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SOLUBILIZATION OF GAMMA-AMINOBUTYRIC ACID RECEPTOR PROTEIN FROM MAMMALIAN BRAIN

Donald V. Greenlee and Richard W. Olsen

Division of Biomedical Sciences and Department of Biochemistry,
University of California,
Riverside, CA 92521 USA

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SUMMARY: Radioactive gamma-aminobutyric acid and muscimol bind to two classes of sites in fractions of bovine brain, with K_D values for gamma-aminobutyric acid of 28 nM and 150 nM and for muscimol of 3 nM and 21 nM; the maximum binding was 0.6 and 1.1 pmol/mg protein respectively for both ligands. Binding activity was solubilized by deoxycholate detergent, with 20-30% yield, assayed by rapid mini-gel filtration columns. Soluble muscimol binding had an apparent K_D of 41 nM and capacity of 0.6 pmol/mg, and was inhibited competitively by gamma-aminobutyric acid (K_I = 68 nM) and by other receptor-specific ligands. Gel filtration revealed a major peak of binding activity with apparent molecular weight of 900,000.

INTRODUCTION. Gamma-aminobutyric acid (GABA) is a prominent neurotransmitter substance in the central nervous system (CNS), with an inhibitory function mediated by a rapid increase in postsynaptic membrane chloride ion permeability (1). Binding sites having receptor-like properties have been measured in membranes of mammalian CNS with [^{3}H]GABA (2-4) or [^{3}H]muscimol (5). Assays with [^{3}H]GABA must be carried out with thoroughly disrupted and washed membranes and under sodium-free conditions, in order to remove endogenous inhibitors of GABA binding (4) and to avoid GABA transport and/or association with nonreceptor sites (2). Such sodium-independent binding is inhibited by low concentrations of receptor-specific GABA analogues, the most potent of which is the natural isoxazole from hallucinogenic mushrooms, muscimol (3), and [^{3}H]muscimol is a specific GABA receptor ligand (5,6). This report describes the first solubilization of GABA receptor-like binding activity from mammalian brain. Some of the results have been presented in abstract form (7).

Abbreviations used: GABA: gamma-aminobutyric acid; DOC: deoxycholate; M.W.: molecular weight; CNS: central nervous system; PPO: 2,5-diphenyloxazole.

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EXPERIMENTAL PROCEDURES. Bovine brain cerebral plus cerebellar cortex membranes, mitochondrial plus microsomal fraction (P_2 + P_3), were prepared as described for rat brain (4), with thorough disruption and multiple washes and freeze-thaws.

Binding Assays: Membranes were resuspended at 0.8 mg/ml protein in 50 mM TRIS-HCl, pH 8.5, 5 mM β-mercaptoethanol for binding assays which were performed in triplicate at 4°C by the pelleting method (2). Binding of [^{3}H]GABA (54-66 Ci/mmol, Amersham) and [^{3}H]muscimol (12.9 Ci/mmol, New England Nuclear) was defined as radioactive ligand pelleted with membranes which was not displaceable by 0.1 mM nonradioactive GABA or 0.01 mM nonradioactive muscimol.

Binding to solubilized proteins was measured with mini-gel filtration columns of Sephadex G-50 FINE (Pharmacia), using low speed centrifugation for rapid elution (8). Triplicate samples of 150 µl of protein (5 mg/ml) which had been equilibrated 15 min at 0° with radioactive ligand (as above) were applied to one ml columns. The void volume (100 µl) was collected and counted in 3 ml of toluene-PPO (5 g/l)-BBS-3 (10%, Beckman), in a Beckman LS-100C scintillation counter (30% efficiency). Separation of bound and free ligand was virtually complete; nevertheless background was determined with 0.1 mM unlabeled GABA. Proteins were determined by the Lowry method (9).

