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Rat Brain Dendrotoxin Receptors Associated with Voltage-Gated Potassium Channels: Dendrotoxin Binding and Receptor Solubilization

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SUMMARY

Venom from the green mamba, *Dendroaspis angusticeps*, contains four polypeptides termed dendrotoxins (DaTXs) that block brain Ca-independent voltage-gated K channels. We compared the binding to rat brain receptors of two of these DTXs, α-DaTX and β-DaTX, which preferentially block inactivating and noninactivating K channels, respectively. ^{125}I-α-DaTX and ^{125}I-β-DaTX bind to single classes of receptor sites on synaptic membranes (K_D = 0.7 and 36 nM for α-DaTX and β-DaTX, respectively), with pH optima of about 6.5. The binding of both iodinated toxins was optimal in solutions containing 150 mM NaCl and decreased as Na was replaced with other alkali metal ions; the rank order for support of toxin binding was Na > K > Li > Rb. Cs (IC_{50} = 5–6 mM) prevented toxin binding, as did the divalent cations Ba and Ca (IC_{50} = 4–6 and 9–13 mM, respectively). The inhibition of ^{125}I-α-DaTX binding by Cs and Ba was noncompetitive. The displacement of ^{125}I-α-DaTX and ^{125}I-β-DaTX binding by the four unlabeled DaTXs was similar; the relative potency was α-DaTX > β-DaTX > γ-DaTX. The displacement curve for δ-DaTX did not parallel the others. When cross-linked with dimethylsuberimidate, both iodinated toxins covalently labeled membrane polypeptides of similar molecular weight (M_r = 65,000). The α-DaTX and β-DaTX receptors were solubilized from rat synaptic membranes. Toxin binding to the soluble receptors was preserved in the presence of K and lecithin and decreased as K was replaced with Rb > Cs > Li > Na. The affinity of ^{125}I-α-DaTX for the solubilized receptor was decreased 10-fold (K_D = 7 nM); the affinity of ^{125}I-β-DaTX was decreased 3.5-fold (K_D = 124 nM). However, the four unlabeled DaTXs retained their relative potencies for the inhibition of ^{125}I-α-DaTX binding. The molecular weight of the solubilized receptor was estimated to be about 270,000 by sucrose density gradient centrifugation. These data raise the possibility that the inactivating ("A-type") and noninactivating voltage-gated K channels in rat brain may have similar subunits and that the channels may be composed of four M_r 65,000 polypeptides.

The venoms of a number of poisonous snakes have neurotoxic actions. For example, the venoms of mamba (*Dendroaspis*) snakes contain polypeptides that produce synaptic facilitation (1, 2). One of the polypeptides from *Dendroaspis angusticeps* venom, often referred to as dendrotoxin (and termed α-DaTX in this article; see Ref. 3), has been shown to facilitate neurotransmitter release at a variety of synapses (1, 2). This facilitatory action is apparently an indirect result of the ability of α-DaTX to block certain Ca-independent voltage-gated K channels in presynaptic nerve terminals. α-DaTX blocks inactivating (A-type), voltage-gated, K channels in the hippocampus (4). Further electrophysiological measurements indicate that α-DaTX and its structural homologue from *Dendroaspis polylepsis polylepsis* venom, toxin I or DTX_I, block voltage-gated K channels in a variety of neurons (5–9).

In addition to α-DaTX, *D. angusticeps* venom contains several other polypeptides that block K channels. Using a tracer (^{86}Rb) flux assay to measure K channel activity in rat brain synaptosomes, Benishin *et al.* (3) identified three such components (dentrotoxins) that also block Ca-independent voltage-gated K channels. These three polypeptides have substantial sequence homologies to α-DaTX. Nevertheless, the four DaTXs have different selectivities; two of the toxins, α-DaTX and δ-DaTX, preferentially block inactivating K channels, whereas the other two, β-DaTX and γ-DaTX, preferentially block noninactivating K channels (3).

High affinity receptor sites for α-DaTX have been identified in brain (10, 11). The present report describes a further characterization of the rat brain α-DaTX binding site and compares the receptor binding characteristics of α-DaTX with the bind-

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ABBREVIATIONS: DaTX, *Dendroaspis angusticeps* toxin or dendrotoxin; DTX_I, *Dendroaspis polylepis polylepis* toxin I; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate.

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ing of a homologue, β-DaTX. The results indicate that the binding sites for these two DaTXs are structurally related. Taken together, the binding data and pharmacological properties are consistent with the idea that the DaTX binding sites are located on K channels. Conditions for the solubilization of the α-DaTX and β-DaTX receptors/putative K channels are also described. A preliminary report of some of these findings has been published (12).

Materials and Methods

Isolation of venom polypeptides. The DaTXs were isolated from *D. angusticeps* venom (Sigma Chemical Co., St. Louis, MO) and assayed for K channel-blocking activity as previously described (3). The iodination of the toxins was a modification of the method of Black *et al.* (10) and will be reported elsewhere.[2]

Toxin binding to membrane receptors. The binding of radioiodinated toxins to rat forebrain synaptic membranes (13) was assayed by a rapid centrifugation technique. The membranes (1 mg of protein/ml) were incubated at 37° in Solution A (145 mM NaCl, 5 mM KCl, 1.4 mM $MgCl_2$, and 20 mM HEPES, pH 7.0) usually containing 1 nM radioiodinated toxin and increasing amounts of the nonradiolabeled toxins or other test compounds. After 30 min, 0.2 ml of the binding mixtures were layered on top of 0.2 ml of dinonyl phthalate/silicone oil (65:35, v/v) in microcentrifuge tubes. The membranes and bound iodinated toxin were collected by centrifugation through the oil mixture at 8700 × *g*, for 4 min, at room temperature in a Microfuge (Beckman Instruments, Palo Alto, CA). The membrane pellets were washed once with Solution A and counted for γ-radiation. Binding data were analyzed by the curve-fitting program LIGAND (Esevier/Biosoft, Milltown, NJ).

