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PRELIMINARY COMMUNICATIONS

BINDING OF GABA RECEPTOR CHANNEL DRUGS TO A PUTATIVE VOLTAGE-DEPENDENT CHLORIDE CHANNEL IN TORPEDO ELECTRIC ORGAN

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In muscle and neuronal cells, there are two major types of Cl^- channels: the voltage-dependent Cl^- channels, which are activated by changes in voltage across the membrane and contribute a major share to the resting membrane conductance (1), and the chemically operated Cl^- channels which are parts of the inhibitory receptor systems of γ-aminobutyric acid (GABA) or glycine, and are activated by binding of the transmitter (2). Although much is known about the biochemical and pharmacological nature of these receptors, very little such information is available on the voltage-dependent Cl^- channels.

The opportunity to study one such channel became feasible when it was discovered that the electric organ of electric rays, which is a rich source for nicotinic acetylcholine (ACh) receptors, may also be rich in Cl^- channels. An anion-selective channel was discovered in the electric organs of *Torpedo californica* as shown by increased anion conductance in reconstituted membrane vesicles in planar phospholipid bilayer (3) and in liposomal membrane vesicles (4), and of *Narke japonica* as shown by changes in osmotic volume and efflux of $^{35}SO_4^-$ (5). These effects were inhibited by the specific Cl^- channel inhibitor 4,4'-diisothiocyano-2,2'-stilbenedisulfonic acid (DIDS). This Cl^- channel was also expressed in frog *Xenopus* oocytes that had been injected with mRNA fraction from *Torpedo* electric organ, while another fraction of mRNA induced the incorporation of ACh receptors (6). This electric organ Cl^- channel is highly anion selective (at least 20-fold more permeable to Cl^- than to K^+) compared to a 2-fold selectivity for a mitochondrial Cl^- channel (3).

The present study was initiated to attempt identification of the Cl^- channel in *Torpedo* electric organ, using [^{35}S]*t*-butylbicyclophosphorothionate (TBPS) as a probe since it binds with a high affinity to the GABA receptor Cl^- channel (7), and to determine and compare the drug specificities of these different types of Cl^- channels.

MATERIALS AND METHODS

Tissue preparation. Electric organs of *Torpedo nobiliana*, obtained from Biofish Assoc., Georgetown, MA, and stored at -90°, were homogenized in 2 vol. of ice-cold 1 mM Tris-HCl buffer (pH 7.4) containing 1 mM EDTA using a Polytron setting at 7 for 1 min twice, with 1 min rest in between. The homogenate was centrifuged at 1,000 *g* for 10 min, the supernatant fraction was collected, and the pellets were rehomogenized in the same buffer and recentrifuged at 1,000 *g* for 10 min again. The supernatant fractions of these two spins were combined and centrifuged at 30,000 *g* for 30 min. The resulting pellet was suspended in the binding assay buffer so that 1 ml of the final preparation represented 1 g of electric organ (1.5 mg protein/ml). These membranes were used immediately.

Binding assay. Binding of [^{35}S]TBPS (sp. act. 77 Ci/mmole, at the start of the study, thereafter adjusted for the short half-life of ^{35}S; $T_{1/2}$ = 87.2 days; from New England Nuclear Corp., Boston, MA) to the electric organ of the *Torpedo* was measured by a filter assay as previously used with rat brain membranes (7,8). *T. nobiliana* membranes (≈0.2 mg protein) were incubated with [^{35}S]TBPS (3 nM) for 60 min at 21° in a total volume of 1 ml of 200 mM KBr, 5 mM Tris-HCl and 1 mM EDTA buffer (pH 7.5). The contents were mixed and the assay was terminated by filtration over a Whatman GF/C glass-fiber filter (which was presoaked in 0.01% poly-L-lysine for 30 min before filtration) using a cell harvester filtration manifold (model M-24R, Brandel, Inc., Gaithersburg, MD) followed by two 5-ml washes with the same buffer (at room temperature). The filters were then placed in a scintillation minivial with 4 ml of a toluene-based scintillation solution, and their radioactivity was counted in a liquid scintillation spectrometer after about 8 hr. Nonspecific binding was determined as the binding measured in the presence of 4 μM unlabeled TBPS. To evaluate the effect of a drug on the specific binding of [^{35}S]TBPS, it was added to the assay buffer in dimethyl sulfoxide (5 μl). The solvent was added into control samples as well and had no effect on binding.