Solubilization: After thorough washing, P_2 + P_3 membrane fragments were suspended to 20 mg protein/ml and a 10% (w/v) DOC solution was added dropwise to obtain a final solution of 2% DOC. Extraction was performed for 15 min at 0-4°C with stirring followed by centrifugation for 45 min at 2 x 10^5 x g. This soluble extract was diluted 10X in buffer containing 0.2% DOC and stirred for 15 min at 0-4°C, and then reconcentrated to its original volume using Amicon CF-50-A Centriflo membrane cones.

Agarose Chromatography: Elution volumes of the binding activity and standard proteins were determined separately by adding 1 ml (50-75 mg protein) or 0.1 ml (1 mg of each standard protein) samples respectively to a 2.1 x 46.2 cm Sepharose 6B-100 column pre-equilibrated with buffer (50 mM TRIS-HCl, pH 8.5, containing 5 mM β-mercaptoethanol, 0.2% DOC, and 100 mM KCl) at 4°C. Soluble receptor was assayed as above on 0.2 ml aliquots of each 1.0 ml column fraction, using 52 nM [^{3}H]muscimol. Ferritin and cytochrome c were determined spectrophotometrically at 410 nm, catalase and trypsin-activated chymotrypsinogen activities were determined as in ref. 10.

RESULTS. The binding of [^{3}H]GABA in Na^+-free buffer to bovine brain membranes gives a Scatchard plot (11) which is best fit by two slopes (affinities), as we observed for rat (4) and human (12) brain. Figure 1A describes a typical GABA binding curve for bovine brain; the average dissociation constants for five experiments were K_{D1} = 28 ± 5 nM and K_{D2} = 150 ± 30 nM. The maximum binding (B_{max}) at saturating ligand concentration for the two classes of sites was 0.7 pmol/mg protein and 1.2 pmol/mg protein, respectively. The same two classes of sites were apparently bound by [^{3}H]muscimol (Figure 1B), which had similar B_{max} values (0.6 pmol/mg and 0.9 pmol/mg, avg. of 3), but

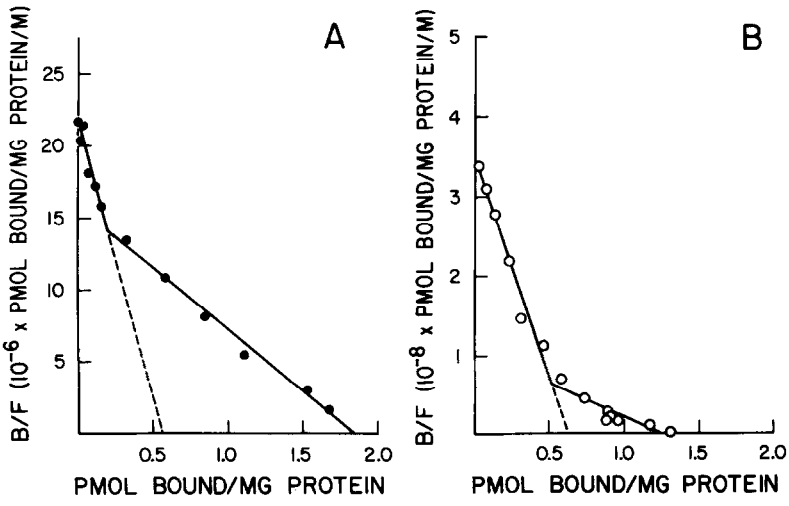

Figure 1. Scatchard Plots of [^{3}H]GABA (A) and [^{3}H]Muscimol (B) Binding to Bovine Brain Membranes. Membranes were thoroughly washed with sodium-free buffer and frozen and thawed. Binding of varying concentrations of ligand was determined by pelleting as described in the text. B/F refers to specifically bound ligand over concentration of free ligand. Solid lines are computer-fitted.

tighter binding affinities (K_{D1} = 3 ± 1 nM and K_{D2} = 21 ± 4 nM). Analogous results (two apparent classes of muscimol binding sites) were also observed by others (5).