Cross-linking. Synaptic membranes were equilibrated with radiolabeled toxin as described above, and the bound toxin was separated from unbound toxin by centrifugation through the dinonyl phthlate/silicone oil mixture. The membranes were washed once with Solution A and then once with 250 mM triethanolamine titrated to pH 8.5 (TE buffer). The washed pellets were resuspended in TE buffer containing 0.1–5.0 mg/ml dimethylsuberimidate and incubated at room temperature for 2–4 hr. The cross-linked material was collected by centrifugation at 8700 × *g*, for 5 min, at room temperature in a Microfuge; the pellets were suspended in SDS-PAGE solubilization buffer (3% SDS, 5% β-mercaptoethanol, 10% glycerol, and 5 mM Tris, pH 8.0). The samples were either kept overnight at room temperature or placed in a boiling water bath for 10 min before the separation of the membrane proteins by SDS-PAGE as described (14). The gels were stained and dried, and autoradiograms were prepared to locate the attached radioiodinated toxin.

Receptor solubilization and assay. The DaTX receptor was solubilized by the following procedure. Synaptic membranes (4 mg of protein/ml) were incubated on ice for 15 min in Solution B (150 mM KCl, 2 mM $MgCl_2$, 10 mM HEPES, pH 7.0) containing 0.25% lecithin (Sigma) and 0.8% zwittergent 3-12 (Pierce Chemical Co., Rockford, IL). The solubilized proteins were subsequently recovered in the supernatant solutions after the mixtures were centrifuged in a Type 70.1 Ti rotor (Beckman) for 30 min at 4° and 171,000 × *g* (r_{av} = 6.12 cm). Additional details and variations in the extraction procedure are described in Results.

To assay for receptor binding, ^{125}I-α-DaTX (usually 1 nM) and other test compounds were added to 0.6-ml aliquots of the undiluted solubilized proteins (final total volume = 0.7 ml). The binding mixtures were incubated at 37° for 30 min. Bound toxin was separated from unbound toxin by a "spun column" method; 0.3-ml aliquots of the binding mixtures were applied to a 2-ml (0.4 × 5 cm) Sephadex G-50 column that had been equilibrated with Solution B containing 0.025% lecithin and 0.08% zwittergent 3-12. The columns were centrifuged at 800 × *g* for 90 sec at room temperature, and the eluate (containing the receptor and bound toxin) was counted for γ-radiation. Under these conditions, the columns retained 93–95% of the unbound toxin, and about 88% of the total applied protein was recovered in the eluate.

Sucrose density gradient. Synaptic membranes were preequilibrated with ^{125}I-α-DaTX as described above. Unbound radioiodinated toxin was removed by centrifugation through the dinonyl phthalate/silicone oil mixture, and the membrane pellets were resuspended in Solution A containing 0.25% lecithin and 1.0% zwittergent 3-12. Following detergent extraction, the solubilized proteins containing the radioiodinated toxin/receptor complex were collected by ultracentrifugation as described above, and the unbound ^{125}I-α-DaTX remaining in the supernatant fraction was removed by the spun column method. An aliquot (0.3 ml) of the spun column eluate, which contained the solubilized toxin/receptor complex, was applied to a 13-ml continuous 5–20% (w/v) sucrose gradient containing 0.1% zwittergent 3-12 and 0.025% lecithin. The gradient was centrifuged at 4° in an SW40 rotor (Beckman) at 200,000 × *g* (r_{av} = 11.27 cm), for 5 hr. Fractions were collected from the gradient and counted for the presence of ^{125}I. Catalase was added as an internal molecular weight marker. The migration of catalase along the sucrose gradient was determined by enzyme assay (15).

Results

^{125}I-α-DaTX and ^{125}I-β-DaTX Binding to Brain Membrane Receptors

Four polypeptides (collectively referred to as DaTXs) that block Ca-independent voltage-gated K channels in rat brain have been isolated from *D. angusticeps* venom (3). Two of the polypeptides, α-DaTX and β-DaTX, which preferentially block inactivating and noninactivating K channels, respectively (3), were radioiodinated and used for the study of their brain receptors. Of critical importance for this study is the evidence that block of K channels by iodo-α-DaTX was comparable to the block produced by native (noniodinated) α-DaTX.[3] Unfortunately, the limited supply of β-DaTX precluded a similar study with this toxin.

Both iodinated DaTXs bound with high affinity to an enriched rat synaptic membrane preparation. This binding depended upon ionic strength. Toxin binding was promoted in solutions containing high salt concentrations (e.g., 150 mM NaCl, or 5 mM KCl plus 145 mM NaCl). Specific binding was reduced by more than 95% in the absence of salt (e.g., when binding was assayed in 20 mM Tris/HEPES, pH 7.0, or in 20 mM sodium phosphate, pH 7.4).

Fig. 1 illustrates the effect of pH on the binding of the two DaTXs. The binding of ^{125}I-α-DaTX (Fig. 1A) and ^{125}I-β-DaTX (Fig. 1B) showed similar pH dependencies; specific binding was maximal at pH 6–7 and decreased with increasing pH. The decrease in specific binding resulted from a decrease in the total binding of the respective toxin to the membranes and an increase in the nonspecific binding (measured in the presence of excess unlabeled toxin). Because the DaTXs are basic proteins, these results suggest that it is the charged forms of the DaTXs that are recognized by the specific membrane receptor sites; this presumably involves ionic interactions with the exposed lysine residues on the toxin molecules (2). All subsequent binding assays were done in solutions buffered to pH 7.0.

^{125}I-α-DaTX (Fig. 2A) and ^{125}I-β-DaTX (Fig. 2B) each labeled a single class of binding sites on brain synaptic membranes. Scatchard analyses gave apparent dissociation constants (K_D values of 0.69 ± 0.09 nM (13 experiments) and 35.9 ± 7.1 nM (seven experiments) for ^{125}I-α-DaTX and ^{125}I-β-DaTX, respec-

[2] R. G. Sorensen and R. S. Rogowski, unpublished observations.

[3] R. G. Sorensen, R. S. Rogowski, and M. P. Blaustein, unpublished data.

