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SOLUBILIZATION AND ANIONIC REGULATION OF CEREBRAL SEDATIVE/CONVULSANT RECEPTORS LABELED WITH [^{35}S] tert-BUTYLBICYCLOPHOSPHOROTHIONATE (TBPS)

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Binding activity for the cage convulsant [^{35}S]-tert-butylbicyclophosphorothionate, which appears to label a site closely associated with the chloride ionophore of the $GABA_A$/benzodiazepine receptor complex has been solubilized from rat cerebral cortex using the zwitterionic detergent CHAPS. Of several detergents screened, only CHAPS and CHAPSO were capable of solubilizing the binding activity with good recovery. The pharmacologic specificity of soluble [^{35}S]-tert-butylbicyclophosphorothionate binding is very similar to the membrane state. In both the membrane and soluble state, [^{35}S]-tert-butylbicyclophosphorothionate binding is enhanced by anions which support inhibitory post-synaptic potentials ("Eccles anions"), suggesting that [^{35}S]-t-butylbicyclophosphorothionate may label chloride channels thought to be involved in these potentials. Since this solubilization procedure also preserves GABA and benzodiazepine binding and their regulation by drugs such as barbiturates, purification and isolation of the macromolecular complex including chloride channel and GABA-benzodiazepine sites may be feasible.

Benzodiazepines, barbiturates and a variety of convulsant drugs are thought to act at closely related sites which are linked with GABA receptors and chloride ion channels that mediate synaptic actions of GABA in the central nervous system (1). A complete understanding of the molecular interactions of GABA, drugs and chloride channels requires purification of the entire macromolecular receptor complex. Several workers have solubilized benzodiazepine and GABA binding activities using a variety of ionic and non-ionic detergents (2-6). However, under detergent conditions used to

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Abbreviations: TPBS - tert-butylbicyclophosphorothionate; CHAPS - 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate; CHAPSO - 3-([(3-cholamidopropyl)-dimethylammonio]-1-[2-hydroxy-1-propanesulfonate]) DHP - α-dihydropicrotoxinin

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extensively purify benzodiazepine receptors (7, 8) receptor interactions of benzodiazepine, barbiturates, and the chloride ionophore are lost (9, 10). Barbiturates are thought to exert their pharmacologic effects via a specific binding site closely related to the recognition site for the convulsant picrotoxin and the chloride ionophore. This site can be labelled with [^{3}H]α-dihydropicrotoxinin ([^{3}H]DHP), but [^{3}H]DHP binding has very poor specific/ non-specific binding characteristics (11). Recently, Squires et al. (12) reported that [^{35}S]-tert-butylbicyclophosphorothionate ([^{35}S]TBPS) labels "sedative/convulsant" sites recognized by picrotoxin and barbiturates; [^{35}S]TBPS binds with much more favorable specific/non-specific ratios than [^{3}H]DHP. [^{35}S]TBPS binding is absolutely dependent upon the presence of appropriate anions (12,13). We now report successful solubilization of the [^{35}S]TBPS binding site from rat cortex together with other components of the GABA-benzodiazepine receptor complex. We demonstrate anionic regulation of both membrane and soluble [^{35}S]TBPS binding consistent with its labelling of chloride channels associated with benzodiazepine/$GABA_A$ receptors.

MATERIALS AND METHODS:

[^{35}S]TBPS (specific activity 103.5 Ci/mmol upon receipt, thereafter corrected for ^{35}S decay) and unlabelled TBPS were obtained from New England Nuclear (Boston, Mass.). CHAPS (Lot No. 73F-5016), Triton X-100, β-octylglucoside, sodium cholate and sodium deoxycholate were from Sigma Chemical Co. (St. Louis, Mo.). CHAPSO was purchased from Pierce Chemical Co. (Rockford, Ill.), and digitonin from Fisher Chemical Co. (Fairlawn, N.J.).