Binding data were analyzed by the EBDA computer program using an IBM PC computer (9) to obtain estimates of affinity constants (K_d values), site densities (B_{max}), Hill coefficients (n_H), and inhibition constants (K_i).

RESULTS

At 3 nM, [^{35}S]TBPS bound to *Torpedo* membranes, but this binding was dependent upon Br^- concentration (Fig. 1), as previously shown for [^{35}S]TBPS binding to the GABA receptor channel (7). Accordingly, binding was measured routinely in the presence of 200 mM KBr (Fig. 1).

An estimated maximal binding capacity of 36 ± 3.5 pmoles/mg protein and an estimated K_d of 1.3 ± 1.5 μM were obtained from the Scatchard analysis of the binding data (Fig. 2). The estimated B_{max} value arrived at here is obtained by extrapolation from the Scatchard plot assuming that there is only one affinity since the Hill coefficient of binding was 0.99 ± .02. The highest concentration of TBPS used (4 μM) gives ≈70% saturation. Higher concentrations of TBPS could not be used because the drug becomes insoluble in the incubation buffer. The possibility that other sites of lower affinity may be present exists, and we are currently modifying the assay condition to allow use of higher concentration of TBPS to reach the saturation level experimentally. Thus the above binding parameters should be regarded as preliminary estimates.

Since [^{35}S]TBPS binds with high affinity to the GABA receptor Cl^- channel (7,8), it was important to determine if *Torpedo* electric organ had GABA receptors. There was no specific binding of [^{3}H]muscimol (10 nM) to a GABA receptor site, using Na^+-free 50 mM Tris-HCl buffer, pH 7.1, or [^{3}H]flunitrazepam (4 nM) to a benzodiazepine receptor site, using 50 mM Tris-citrate buffer, pH 7.0. Furthermore, GABA, bicuculline or flunitrazepam (from 10 nM to 100

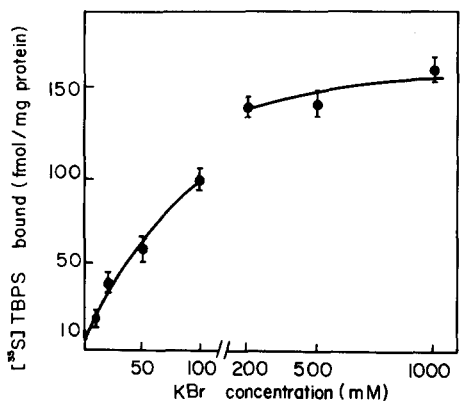

Fig. 1. Effect of KBr concentration on the specific binding of [^{35}S]TBPS at 3 nM to *Torpedo* membranes. Symbols and bars represent means ± S.D. of triplicate experiments.

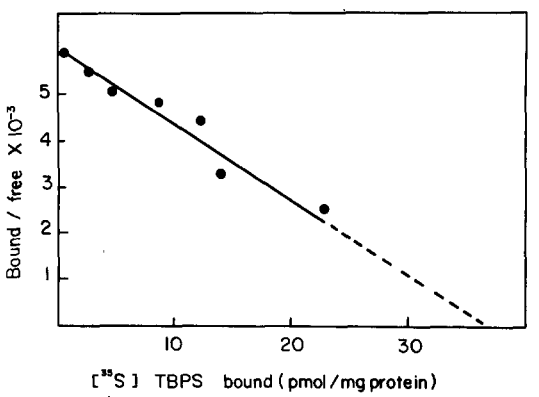

Fig. 2. Scatchard plot of [^{35}S]TBPS binding to *Torpedo* electric organ membranes measured in the presence of 200 mM KBr at 21°. Nonspecific binding was that observed in the presence of 4.5 μM unlabeled TBPS. The points are the means of three experiments, each done in triplicate.