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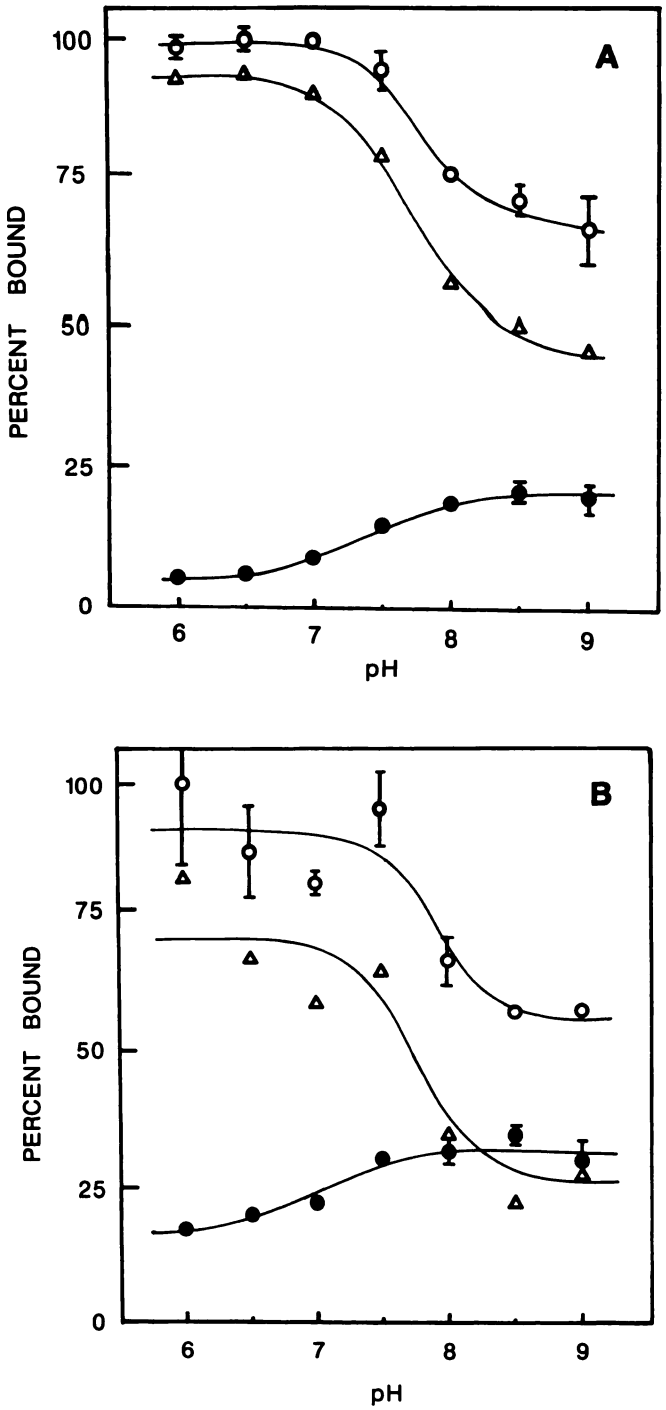

Fig. 1. pH Dependence of DaTX binding. The binding of ^{125}I-α-DaTX (A) and ^{125}I-β-DaTX (B) to synaptic membranes was assayed by the microcentrifugation method in the presence of Solution A, containing 1 nM radioiodinated toxin, at the pH indicated. A 500-fold excess of the respective unlabeled DaTX was used to determine nonspecific binding. The graphs show total binding (○), nonspecific binding (●), and specific binding (△). *Error bars* indicate ± standard errors of triplicate determinations.

tively; the calculated binding site densities (B_{max} values were 1.8 ± 0.2 and 8.6 ± 1.7 pmol of ligand bound/mg of protein, respectively.

Modulators of ^{125}I-α-DaTX and ^{125}I-β-DaTX binding

Displacement by unlabeled DaTXs. The four K channel blockers from *D. angusticeps* venom [α-, β-, γ-, and δ-DaTX (3)] inhibited the binding of ^{125}I-α-DaTX (Fig. 3A) and ^{125}I-β-

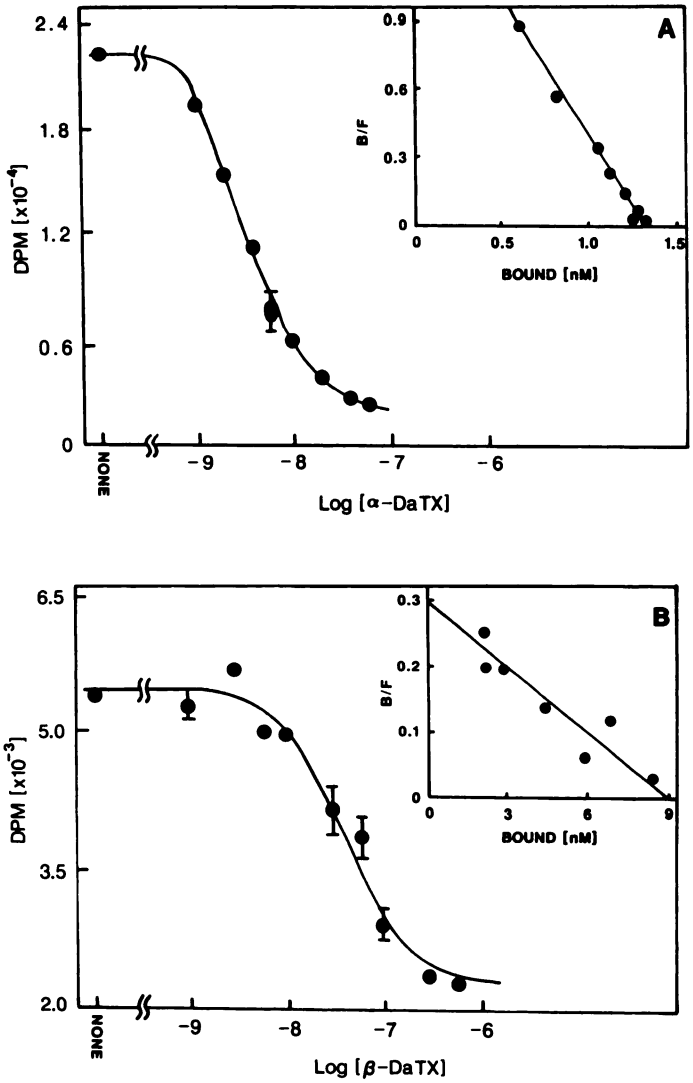

Fig. 2. DaTX binding to synaptic membranes. The *main graphs* illustrate the competitive displacement of bound ^{125}I-α-DaTX (A) and ^{125}I-β-DaTX (B) by increasing amounts of the respective unlabeled toxin. The data were obtained by the microcentrifugation method. The corresponding Scatchard plots are presented in the *insets*. Each *point* represents the mean of triplicate determinations (*error bars* indicate ± standard errors).

DaTX (Fig. 3B) to synaptic membranes. The displacement profiles for the two iodinated DaTXs were similar: α-DaTX > β-DaTX > γ-DaTX. The displacement curves produced by δ-DaTX did not parallel the others; this may indicate that δ-DaTX has complex interactions at the toxin binding sites.