Solubilization Procedure: Cerebral cortices from male Sprague-Dawley rats sacrificed by decapitation were homogenized in 20 volumes ice-cold 0.32 M sucrose with a Teflon-glass homogenizer. The homogenate was spun 10 min x 1,000 g and the supernate spun 30 min x 150,000 g in a preparative ultracentrifuge. The resulting pellet was resuspended in 20 volumes ice-cold distilled water using a Polytron (Brinkmann instruments, Westbury, N.Y.) and the homogenate spun 30 min x 150,000 g. The resulting water-lysed crude P_2 pellet was washed once in ice-cold 50 mM Tris-citrate (pH 7.5 at 0° C). The resulting pellet was resuspended in 3 volumes based on original wet weight) 0.8°/° (w:v) CHAPS in ice-cold 50 mM Tris-citrate by Polytron. After 20 min extraction at 4° C with constant agitation, the mixture was spun 60 min x 150,000 g. The resulting supernate was used in soluble binding assays. Tissue preparations to assess particulate [^{35}S]TBPS binding were the same except for omission of the detergent extraction step.

Soluble and membrane [^{35}S]TBPS binding assays: To 100 µl of soluble extract or membrane preparation, 50 µl of [^{35}S]TBPS was added to a final concentration of 2 nM. The [^{35}S]TBPS was made in 1M NaBr unless otherwise noted. Fifty µl of 50 mM Tris-citrate (pH 7.1 at 25° C) was added to define total binding, or 10^{-5} M TBPS in the same buffer to define non-specific

binding. The mixture was incubated 90 min at 21° C (room temperature), and then filtered over glass-fiber filters (Schleicher and Schuell 32) which had been briefly (5-10 sec) immersed in a solution of 0.3°/° polyethylenimine (PEI) in distilled water. The filters were washed three times with 3 ml 0.9°/° NaCl equilibrated to room temperature. Filters were counted in 4 ml Formula 947 (New England Nuclear, Boston, Mass.) by liquid scintillation spectrometry. Using these conditions (and upon receipt of the radioligand) specific binding was typically about 1300 cpm and non-specific binding 200 cpm, for both membrane and soluble preparations. The PEI-coated filtration assay described here is a minor modification of that previously described by Bruns et al. (14) for a wide variety of soluble binding activities. The PEI-coated filtration assay gave very similar results for [^{35}S]TBPS as a somewhat more tedious polyethylene glycol/γ-globulin assay previously described for soluble benzodiazepine receptor binding (2). The soluble [^{35}S]TBPS binding activity described here was stable for at least 24 hrs at 4° C with negligible loss of activity. Soluble extract could be frozen for at least four months at -80° C with no loss in activity.

Soluble and membrane [^{3}H]Ro-15-1788 binding assays: Soluble and membrane binding of [^{3}H]Ro-15-1788, a radiolabelled benzodiazepine antagonist, was performed in a similar fashion to the [^{35}S]TBPS assays with the exception that 1) the final [^{3}H]Ro-15-1788 concentration was 12.5 nM 2) 10^{-5} M flunitrazepam was used to define non-specific binding 3) NaBr was not present during the assay and 4) the incubation period was 60 min at 0° C.

RESULTS AND DISCUSSION

Of numerous detergents screened [each at 1°/° (w:v)], only the zwitterionic detergents CHAPS and CHAPSO provide high levels of specific binding of [^{35}S]TBPS in the soluble state (Table 1). By contrast, most of the detergents screened can solubilize benzodiazepine binding activity, as assayed with [^{3}H]Ro-15-1788. As CHAPS provides the most nearly quantitative recoveries of [^{35}S]TBPS binding activities, this detergent was used exclusively in further studies.

The amount of [^{35}S]TBPS binding activity extractable with CHAPS rises sharply above 0.4°/° (w:v) CHAPS concentration but plateaus at 0.8°/° (w:v) CHAPS at approximately 40°/° of the total binding activity (Fig. 1a). [^{3}H]Ro-15-1788 binding activity solubilized closely parallels [^{35}S]TBPS activity, as expected if the two sites are in molecular association in the soluble state. If one extracts with 0.8°/° (w:v) CHAPS in the presence of varying concentrations of NaCl (Fig. 1b), larger quantities of both [^{35}S]TBPS (and [^{3}H]Ro-15-1788) binding sites can be extracted. Two classes of benzodiazepine receptors are physically separated by reponse to detergents, Type II sites readily solubilized by detergent alone and Type I

Table 1. Comparison of Detergents for Solubilization of [^{35}S]TBPS and [^{3}H]Ro-15-1788 Binding Activities