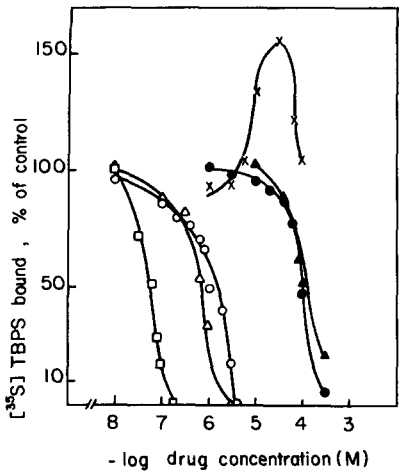

Fig. 3. Effects of drugs on the specific binding of [^{35}S]TBPS (3 nM) to *Torpedo* electric organ membranes. The tissue was added last to the buffer-KBr (200 mM) solution and [^{35}S]TBPS and then incubated for 60 min at 21° before filtration. Key: lindane (□), endrin (Δ), TBPS (o), picrotoxinin (●), pentobarbital (▲), and DIDS (x).

μM) had no effect on [^{35}S]TBPS binding to the *Torpedo* membranes. On the other hand, picrotoxinin and pentobarbital displaced the specific binding of [^{35}S]TBPS with a K_i of 100 ± 18 μM and 110 ± 5 μM respectively. Phenobarbital was ineffective at 100 μM. The specific inhibitor of Cl^- transport, DIDS, increased the specific binding of [^{35}S]TBPS in a concentration-dependent manner in the concentration range of 1 to 30 μM (Fig. 3), but at higher concentrations of DIDS this effect was reversed. Endrin, a cyclodiene chlorinated hydrocarbon insecticide, displaced the specific binding with a K_i of 0.75 ± 0.07 μM, while lindane, another chlorinated hydrocarbon insecticide, was more potent, having a K_i of 0.04 ± 0.01 μM (Fig. 3, Table 1).

The more toxic chlorinated hydrocarbon stereoisomers were more potent in inhibiting [^{35}S]TBPS binding to the electric organ membranes: 1 μM dieldrin and endrin inhibited binding by 40 and 100%, respectively; 1 μM endosulfan II and endosulfan I by 42 and 72%, respectively, and β-BHC and γ-BHC (lindane) by 0 and 100%, respectively. Furthermore, the more toxic epoxides were more potent, such as 1 μM aldrin and dieldrin inhibiting 6 and 40%, respectively, isodrin and endrin inhibiting 10 and 100%, respectively, and 1 μM heptachlor and heptachlorepoxide inhibiting 27 and 58%, respectively. Furthermore, the anthelmintic and insecticidal avermectin B_{1a}, from 1 nM to 50 μM, had no effect on [^{35}S]TBPS binding to *Torpedo* electric organ.

Cholinergic drugs at 100 μM had no effect on [^{35}S]TBPS specific binding to the electric organ membranes; neither did the agonist carbamylcholine, the competitive antagonist *d*-tubocurarine or the noncompetitive blockers of the nicotinic ACh receptor, perhydrohistrionicotoxin and phencyclidine. On the other hand, amantadine at 0.1 mM inhibited 46% of [^{35}S]TBPS binding. Furthermore, binding of 2 nM [^{3}H]perhydrohistrionicotoxin to the channel sites of the nicotinic ACh receptor was unaffected by 4 μM TBPS. But picrotoxinin and pentobarbital at 100 μM inhibited [^{3}H]perhydrohistrionicotoxin binding by 25 and 35%, respectively.