The interactions between the polypeptides at the DaTX receptors was studied further by Scatchard analysis. As expected, unlabeled α- and β-DaTX competitively inhibited the binding of their respective iodinated forms (data not shown). Similarly, as illustrated in Fig. 4A, the apparent affinity of ^{125}I-α-DaTX for its receptor decreased, with only a small effect on binding site density, when the binding of this toxin was measured in the presence of unlabeled β-DaTX. This indicates that β-DaTX is a competitive inhibitor at the α-DaTX receptor.

In contrast, α-DaTX appears to have a more complex (allosteric) interaction at the β-DaTX receptor. In the presence of unlabeled α-DaTX, there was a decrease in the total number of ^{125}I-β-DaTX binding sites as well as in the apparent affinity of ^{125}I-β-DaTX for its receptor (Fig. 4B).

Effects of alkali metal and alkaline earth cations. As

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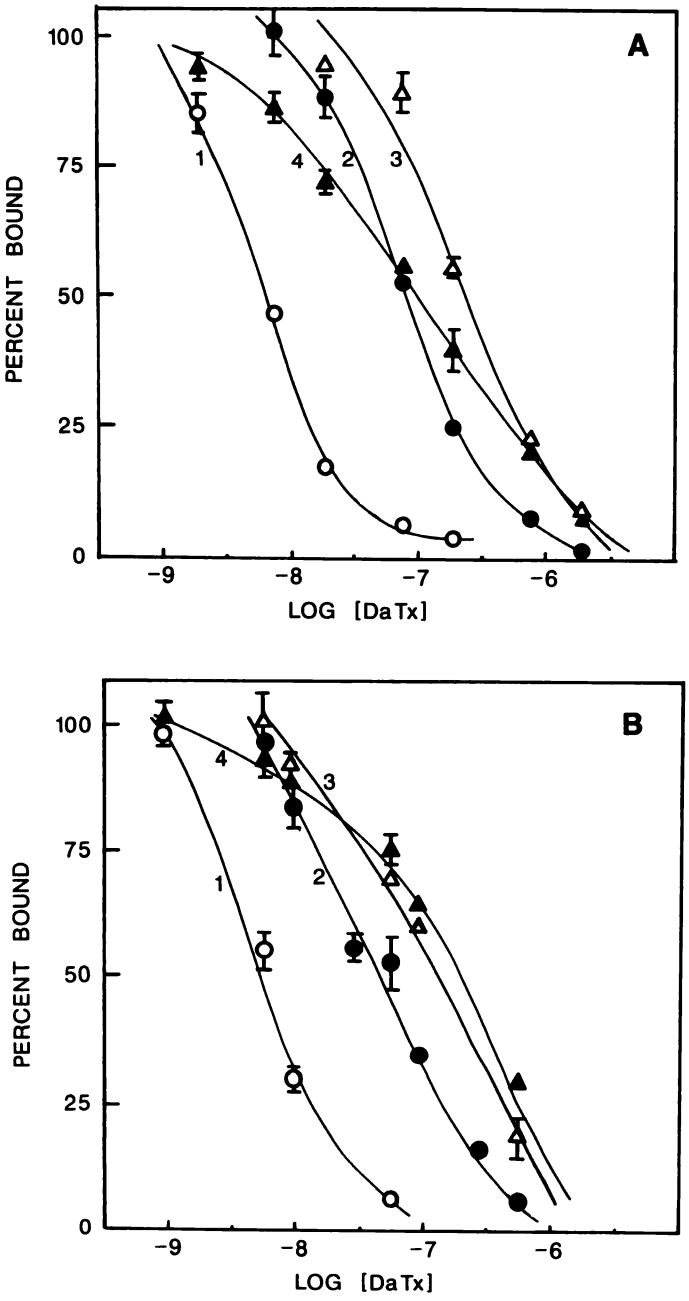

Fig. 3. Inhibition of iodinated α- and β-DaTX binding to synaptic membranes by unlabeled DaTXs. The *graphs* show the inhibition of ^{125}I-α-DaTX (A) and ^{125}I-β-DaTX (B) binding by increasing concentrations of the four unlabeled dendrotoxins. *1*, α-DaTX; *2*, β-DaTX; *3*, γ-DaTX; and *4*, δ-DaTX. *Error bars* indicate ± standard errors of triplicate determinations.