Detergent	[^{35}S]TBPS binding activity		[^{3}H]Ro-15-1788 binding activity	
	°/°Solubilized*	°/°Recovered+	°/°Solubilized*	°/°Recovered+
None	0.9	100	1.4	100
CHAPS (1°/° (w:v))	46	93	33	78
CHAPSO (1°/° (w:v))	41	56	25	90
β-octylglucoside (1°/° (w:v))	13	24	5.9	88
Na Deoxycholate (1°/° (w:v))	8	13	25	91
Na Cholate (1°/° (w:v))	10	19	29	56
Digitonin (1°/° (w:v))	0	15.4	31	86
Triton X-100 (1°/° (w:v))	37	5.7	47	79

Values are from a representative experimental which was replicated, with very similar results

* - "°/° solubilized" is calculated as the ratio of specific binding in the supernate to the sum of that in the supernate and pellet, expressed as a percent

+ - "°/° recovered" is calculated as the ratio of the sum of specific binding in the supernate and pellet to the same sum under conditions where no detergent was present, expressed as a percent.

requiring salt plus detergent (4). Apparently, multiple classes of [^{35}S]TBPS binding sites can also be defined based on ease of detergent solubilization. At least some of [^{35}S]TBPS binding sites resistant to detergent solubilization can be solubilized by a combination of detergent and salt. In this communication, only the detergent-soluble population of [^{35}S]TBPS binding sites has been further characterized.

[^{35}S]TBPS binding to the solubilized receptor preparation is saturable and of high affinity. Scatchard analysis indicates a single apparent population of soluble [^{35}S]TBPS sites with K_D = 96 nM (data not shown). The pharmacologic specificity of membrane and soluble [^{35}S]TBPS binding sites are very similar (Table 2, Figure 2a and b) and resemble reports for particulate preparations (12, 16). In both the membrane and soluble states the cyclopyrrolone anxiolytic suriclone (15) is the most potent inhibitor of [^{35}S]TBPS binding, manifesting biphasic effects with an IC-50 for the high affinity component of 2-4 nM, as we have found recently in particulate

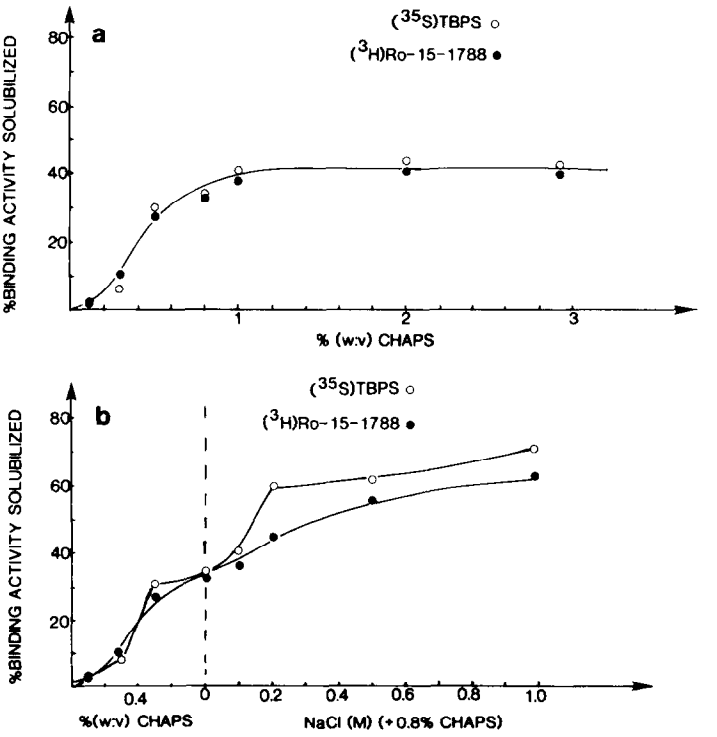

Figure 1. Solubilization of [^{35}S]TBPS and [^{3}H]Ro-15-1788 binding activity from rat cerebral cortical membranes. (a) extraction at various CHAPS concentrations. (b) extraction with 0.8°/° CHAPS containing varying concentrations of NaCl. The percent binding activity solubilized is the ratio of [^{35}S]TBPS binding activity in the soluble extract to the sum of [^{35}S]TBPS binding activity in the soluble extract and that remaining in the pellet. Pellets and supernates were assayed under similar ionic conditions, as described in Materials and Methods. Data is from a representative experiment replicated twice.