Bovine brain membranes were subjected to various mild detergents, chaotropic salts, and enzyme treatments in attempts to solubilize GABA/muscimol binding activity. Membranes were incubated at 0° for 30 min with solubilizing agents, followed by centrifugation for 30 min at 200,000 x g. Supernatant fractions were assayed for [^{3}H]muscimol binding by a rapid mini-gel column separation of bound and free ligand (8). The best yield (22 ± 2%) of soluble binding activity of the methods tested was obtained with 2% sodium deoxycholate (DOC). Numerous other detergents yielded little if any soluble binding activity. Muscimol/GABA binding to DOC extracts was improved three-fold by dialyzing the extract in Amicon membrane-filter centrifuge cones, which retain proteins of M.W. >50,000 (see "Procedures").

Figure 2 depicts a Scatchard plot for [^{3}H]muscimol binding to solubilized membrane protein, yielding one apparent class of sites with K_D = 41 ±

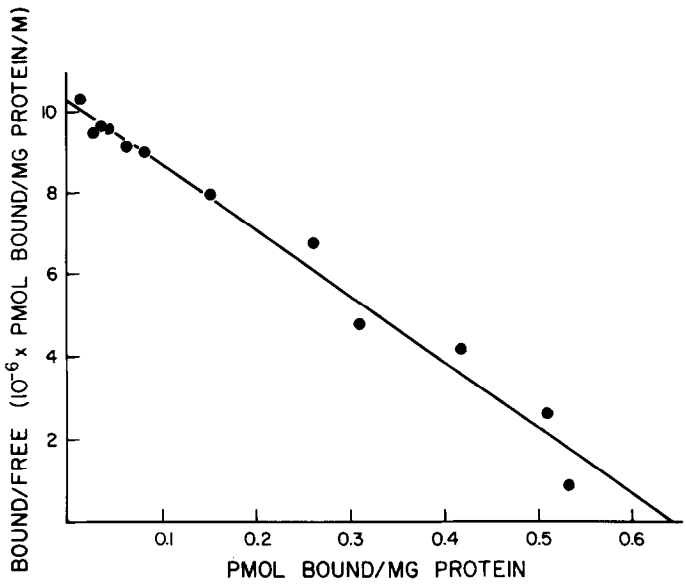

Figure 2. Scatchard Plot of [^{3}H]Muscimol Binding to Detergent-Solubilized Bovine Brain Membranes. Deoxycholate supernatants were dialyzed and assayed with varying concentrations of ligand by rapid mini-gel filtration columns as described in the text. The solid line is a compute-fitted linear regression.

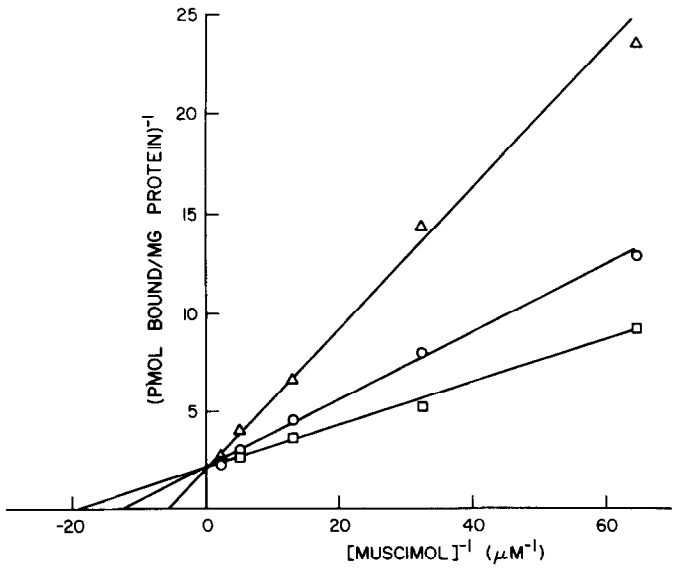

Figure 3. Double Reciprocal Plot: Competitive Inhibition by GABA of Soluble [^{3}H]Muscimol Binding. Muscimol binding to non-dialyzed deoxycholate supernatant was assayed by the gel filtration method, using varying concentrations of [^{3}H]muscimol (protein = 0.61 $\pm$ 0.03 mg/ml), and constant [GABA]: (□) 0 nM; (○) 40 nM; (△) 200 nM.