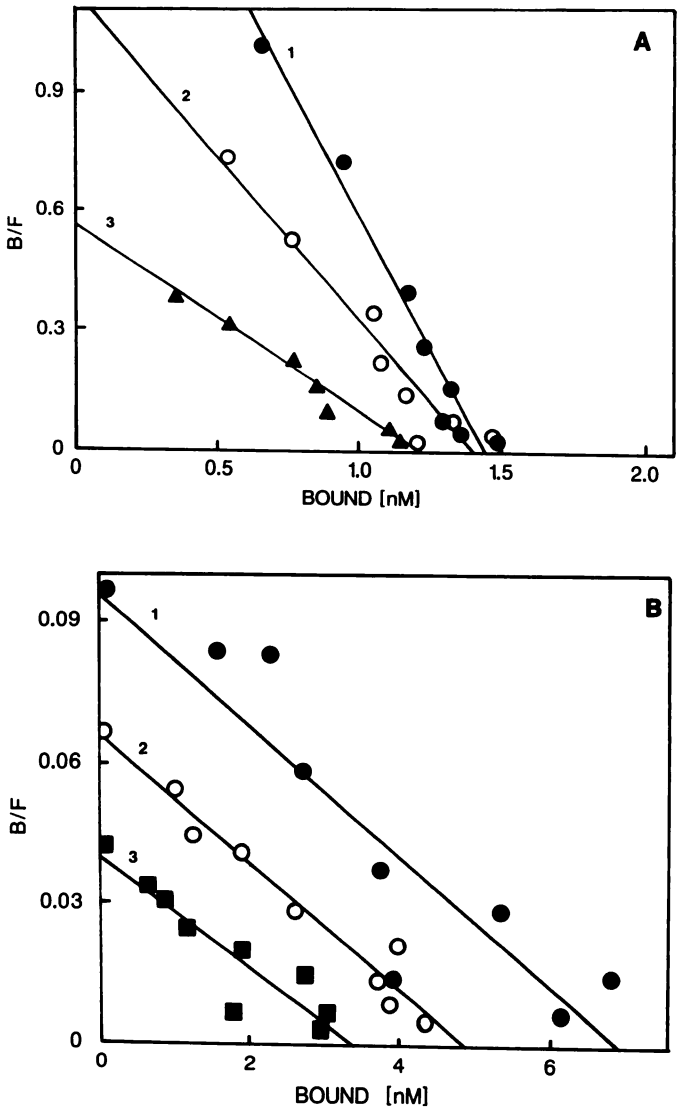

Fig. 4. Scatchard analysis of the inhibition of iodinated α- and β-DaTX binding by the unlabeled toxins. A, ^{125}I-α-DaTX binding to synaptic membranes was measured by competitive displacement with increasing concentrations of unlabeled α-DaTX in the presence of 0 (*1*), 47.6 (*2*), and 95.2 nM β-DaTX (*3*). B, ^{125}I-β-DaTX binding to synaptic membranes was measured by competitive displacement with increasing concentrations of unlabeled β-DaTX in the presence of 0 (*1*), 2.2 (*2*), and 5.5 nM α-DaTX (*3*). *Data points* represent the means of triplicate determinations.

noted above, DaTX binding could be measured only in solutions with a high salt concentration. To determine whether this was a manifestation of a nonspecific ionic strength requirement for toxin binding or whether binding required specific monovalent or divalent cations, toxin binding was assayed in the presence of various alkali metal and alkaline earth cations.

The binding of both ^{125}I-α-DaTX and ^{125}I-β-DaTX was maximal in the presence of 150 mM NaCl (the highest concentration tested). The amounts of the two radioiodinated DaTXs bound to synaptic membranes decreased, relative to the levels bound in the presence of Na, as Na was replaced isosmotically by (in order of increasing loss of toxin binding) K > Li > Rb > Cs (Table 1). Cs (IC_{50} = 5–6 mM) was the only monovalent cation tested that completely prevented the binding of the two radiolabeled toxins (Table 1).

The divalent cations Ba and Ca were also tested. Ba (IC_{50} = 4–6 mM) was more effective than Ca (IC_{50} = 9–13 mM) in inhibiting both ^{125}I-α-DaTX and ^{125}I-β-DaTX binding (Table 1).

The inhibition of ^{125}I-α-DaTX binding by Cs and Ba was examined further by Scatchard analysis. In the presence of either Cs (Fig. 5A) or Ba (Fig. 5B), both the apparent K_D for ^{125}I-α-DaTX binding to its membrane receptor and the B_{max} decreased. Thus, Cs and Ba are both probably allosteric inhibitors at the DaTX binding sites.

Cross-linking of the Iodinated DaTXs to their Membrane Receptors

^{125}I-α-DaTX and ^{125}I-β-DaTX were attached covalently to their membrane receptor sites with the cross-linking reagent

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TABLE 1

Effects of ions on ^{125}I-α-DaTx and ^{125}I-β-DaTx binding to rat synaptic membranes

^{125}I-α-DaTX and ^{125}I-β-DaTX binding was assayed in solutions containing either: (i) for the monovalent cations, 75 mM NaCl plus 75 mM test ion (as the chloride salt), or 150 mM test ion, or (ii) for the divalent cations, 150 mM NaCl and increasing amounts of $BaCl_2$ or $CaCl_2$; all solutions also contained 1.4 mM $MgCl_2$ and 10 mM HEPES (pH 7.0). Values are the averages from three to five experiments, each performed in triplicate. The data are presented as the percentage of neurotoxin bound in the presence of the test cation, relative to the amount bound (taken as 100%) in the presence of 150 mM NaCl. Cs, Ba, and Ca completely blocked neurotoxin binding; the concentrations of these ions that produced 50% block (IC_{50} values) are given in parentheses.

	Binding	
	^{125}I-α-DaTx	^{125}I-β-DaTx
	%	
Monovalent cations		
Na, 150 mM	100	100
K, 75 mM	80.0	79.1
K, 150 mM	76.5	71.3
Li, 75 mM	82.4	52.2
Li, 150 mM	64.9	45.0
Rb, 75 mM	52.8	42.4
Rb, 150 mM	46.4	29.5
Cs	0 (5.0 mM)	0 (6.0 mM)
Divalent cations		
Ba	0 (4.0 mM)	0 (6.0 mM)
Ca	0 (13.0 mM)	0 (9.0 mM)

dimethylsuberimidate. The labeled polypeptides were separated by SDS-PAGE and autoradiograms were prepared (Fig. 6). The results indicate that both radioiodinated toxins labeled polypeptides that migrated with apparent molecular weights of 72,000. This corresponds to apparent molecular weights of 65,000 for both receptors, after correction for the molecular weights of the bound toxins.

Solubilization of DaTX Receptors

Favorable conditions for the solubilization of the DaTX receptors were established by comparing the ability of several detergents to solubilize membrane receptor proteins that had been preequilibrated with ^{125}I-α-DaTX. The following procedure was used. Synaptic membranes were equilibrated with ^{125}I-α-DaTX and the unbound toxin was removed by microcentrifugation (see Materials and Methods). The radiolabeled membranes were then incubated on ice in the presence of a detergent; after 15 min, the suspensions were subjected to ultracentrifugation and the soluble proteins were recovered in the supernatant. To determine the amount of radioiodinated toxin that remained bound after solubilization of the receptor proteins, aliquots of the supernatant solutions containing the solubilized proteins were passed through a Sephadex G-50 column and eluted by low speed centrifugation (spun column method). The radioactivity in the eluate collected from the spun columns was taken as the amount of ^{125}I-α-DaTX bound to the receptor protein after detergent extraction.

The detergents tested, at concentrations ranging from 0.1 to 1%, included Triton X-100, deoxycholate, cholate, octylglucoside and CHAPS. Each of these detergents solubilized 50–60% of the radioactivity initially bound to the membranes as well as 50–60% of the membrane protein. However, little of the solubilized radioactivity (≤5%) remained bound to the solubilized protein when the free toxin was subsequently separated from the solubilized protein by the spun column method. This indicates that there was little recovery of functional receptor (defined as soluble protein retaining bound toxin).