Table 2. Inhibition by Drugs of [^{35}S]TBPS Binding to Rat Cerebral Cortex Membranes and Soluble Extract

Drug	IC_{50} (M) Membrane State	IC_{50} (M) Soluble State
Suriclone*	0.003, 3	0.003, 3
Zopiclone*	0.01, 3	0.01, 3
Picrotoxinin	0.21	0.21
Muscimol	0.49	2.3
CL 218, 872	4.5	3.2
Flunitrazepam	5.8	6.2
β-CCM	6.2	5.6
Pentobarbital	83.2	76
Ro-15-1788	100	100

Values are from a representative experiment replicated two times with less than 20°/° variation.

*, - Inhibition of [^{35}S]TBPS binding by zopiclone and suriclone appears multiphasic. Smaller and larger IC_{50} values refer to inhibition of the high and low-affinity components of inhibition of [^{35}S]TBPS binding by these drugs, respectively (see Fig. 3).

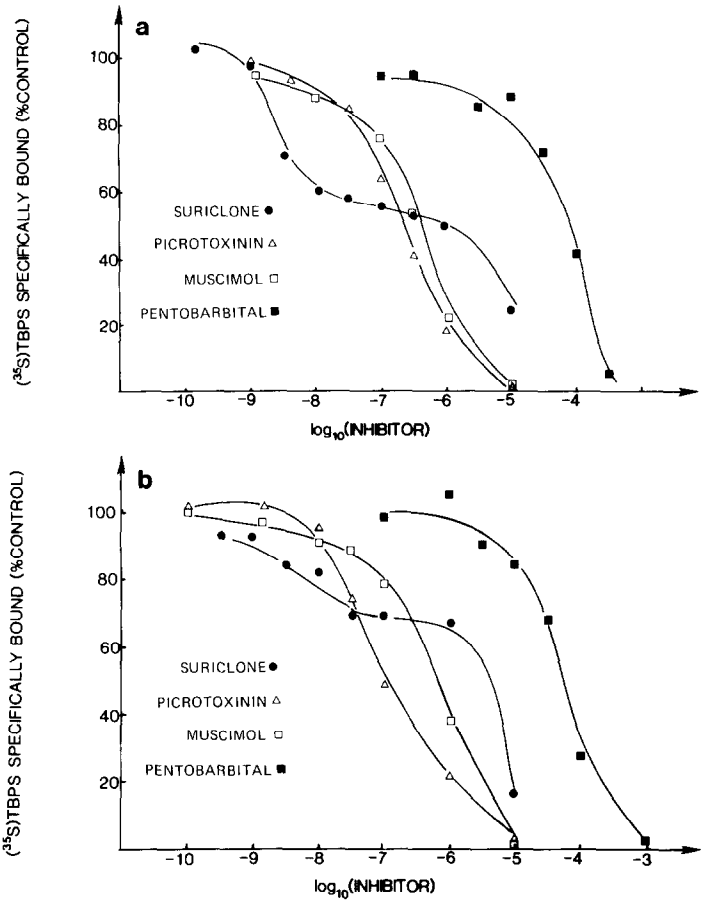

Figure 2. Pharmacologic specificity of (a) membrane and (b) souble [^{35}S]TBPS binding activity from rat cerebral cortical membranes. Binding assays are described in Materials and Methods. Data are from a representative experiment replicated twice.

preparations (Trifiletti, Snowman and Snyder, in preparation). In both the membrane and soluble states, inhibition of [^{35}S]TBPS binding by suriclone, muscimol and pentobarbital, but not picrotoxinin, is reversed by 1 μM bicuculline (data not shown).

One important line of evidence which suggests that [^{35}S]TBPS labels sites associated with chloride ionophores is the anion specificity of [^{35}S]TBPS binding. In both the membrane and soluble states, [^{35}S]TBPS binding is absolutely dependent on the presence of appropriate anions (Fig. 3a and b). Bromide ion maximally supports [^{35}S]TBPS binding as do chloride and nitrite ion (data not shown). Both iodide and thiocyanate ions support [^{35}S]TBPS binding to intermediate levels. All of these ions permeate

