [^{3}H]GABA binding was inhibited by high concentrations (500 μM) of purines like inosine and hy-

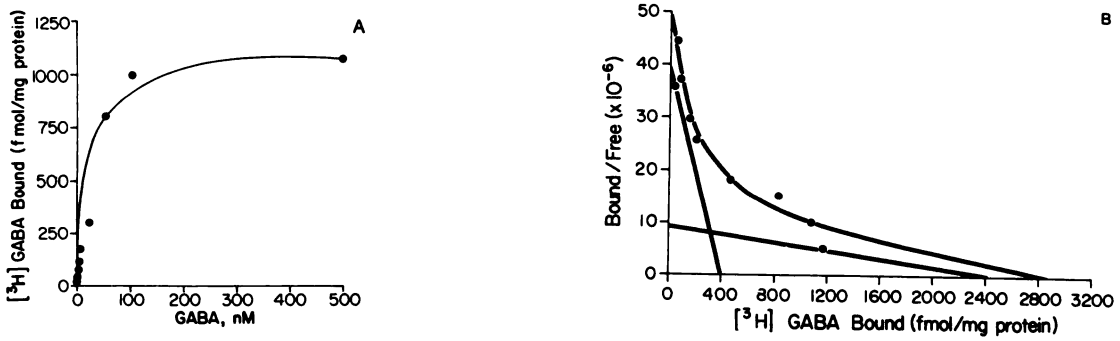

FIG. 2. *Saturation (A) and Scatchard (B) plots of [^{3}H]GABA binding to cultured brain cells*
Cultured brain membranes (as described in Materials and Methods) were incubated with various concentrations of [^{3}H]GABA (0.25–504 nM) for 10 min at 0–4°C and assayed by a centrifugation assay as described in the text. Background was obtained in the presence of 0.1 mM nonradioactive GABA and subtracted from each point. Binding data were plotted as a saturation isotherm (A) and as a Scatchard plot (B). The affinities (K_D's) and binding capacities (B_{max}'s) were obtained by subjecting the Scatchard plots to the graphic analyses of Rosenthal (18) and Feldman (19). Results are the mean values of one experiment (in triplicate) which was repeated three times with similar results.

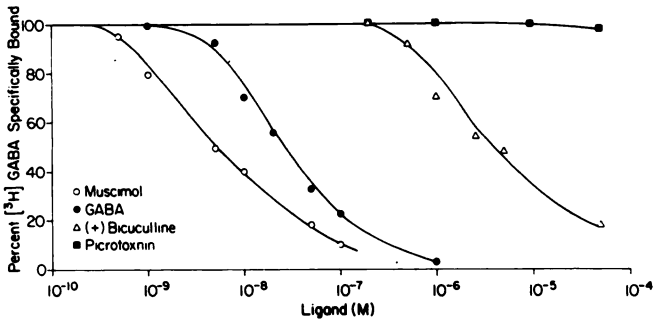

FIG. 3. *Displacement of specifically bound [^{3}H]GABA to cultured brain cells by various ligands*

Cell membranes were incubated with 2 nM [^{3}H]GABA and various concentrations of ligands and with and without 0.1 mM nonradioactive GABA for 10 min at 0–4°C. At each ligand concentration the total binding and background were determined in triplicate. Binding assays were performed as described in the text.

poxanthine in both cultured brain cells and rat brain membranes (Table 2).

DISCUSSION

GABA binding to mammalian brain homogenates has been extensively studied (9, 13–15, 17). Triton-treated brain membranes (9, 17) or freeze–thawed and extensively washed membranes (non-detergent treated) bind GABA to two classes of binding sites (13–15). The heterogeneity of GABA receptor sites has been observed in mouse, rat, bovine, and human (13–15) brains. Neurophysiological and biochemical studies have demonstrated a possible interaction between centrally acting drugs, like benzodiazepines (4) and barbiturates (5, 6), and GABA synaptic events. However, due to the complex and heterogeneous nature of the CNS, it has not been possible biochemically to demonstrate either GABA receptor-linked chloride permeability changes (and possible coupling mechanisms involved) or the exact molecular mechanisms by which drugs affect GABAergic transmission. Neuronal cells grown in tissue culture offer us simple systems for analyzing these events. Cultured brain cells have not yet been characterized by electrophysiological techniques. However, spinal cord cultures grown under identical conditions have electrophysiological properties similar to those of CNS neurons (6, 7, 11, 12, 20).