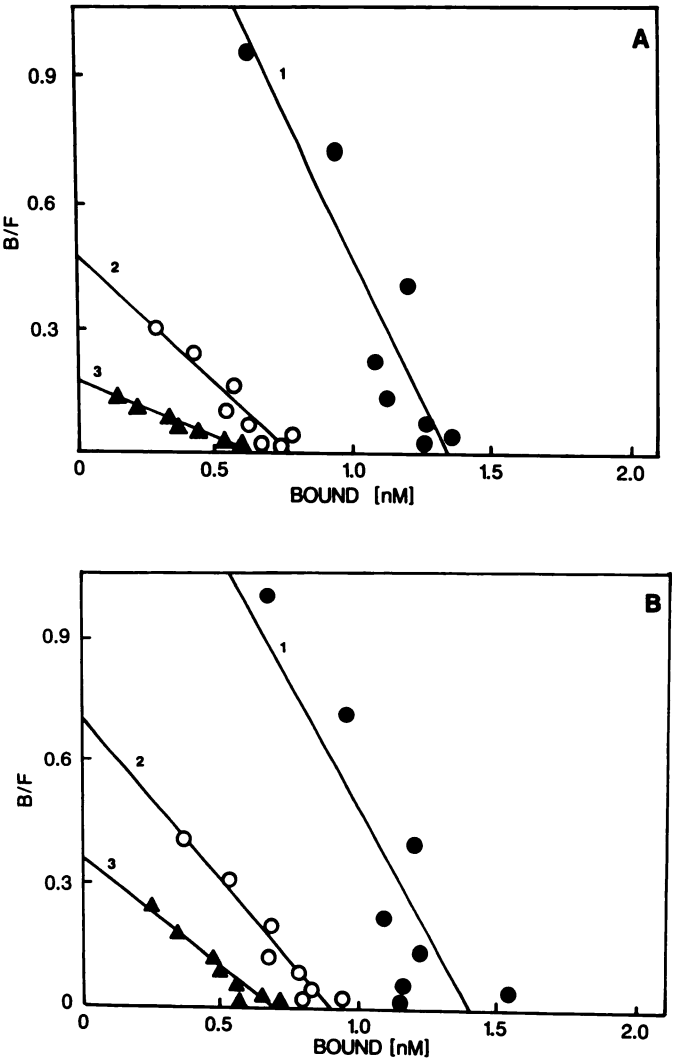

Fig. 5. Scatchard analysis of Cs and Ba inhibition of α-DaTX binding. ^{125}I-α-DaTX binding to synaptic membranes was measured by competitive displacement with unlabeled α-DaTX in the presence of several concentrations of CsCl (A) or $BaCl_2$ (B). The corresponding Scatchard plots are shown for binding measured in the presence of 0 (*1*), 2.7 (*2*), or 8.0 mM Cs (*3*) (A) and 0 (*1*), 1.4 (*2*), or 4.8 mM BA (*3*) (B). *Data points* represent the means of triplicate determinations.

The zwitterionic detergents zwittergent 3–12 and zwittergent 3–14 yielded better results; in the presence of either zwittergent (1% w/v), 50–60% of the radioactivity initially bound to the membranes and 75–80% of the total membrane protein was routinely solubilized. Furthermore, 20–25% of the radioactivity in the supernatant remained bound to soluble protein, as determined by the spun column method. This corresponds to a 10–15% recovery of functional receptor from synaptic membranes. Higher concentrations (2% of the zwittergents increased the amounts of both radioactivity and solubilized membrane proteins by 10%, but the total recovery of functional receptor remained at approximately 10–15%. All subsequent studies were done using zwittergent 3–12.

Phospholipids further stabilized the toxin-binding activity. The presence of 0.25% phosphatidylcholine (lecithin) in the solubilization solutions resulted in the recovery of 45–50% of the membrane-associated radioactivity in the soluble protein fraction. Fifty to fifty-five percent of this radioactivity was recovered from the spun column, giving a 2-fold increase (to

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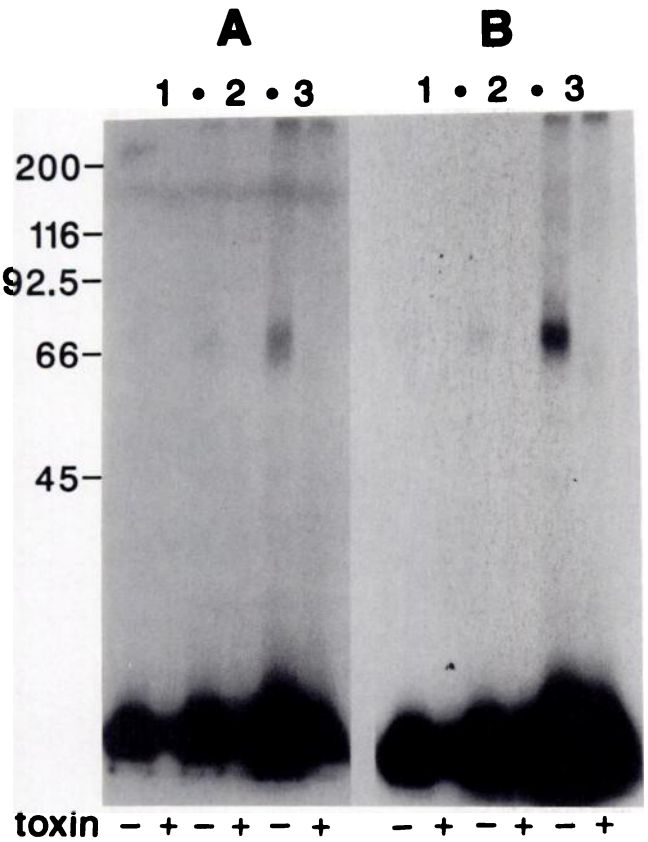

Fig. 6. Cross-linking of ^{125}I-α-DaTX and ^{125}I-β-DaTX to synaptic membrane proteins. ^{125}I-α-DaTX (A) and ^{125}I-β-DaTX (B) were covalently attached (by cross-linking in the presence of 0.1 mg/ml dimethylsuberimidate) to membrane polypeptides in the absence (−) and presence (+) of a 500-fold excess of the respective unlabeled DaTX. In A, paired *lanes 1, 2,* and *3* contained membranes incubated with 5.6, 11.1 and 16.7 nM ^{125}I-α-DaTX, respectively. In B, paired *lanes 1, 2,* and *3* contained membranes incubated with 4.9, 9.7, and 14.6 nM ^{125}I-β-DaTX, respectively. Migration distances of standard proteins are shown along the *ordinate.*

22–25%) in the amount of soluble functional receptor recovered. There was no requirement for any particular phospholipid; the addition of 0.25% purified phosphatidylserine or phosphatidylethanolamine also resulted in the recovery of 23–25% functional receptors. However, in the presence of phosphatidylserine and phosphatidylethanolamine, more (65%) of the membrane-associated radioactivity was solubilized but less (35%) remained attached to the soluble protein. Solutions containing 0.8% or 1.0% zwittergent 3–12 (see Table 2 and figure legends) and 0.25% lecithin were chosen for subsequent studies of the solubilized DaTX receptors.

