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SOLUBILIZATION OF CONVULSANT/BARBITURATE BINDING ACTIVITY ON THE γ-AMINOBUTYRIC ACID/BENZODIAZEPINE RECEPTOR COMPLEX

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Summary: Binding activity of the radioactive cage convulsant [^{35}S]t-butyl-bicyclophosphorothionate was solubilized from rat brain membranes using the zwitterionic detergent 3-[(3-cholamidopropyl)-dimethylammonio] propanesulfonate. Binding (K_D = 26 nM, Bmax = 0.4 pmol/mg protein) was inhibited by picrotoxin and related convulsants and by barbiturates and related depressants that interact with γ-aminobutyric acid and benzodiazepine receptors via the picrotoxinin binding site. The convulsant/ barbiturate binding activity chromatographed on gel filtration as a single peak coinciding with the benzodiazepine/γ-aminobutyric acid receptor protein complex.

Introduction: Convulsant drugs related to the naturally-occurring picrotoxinin (1) and the synthetic cage convulsants (2) inhibit neuronal membrane chloride channels activated by the major inhibitory neurotransmitter, GABA*. Binding sites for these convulsant drugs have been assayed *in vitro* with a radioactive picrotoxin analog, [^{3}H]DHP, the binding of which is competitively inhibited not only by convulsant drugs which block GABA function, but also by depressant drugs such as barbiturates, which enhance GABA function (1, 3-5). The convulsant/barbiturate receptors thus defined appear to be tightly coupled to both GABA and benzodiazepine receptor sites in neuronal membranes by virtue of reciprocal chloride-dependent allosteric interactions between the three receptor classes (1).

GABA and benzodiazepine receptor binding activities have been solubilized with mild detergents and the two were found to co-migrate during a

*Abbreviations used: CHAPS: 3-[(3-cholamidopropyl)-dimethylammonio] propanesulfonate; GABA: γ-aminobutyric acid; TBPS: t-butyl-bicyclophosphorothionate; DHP: α-dihydropicrotoxinin.

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variety of protein fractionation steps (6-9), including benzodiazepine affinity column purification (8-10). Solubilization of [^{3}H]DHP binding activity has been reported (11), but the use of this ligand is hampered by a low degree of specific binding (3-5). We were able to demonstrate a chloride-dependent and picrotoxin-sensitive enhancement by barbiturates of benzodiazepine and GABA receptor binding solubilized with the mild zwitterionic detergent CHAPS (12). Recently, the picrotoxin site has been assayed with a new ligand, the cage convulsant [^{35}S]TBPS (13). This binding is competitively inhibited by cage convulsants, picrotoxin, and barbiturates, and allosterically by GABA agonists (13,14), and its brain regional distribution is similar to that of GABA receptors (15). We now report solubilization with CHAPS detergent of the [^{35}S]TBPS binding activity and demonstrate that it appears to be associated with the GABA/benzodiazepine receptor protein complex.

Materials and Methods: Bicyclophosphate compounds were a kind gift of J. Casida, Berkeley, CA; tutin was from P. Buckley, Palmerston North, New Zealand; etomidate isomers were from Janssen Pharmaceuticals; the convulsant benzodiazepine Ro5-3663 from Hoffmann-LaRoche; etazolate from Squibb & Sons; 5-ethyl, 5-(1,3-dimethylbutyl) barbituric acid from Lilly; and picrotoxinin was prepared from picrotoxin by M. Ban, Riverside, CA. [^{35}S]t-Butylbicyclophosphorothionate (105.7 Ci/mmol) and [N-Methyl-^{3}H]flunitrazepam (73.0 Ci/mmol) were from New England Nuclear, Boston, MA. CHAPS was from Serva Chemicals (Garden City Park, NY).

Sprague-Dawley rats (200-300 g) were decapitated, and the brains rapidly removed and bathed in ice-cold 0.32 M sucrose. Cerebral cortex was then removed and homogenized to prepare thoroughly washed, osmotically shocked crude mitochondrial, synaptosomal, and microsomal membranes as previously described (6). The pellet was then resuspended in 200 mM KCl, 5 mM Tris-HCl, pH 8.0, and frozen. All preparations were done in the presence of the following protease inhibitors: 1 mM EDTA, 0.1 mM benzethonium chloride, 0.1 mM phenylmethyl sulfonyl fluoride, 2 mM benzamidine hydrochloride, bacitracin (20 µg/ml), and 0.02% (w/v) NaN_3. The frozen membrane suspension was thawed and washed extensively, resuspended to the desired volume (1 g tissue/3 ml buffer) and incubated with CHAPS (20 mM final concentration) for 30 min at 4° C, then centrifuged at 40,000 rpm (100,000 x g) for 60 min. Soluble receptor activity was measured by bovine γ-globulin-poly(ethyleneglycol) precipitation-centrifugation or filtration techniques as previously described (6,12). Protein was estimated by the method of Lowry et al. (16).