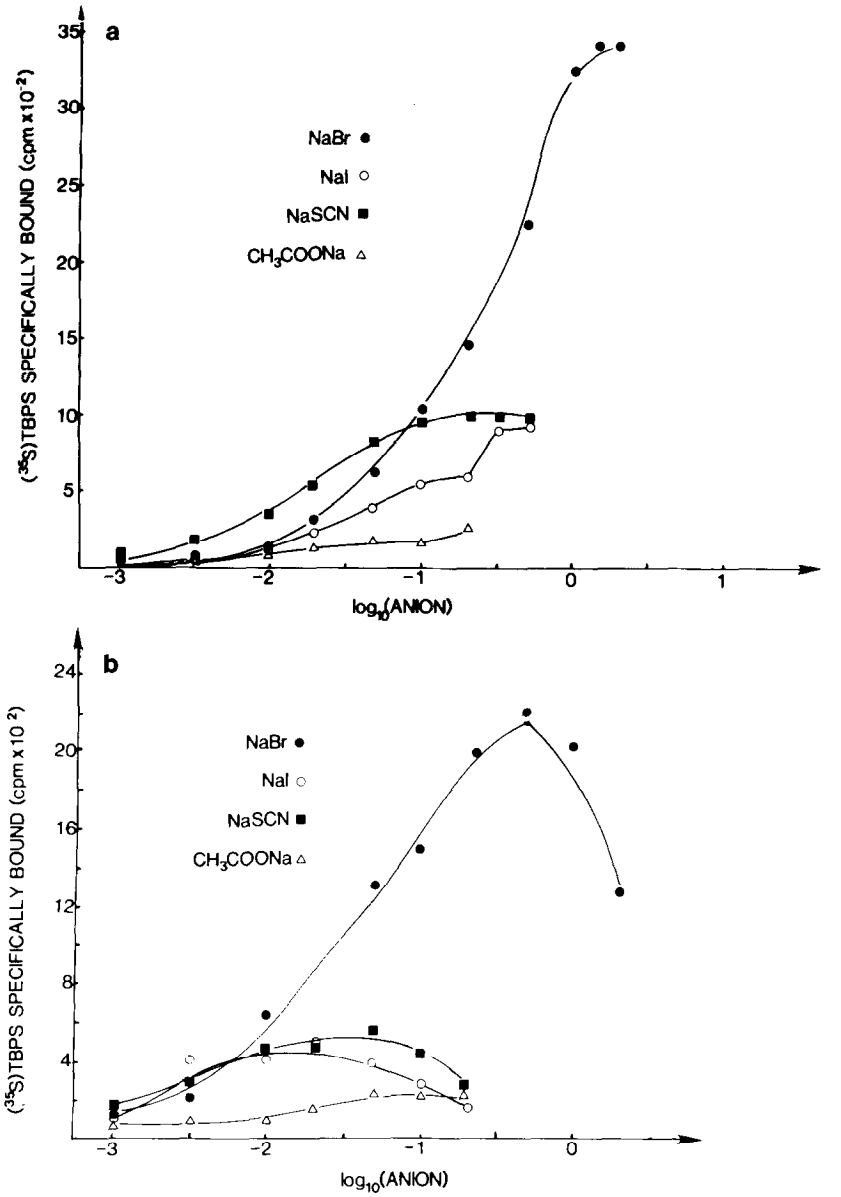

Figure 3. Anion regulation of (a) membrane and (b) soluble [^{35}S]TBPS binding activity from rat cerebral cortical membranes. To 100 µl of membranes or 0.8 % CHAPS extract prepared as described in Material and Methods, were added 50 µl of [^{35}S]TBPS in 50 mM Tris citrate (pH 7.1 at 25° C) and 50 µl of the sodium salt of appropriate anion (which sometimes contained 10^{-5} M TBPS to define non-specific binding), in 50 mM Tris citrate buffer. The mixture was then processed as described in Materials and Methods. Data is from a representative experiment replicated twice.

chloride channels in electrophysiological tests (17). In sharp contrast to bromide ion, the channel-impermeable anions acetate (Fig. 3A and 3B and data not shown) succinate, and perchlorate do not support [^{35}S]TBPS binding in either the membrane or soluble states.

Two groups have reported extensive purification of benzodiazepine binding sites (7, 8) extracted with sodium deoxycholate (7) or Triton X-100 (8). Neither of these solubiilzation procedures yield preparations with appreciable [^{35}S]TBPS binding activity (Table 1). Thus, the purified preparations of benzodiazepine receptor reported by these groups may lack the chloride ionophore. Receptor isolation with the procedures described here may preserve intact the macromolecular GABA-benzodiazepine-chloride channel receptor complex.

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