Proteins solubilized under these conditions were assayed directly for toxin-binding activity. The aforementioned experiments on the solubilized receptor were carried out in solutions containing 150 mM Na because Na promoted maximal binding of ^{125}I-α-DaTX to native synaptic membranes (Table 1). However, when the soluble proteins were directly assayed for ^{125}I-α-DaTX binding in the presence of Na, the binding was variable. It became apparent that the ionic composition of the solubilization solution affected the retention of toxin binding activity by the soluble receptor.

The effects of several monovalent cations on receptor solubilization are shown in Table 2. Maximal and reproducible ^{125}I-α-DaTX binding was obtained in solutions containing 150 mM

TABLE 2

Effects of cations on α-DaTx receptor solubilization

Synaptic membrane proteins were solubilized (see Materials and Methods) in solutions containing 10 mM HEPES, pH 7.0, 1.4 mM $MgCl_2$, 0.25% lecithin, 0.8% zwittergent 3-12, and 150 mM concentrations of an alkali metal ion (as the chloride salt; see table). The soluble protein was assayed for ^{125}I-α-DaTX binding by the spun column method. Values are presented as the amount of neurotoxin bound in the presence of the test cation, relative to the amount bound in the presence of 150 mM KCl (taken as 100%). The data are the averages from two experiments, each performed in triplicate.

Cation	Bound toxin
	%
K	100
Rb	54.8 ± 0.1
Cs	44.0 ± 1.6
Li	37.3 ± 8.8
Na	24.6 ± 12.1

K. Other alkali metal ions could substitute for K, but the retention of toxin binding activity was reduced. The relative effectiveness of these cations was K > Rb > Cs > Li > Na. This sequence is different from the order of potency of these monovalent cations for supporting ^{125}I-α-DaTX binding to the native membrane receptor (Table 1), Na > K > Li > Rb > Cs.

^{125}I-α-DaTX and ^{125}I-β-DaTX Binding to the Solubilized Receptor

Scatchard analysis (Fig. 7A) indicated that the affinity of ^{125}I-α-DaTX for its receptor decreased 10-fold (K_D = 7.0 ± 1.3 nM; six experiments) after receptor solubilization; 30% of the total membrane receptors (B_{max} = 0.56 ± 0.11 pmol/mg of protein) retained their ability to bind toxin after solubilization. This percentage is similar to that determined from the solubilization of the toxin/receptor complex described above (22–25%). For ^{125}I-β-DaTX binding, Scatchard analysis (Fig. 7B) indicated a 3.5-fold decrease in apparent affinity (K_D = 124 ± 5 nM; two experiments) and a recovery of 23% of the binding sites (B_{max} = 2.0 ± 0.1 pmol/mg of protein) after receptor solubilization.

As was the case with the native membrane receptor (Fig. 3), the four unlabeled DaTXs also inhibited ^{125}I-α-DaTX binding to the soluble receptor. The same relative order of potency for inhibition of ^{125}I-α-DaTX binding was observed: α-DaTX > β-DaTX > γ-DaTX (Fig. 8). In this case, too, the displacement curve produced by δ-DaTX did not parallel the other displacement curves.

Sucrose Gradient Centrifugation of the Solubilized α-DaTX Receptor

^{125}I-α-DaTX was preequilibrated with synaptic membranes, and the toxin/receptor complex was solubilized as described above. Aliquots of the solubilized ^{125}I-α-DaTX were eluted through a Sephadex G-50 spun column to remove unbound and dissociated toxin. The eluate, containing only receptor-associated ^{125}I-α-DaTX, was applied to 5–20% continuous sucrose density gradients containing 0.1% zwittergent and 0.025% lecithin. Following centrifugation (200,000 × *g* for 5 hr), sucrose gradient fractions were collected to determine the location of the ^{125}I-α-DaTX in the gradient. As shown in Fig. 9, the location of the tracer indicated that the toxin/receptor complex migrated slightly faster on the sucrose gradient than did catalase (M_r = 250,000). From these data, the molecular weight for the α-DaTX receptor was estimated to be about 270,000. Similar

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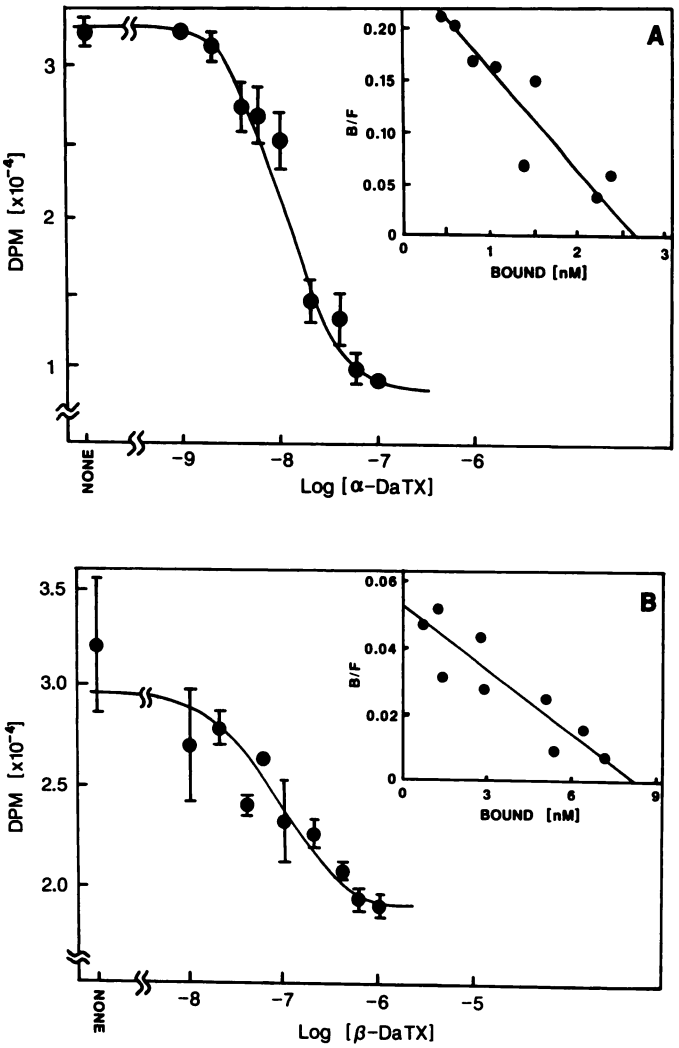

Fig. 7. DaTX binding to soluble synaptic membrane receptors. The *main graphs* illustrate the competitive displacement of bound ^{125}I-α-DaTX (A) and ^{125}I-β-DaTX (B) from the solubilized membrane receptors by increasing amounts of the respective unlabeled toxin. The corresponding Scatchard plots are presented in the *insets*. Each *point* represents the mean of triplicate determinations (*error bars* indicate ± standard errors).