Results: Treatment of rat brain membranes at 4° with the detergent CHAPS (20 mM) resulted in the recovery of [^{35}S]TBPS binding activity in the supernatant fraction of a 60 min centrifugation at 100,000 x g. Fig. 1 depicts a Scatchard plot for the saturable binding of [^{35}S]TBPS in the CHAPS extract (displaceable by 200 µM picrotoxinin). The plot shows a single apparent

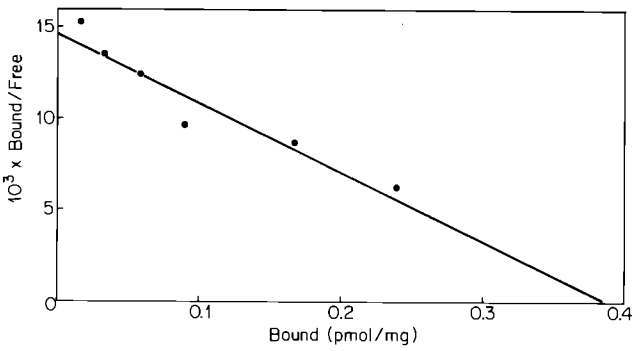

Fig. 1. Scatchard Plot of [^{35}S]TBPS Binding to CHAPS Extract of Rat Cortex Membranes. Triplicate assays contained [^{35}S]TBPS (1-40 nM), with nondisplaceable background determined with 0.2 mM picrotoxinin. Preparation of membranes, solubilization, and assay methods are described in the Materials and Methods section.

binding affinity (K_D = 26 nM) and a specific activity of 0.4 pmol/mg protein. This corresponds to a yield of approximately 50% of the binding sites detected in membranes, with no significant change in affinity (B_{max} = 0.8 pmol/mg and K_D = 30 nM, not shown). The yield was not improved by longer solubilization times, extraction at 21° instead of 4°, higher ionic strength, nor by substituting the detergents Triton X-100 or sodium deoxycholate (1% w/v).

Table 1 lists the potencies for several compounds to displace the binding of [^{35}S]TBPS to rat brain membranes and the CHAPS extract. The same amount of specific binding was displaced by excess concentrations of picrotoxinin, ethyl bicyclophosphate, as well as pentobarbital. This amounted to approximately 60% displacement using the centrifugation assay and 90% using the filtration assay method for both preparations. The pharmacological specificity of drug displacement, including stereospecificity, was similar for membrane-bound and solubilized [^{35}S]TBPS binding, and agreed with published reports (13, 14). The same specificity was seen as for drugs active in enhancing the binding of GABA receptors and benzodiazepine receptors (1,5,10,17,18), in membranes and in solution (12). Furthermore, GABA inhibited the binding of [^{35}S]TBPS binding in solution as in membranes (Table 1). The GABA receptor antagonist bicuculline reversed this GABA

TABLE 1: INHIBITORS OF [^{35}S]TBPS BINDING IN RAT BRAIN

Compound	IC_{50} (μM)	
	Membranes	Soluble
Bicyclophosphates:		
t-Butyl	0.3	0.2
Isopropyl	4	--
Ethyl	1	1.8
p-Chlorophenyl Silatrane	11	100
Picrotoxinin	0.6	0.2
Tutin	0.11	0.12
Benzodiazepine Ro5-3663	37	20
Etazolate	--	1.5
(+)Etomidate	7	4
(-)Etomidate	155	300
Dimethylbutyl barbiturate	100	40
Pentobarbital	40	45
GABA	2.5	0.5

[^{35}S]TBPS binding was measured by filtration (Whatman GF/B) following 90 min incubation at 21° C in 5 mM Tris-HCl buffer, pH 8.0, 0.2 M KCl, containing six concentrations of displacing agent. Non-displaceable background (5-10% of total) was determined with 0.2 mM picrotoxinin (see Materials and Methods section for preparation of membranes and CHAPS extract and assay of binding in the soluble extract).

inhibition (not shown) but did not enhance [^{35}S]TBPS binding, probably due to the absence of any significant endogenous GABA.

The CHAPS extract was chromatographed on a column of Sepharose 6B. Fig. 2 shows the elution profile for binding of both [^{35}S]TBPS and [^{3}H]flunitrazepam. A single peak of activity was seen for both ligands, and the two coincided. Calibration with protein standards showed both binding activities to have an apparent molecular weight in the range of 900,000 (larger than ferritin and significantly larger than catalase). Of course, the species measured could contain considerable amounts of bound detergent. This gel filtration behavior is similar to that previously reported for benzodiazepine and GABA receptor binding activity (6,7,12,16) and is consistent with the presence of the convulsant/barbiturate receptor sites on the same macromolecular complex which contains GABA/benzodiazepine receptor sites.