Table 1. Inhibition of [^{35}S]TBPS binding to the putative voltage-dependent Cl^- channel in *Torpedo* electric organ and the GABA receptor Cl^- channel in rat brain

Drug	K_i (μM)	
	Torpedo electroplax	Rat brain*
TBPS	1.37	0.05
Picrotoxinin	100.00	0.20
Pentobarbital	110.00	58.00†
Endrin	0.75	0.03
Lindane	0.04	0.15

*Data calculated from Ref. 10.
†Data from Ref. 7.

DISCUSSION

The specific binding of [^{35}S]TBPS to *Torpedo* electric organ membranes is obviously not to GABA receptors, since these membranes do not bind GABA receptor ligands such as [^{3}H]muscimol or [^{3}H]flunitrazepam, nor is [^{35}S]TBPS binding affected by GABA, unlike its effect on [^{35}S]TBPS binding to the GABA receptor channel in mammalian brain (6). This convulsant inhibits GABA transmission presumably by binding to the picrotoxinin binding site of the receptor (7,8).

It is also unlikely that [^{35}S]TBPS is binding to the nicotinic ACh receptor, which is found in high concentrations in this tissue, since binding is unaffected by carbamylcholine, *d*-tubocurarine and several noncompetitive inhibitors of this receptor and TBPS has no effect on [^{3}H]perhydrohistrionicotoxin binding.

The sensitivity of [^{35}S]TBPS binding to Br^- (Fig. 1) and DIDS (Fig. 3) suggests that the binding protein may be to the Cl^- channel, which is known to exist in *Torpedo* electric organs (3). The effect of DIDS is interesting in that at lower concentrations it potentiated [^{35}S]-TBPS, thus is suggested to bind to another site on this putative Cl^- channel. The TBPS binding site identified in *Torpedo* electric organs is of far lower affinity than that identified in rat brain in association with the GABA receptor (7). There are several similarities between [^{35}S]TBPS binding to the putative voltage-dependent Cl^- channel in *Torpedo* membranes and its binding to the GABA receptor Cl^- channel in mammalian brain (7,8,10,11). They are both sensitive to Br^- concentration, are inhibited by convulsants such as picrotoxinin, cyclodiene insecticides and lindane as well as depressants such as pentobarbital, and the inhibition is stereospecific. However, there are differences in drug sensitivity of TBPS binding between this putative voltage-dependent Cl^- channel and that of the $GABA_A$ receptor to various ligands. TBPS and most convulsants, except for lindane, have higher potency in inhibiting [^{35}S]TBPS binding to the GABA receptor Cl^- channel than the *Torpedo* putative Cl^- channel (Table 1). It raises the possibility that the insecticidal action of lindane, which has lower affinity for the GABA receptor Cl^- channel, may be due primarily to its action on a voltage-dependent Cl^- channel. The lack of effect of GABA, bicuculline or benzodiazepine is explainable by the absence of their binding sites on this putative voltage-dependent Cl^- channel. This study shows that the site of binding of TBPS in *Torpedo* electric organs is not a protein identical to the protein possessing the GABA/benzodiazepine sites, thus raising the possibility that these sites developed separately from the chloride channel protein. The lack of effect of avermectin B_{1a} on [^{35}S]TBPS binding to this putative voltage-dependent Cl^- channel, yet its inhibition of [^{35}S]TBPS binding to the GABA receptor Cl^- channel (11), suggests that the action of avermectin B_{1a} on the GABA receptor may be via other sites.

It should be made clear that no claims are made at this time that the TBPS binding sites of *Torpedo* electric organs are in fact the voltage-dependent chloride channels of that tissue. Verification of the identity of the [^{35}S]TBPS binding protein in *Torpedo* electric organ as the voltage-dependent Cl^- channel awaits studying the effects of drugs, that activate or inhibit [^{35}S]TBPS binding, on Cl^- flux across membrane preparation of *Torpedo* electric organs. These experiments are currently in progress.

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