TABLE 1. Comparison of Drug Specificities Between Soluble and Membrane-Bound GABA Receptor

Ligand	IC_{50} (nM) Soluble	Membranes
Muscimol	41[a]	3 and 21[a]
GABA	54	28 and 150[a]
3-Aminopropane sulfonate	118	70
trans-1-Aminocyclopentane-3-carboxylate	207	270
cis-1-Aminocyclopentane-3-carboxylate	3500	5200
Isoguvacine	150	160
Imidazole acetate	4900	900
-Alanine	$39,000_5$	42,000
L-Glutamate	$> 10^5$	$150,000_5$
DL-Nipecotic acid	$> 10^5$	$>10^5$
L-2,4-Diaminobutyrate	$> 10^5$	$>10^5$

[a] K_D values from bovine brain. [^{3}H]Muscimol binding to soluble bovine brain receptor was measured by the mini-gel filtration method described in text, with 15 nM [^{3}H]muscimol (12.9 Ci/mmol) and 5-8 mg/ml protein, and varying concentrations of displacing agent (triplicates). Each IC_{50} value is the mean of three experiments, standard deviations less than 40%. Solubilized binding protein solutions were dialyzed (see text) prior to assay. IC_{50} values were corrected for the high concentration of radioactive ligand and receptor according to equation 10 of ref. 13. [^{3}H]GABA binding to rat brain membrane fragments was determined by the pellet assay using 2 nM [^{3}H]GABA (54 Ci/mmol), 0.8-1.5 mg/ml protein, and varying concentrations of displacing agents (triplicates). Values are means of at least three experiments. There were no differences in IC_{50} values between rat and cow brain membranes (our observations).

22 nM (av. of 7), B_{max} = 0.55 $\pm$ 0.10 pmol/mg (av. of 3). Figure 3 shows that this [^{3}H]muscimol binding was inhibited competitively by nonradioactive GABA, with a K_I value of 68 $\pm$ 9 nM (av. of 4). Binding was also inhibited by other GABA analogues (Table 1), with a specificity expected of GABA receptors (3,6): GABA agonists 3-aminopropane sulfonate, trans-1-aminocyclopentane-3-carboxylate, isoguvacine, and imidazole-acetate were good inhibitors, and GABA uptake blockers nipecotic acid and L-2,4-diaminobutyrate did not inhibit binding. Thus there is little doubt that the solubilized binding activity has GABA receptor-like properties.

The soluble GABA binding activity decayed in several hours in 2% DOC, but was stable in 0.2% DOC for at least 24 hours. Figure 4 shows the pattern from a calibrated agarose column run in 0.2% DOC; the bulk of binding activity