Discussion: Solubilization and fractionation ought to allow determination of the number of proteins that are involved in GABA receptor function including recognition sites for GABA, the chloride channel regulated by GABA, and drug

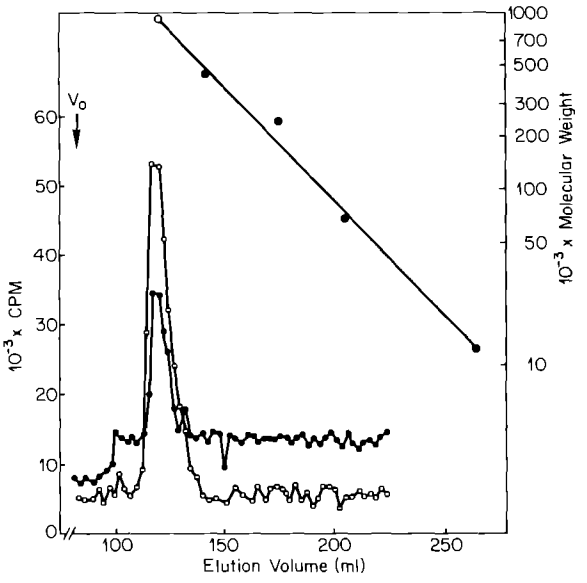

Fig. 2. Gel Filtration Column Chromatography of [^{35}S]TBPS Binding Activity. The CHAPS extract was concentrated approximately 2-fold on Amicon Centriflo membrane cones, and applied to a Sepharose 6B column (78 cm x 2.5 cm) equilibrated and eluted at 4° C with the buffer described, containing protease inhibitors and 0.25% CHAPS. Fractions of 3.2 ml were collected at a flow rate of 25 ml/h and aliquots of 0.4 ml in duplicate were assayed for [^{3}H]flunitrazepam (O) and [^{35}S]TBPS (●) binding activity. The calibration of the column was done using (from left to right): ferritin (5 mg), catalase (3 mg), bovine serum albumin (2 mg), and cytochrome C (2 mg) as molecular weight markers. The plot above shows column elution volume as a function of the molecular weight standards (log scale), with the receptor indicated by the open circle.

modulatory sites for benzodiazepines, barbiturates, and picrotoxin/cage convulsants. GABA receptor binding was first solubilized with deoxycholate (19), and subsequently shown to co-purify with benzodiazepine receptor binding activity (6-10). Drugs which inhibit [^{3}H]DHP binding, picrotoxin, cage convulsants, and barbiturates, show allosteric interactions with GABA and benzodiazepine receptor sites in membranes (1), but barbiturate-sensitive [^{3}H]DHP binding has not been described to be associated with the partially-purified GABA/benzodiazepine receptor complex. Davis and Ticku reported solubilization of [^{3}H]DHP binding with the detergent Lubrol (11) and separation of this activity from benzodiazepine binding by gel filtration chromatography, with subsequent loss of the barbiturate allosteric enhancement of benzodiazepine binding (20). However, these workers reported an apparent molecular weight for native [^{3}H]diazepam binding protein (60,000)

much smaller than that observed by numerous other workers of $\geq$ 200,000 (6-10). Their apparent molecular weight for [^{3}H]DHP binding activity was about 200,000 (20).

The allosteric barbiturate interactions also were not described for highly purified GABA/benzodiazepine receptor preparations in Triton X-100 which had a size of > 200,000 (9). However, we found barbiturate allosteric interactions with the GABA/ benzodiazepine receptor sites to be unstable in deoxycholate and Triton X-100, but moderately stable in CHAPS extracts, persisting in gel filtration fractions associated with a protein of > 200,000 molecular weight (12). CHAPS was also more effective than the other detergents in solubilizing [^{35}S]TBPS binding activity.

That this solubilized [^{35}S]TBPS binding represents the pharmacologically relevant convulsant site on the GABA receptor-ionophore is demonstrated by the specificity of drug inhibition. As in membranes, soluble [^{35}S]TBPS binding is inhibited by micromolar levels of picrotoxinin, its analog tutin, cage convulsants including t-butyl and ethyl bicyclophosphate, the convulsant benzodiazepine Ro5-3663, and by barbiturates and related depressants such as the pyrazolopyridine etazolate, and etomidate, which shows the appropriate stereospecificity. These compounds are all active in modulating GABA synaptic function and in modulating GABA and benzodiazepine receptor binding *in vitro*. As in membranes, GABA inhibits the binding of [^{35}S]TBPS, and this is reversed by the GABA antagonist bicuculline, indicating a retention of the allosteric coupling of the solubilized [^{35}S]TBPS convulsant/barbiturate receptor sites with GABA receptors.

This coupling is further suggested by the co-migration on gel filtration column chromatography of the [^{35}S]TBPS binding in a single peak with [^{3}H]flunitrazepam binding, which has in turn been shown previously to be enhanced by pentobarbital (12) and to copurify with GABA receptor activity (6-10). Indeed, preliminary observations suggest that [^{35}S]TBPS binding and allosteric interactions of the convulsant/barbiturate receptor sites with GABA and benzodiazepine receptor sites persist in a single protein complex

following extensive purification on a benzodiazepine affinity column (10). It would appear that the use of the detergent CHAPS provides optimal preservation of this activity.

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