TABLE 2

IC_{50} values for inhibition of [^{3}H]GABA binding to cultured brain neurons

IC_{50} values were obtained by using 2 nM [^{3}H]GABA and 4–6 concentrations of the displacing ligand. Values are the averages of two experiments, each done in triplicate.

Ligand	IC_{50}	
	μM	
Muscimol	0.005	
GABA	0.025	
Isoguvacine	0.11	
THIP hydrate	0.23	
(+)-Bicuculline	4	
(±)-Nipecotic acid	>100	
Picrotoxinin	>100	
Pentobarbital	>200	
	% Inhibition at 500 μM	
	Cultured brain cells	Rat brain
Inosine	21 ± 9	31 ± 13
Hypoxanthine	31 ± 6	34 ± 14

The supernatant of the primary cultured brain cells, like rat brain membranes (9, 13, 21), contained a GABA receptor binding inhibitory substance(s). The nature of the inhibitory material is still a matter of debate; however, GABA (13), protein (9), and phospholipids (21) are possible candidates. The presence of the inhibitory substance(s) makes it necessary to wash the cultured brain cells extensively prior to binding studies. Similar procedures are needed for GABA binding studies in mammalian brain (13–15). Routinely, we subjected the tissue (100,000*g* pellet) to two osmotic shock treatments, two freeze–thaw cycles, and four washes with 0.05 M Tris–citrate (pH 7.1) buffer prior to GABA binding studies (see Materials and Methods; 13, 15). This procedure was adequate in getting consistent and highly reproducible results, and also eliminates the need for using Triton X-100 during tissue preparation (13–15).[2]

Extensively washed cultured brain cells bind GABA to two classes of binding sites with affinities (K_D's) of 9 ± 3 and 250 ± 30 nM and binding capacities (B_{max}'s) of 380 ± 35 and 2400 ± 125 fmol/mg protein, respectively. The affinities of the two GABA binding sites in primary brain cultures are similar to that of whole rat brain (13–15, 17). It may be noted that the culture used in the present study is not a cell line but a heterogeneous population of CNS cell types. GABA binding in cultured brain cells, like mammalian brain membranes, is displaced by GABA agonists in a rank order (muscimol > GABA > isoguvacine) correlating with their reported neurophysiological and binding displacement activity (22). Specifically bound [^{3}H]GABA was inhibited by (+)-bicuculline with an IC_{50} value of 5 μM, a value similar to that reported for mammalian brain (13–15, 17). These results show a good agreement between the GABA binding properties of brain cultured cells and mammalian brain. Since the binding of GABA to its receptor sites is complex, it is not clear from the displacement curves whether the displacing ligands preferentially interact with one of the two sites. The inability of GABA uptake blockers like nipecotic acid to inhibit specifically bound GABA further supports the receptor-like nature of the GABA binding sites in these cells.

GABA binding was not inhibited by the GABA syn-

aptic antagonist, picrotoxin, or its active ingredient, picrotoxinin, consistent with the inability of these ligands to inhibit GABA binding in mammalian brain (13–15, 17). Several lines of evidence indicate that picrotoxin binds at a site distinct from the GABA recognition site at the GABA synapse (23, 24). Pentobarbital, which facilitates GABAergic transmission (5, 6), did not significantly inhibit GABA binding in cultured brain cells. Similar results have been observed in mammalian brain (14). However, pentobarbital (10–200 μM) inhibited the binding of [^{3}H]dihydropicrotoxinin (picrotoxin analogue) in the cultured brain cells,[2] and these findings are consistent with our earlier observations that barbiturates inhibit picrotoxin binding in mammalian brain and that they may act via the picrotoxin-sensitive site at the GABA receptor–ionophore system (25). GABA binding to cultured brain cells and rat brain membranes was inhibited by high concentrations of inosine and hypoxanthine (Table 2). These ligands, at similar concentrations, inhibit [^{3}H]diazepam binding and have been postulated to be possible endogenous ligands for benzodiazepine receptors (26). Inosine and hypoxanthine also inhibited the binding of α-[^{3}H]dihydropicrotoxinin to rat brain membranes (27).[2] Thus, the exact interaction of inosine and hypoxanthine with benzodiazepine receptors and GABA receptor–ionophore systems needs to be investigated further.

In summary, cultured brain neurons contain GABA receptors which have properties similar to those of mammalian brain. These primary cultured brain cells should prove a useful model system for analyzing GABA receptor–ionophore functions, receptor-stimulated chloride responses, possible coupling mechanisms involved in stimulus–receptor responses, and molecular interactions of barbiturates, benzodiazepines, purines, alcohol, and endogenous inhibitors with GABA postsynaptic events.

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