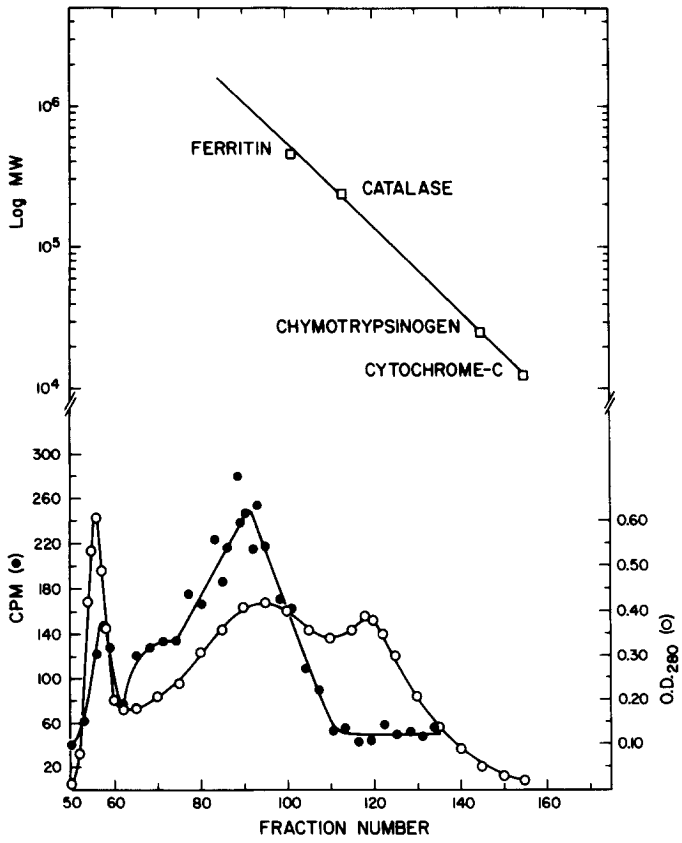

Figure 4. Molecular Sieve Column Chromatography of Soluble Muscimol Binding. A sample of deoxycholate supernatant was applied to a Sepharose 6B column equilibrated and calibrated as described in the text. Standard proteins of known M.W. were chromatographed separately. Fractions were assayed for enzymatic activity, protein, and [^{3}H]muscimol binding as previously described.

eluted with an apparent M.W. of 900,000, with a possible shoulder of activity on the higher M.W. side, and a small amount of activity in the void volume.

DISCUSSION.

GABA is a major transmitter in the CNS and thus important in normal and abnormal CNS function (1). We have characterized receptor-like binding sites for GABA in the CNS (3,4) and in crayfish muscle (14), and also sites for the synaptic antagonist, picrotoxinin (15), which appear to be related to GABA-regulated chloride channels and perhaps to barbiturate action (16). The present study demonstrates solubilization by aqueous detergent solutions of GABA receptor binding sites from bovine CNS.

[^{3}H]Muscimol binding activity was soluble in 2% DOC by the criteria of centrifugation and by inclusion in a molecular sieve column chromatogram, which revealed a major peak of activity with apparent M.W. of 900,000. Detergent molecules and perhaps proteins other than the GABA receptor may contribute to the large size of this binding species. A small amount of activity detected on the (high M.W.) shoulder of the major peak and in the column void volume may represent incompletely dispersed protein, or perhaps other minor species.

One major peak of activity would be consistent with the one apparent binding affinity for [^{3}H]muscimol in the DOC supernatant, but not with the two apparent affinities in membrane fragments (Fig. 1). It is not yet clear whether detergent selectively solubilizes one of two (or more) discrete species, or whether detergent modifies properties of the soluble binding sites so that the differences in affinity between the two classes becomes less distinct. Although binding affinities of various ligands are slightly altered, the overall drug specificity of soluble binding is very similar to that of membranes and clearly shows receptor-like specificity. Dialysis of the supernatants to remove molecules of M.W. under 50,000 results in an improvement of binding, both with respect to the affinity for all ligands, and in the B_{max}. This effect could be due to detergent, since the concentration of DOC was probably lowered by this process from 2% to 0.4% minimum. However, at least part of the effect seems to reflect removal of some endogenous inhibitory material (4,17) solubilized from the membranes. Binding affinities and B_{max} values may still be underestimated if all of the inhibitor(s) has not been washed out. Furthermore, some muscimol binding curves on well-dialyzed samples suggest a best fit of two classes of sites rather than one (Fig. 2).

Nevertheless, GABA receptor binding activity has been solubilized in fair yield and with retention of reasonably normal binding properties. B_{max} values suggest a considerable purification factor will be necessary to

achieve a homogeneous receptor, but attempts can now be made with the solubilized preparation. Further clarification on the question of possible heterogeneity should also be rapidly obtained.

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