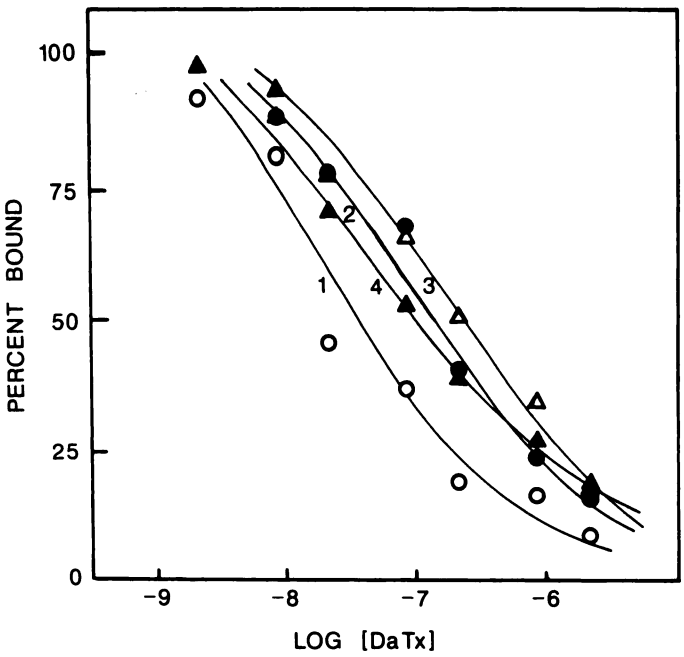

Fig. 8. Inhibition of iodinated α-DaTX binding to solubilized synaptic membrane receptors by unlabeled DaTXs. The *graphs* show the inhibition of ^{125}I-α-DaTX binding by increasing concentrations of the four unlabeled dendrotoxins. *1*, α-DaTX; *2*, β-DaTX; *3*, γ-DaTX; *4*, δ-DaTX. *Symbols* represent the means of triplicate determinations.

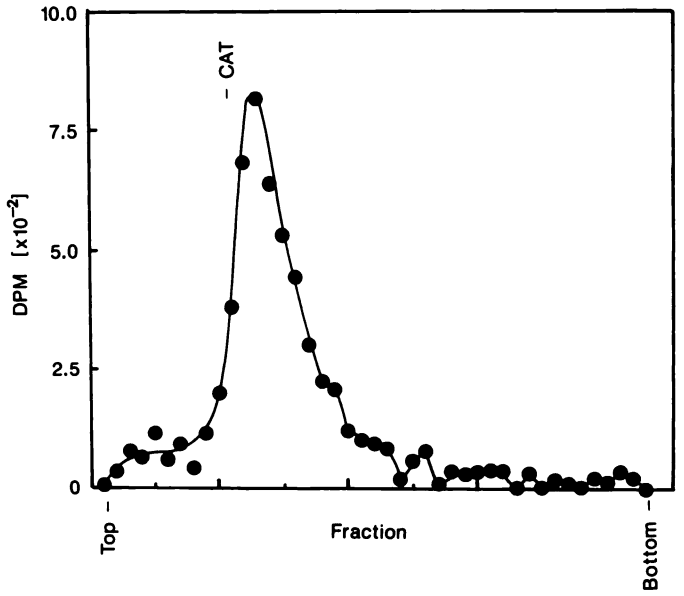

Fig. 9. Sucrose gradient centrifugation of the soluble ^{125}I-α-DaTX/receptor complex. ^{125}I-α-DaTX was equilibrated with synaptic membranes, solubilized with 1.0% zwittergent in Solution A containing 0.25% lecithin, and passed through a spun column to remove unbound and dissociated toxin. An aliquot of the solubilized α-DaTX/receptor complex was applied to a 5–20% (w/v) continuous sucrose gradient and centrifuged at 200,000 × *g* for 5 hr. The amount of ^{125}I-α-DaTX recovered in 0.3-ml fractions collected from the gradient are shown. *CAT* indicates the position to which catalase (M_r = 250,000) migrated.

results were obtained after centrifugation of the sucrose gradients for 10 or 15 hr (not shown).

Discussion

α-DaTX blocks a variety of inactivating, voltage-gated K channels in excitable cells with high affinity (IC_{50} < 100 nM) (3, 9). This has fostered the idea that α-DaTX may be a good ligand to use for the identification, purification, and characterization of some K channel proteins. With this goal in mind, we have characterized the rat brain α-DaTX receptor and have solubilized this receptor protein. For comparison, we also studied the binding of a second dendrotoxin, β-DaTX, that preferentially blocks a different (noninactivating) voltage-gated K channel (3). These results provide new information about the possible structures of these dendrotoxin receptor/putative K channels in rat brain.

α-DaTX and β-DaTX binding sites. Despite their block of different K channels, α-DaTX and β-DaTX have some similarities. The two dendrotoxins have at least 35% homology, with the highest concentrations of conserved residues at their carboxy terminals (3). Moreover, although at low concentrations they preferentially block different K channels, they are less selective at higher (but submicromolar) concentrations (3). They also exhibit similarities as well as differences in their receptor binding properties.

^{125}I-α-DaTX and ^{125}I-β-DaTX both bound specifically, and

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with high affinity (K_D = 0.69 and 35.9 nM, respectively), to rat brain synaptic membranes. These K_D values are of the same order of magnitude as their respective IC_{50} values for block of voltage-gated K channels (3, 9). The fact that the two toxins preferentially block different voltage-gated K channels (3) suggests that they have different binding sites. Indeed, Scatchard analysis indicated that α-DaTX bound to only 1.8 pmol of sites/mg of protein, whereas β-DaTX bound to 8.6 pmol of sites. However, the evidence that the two toxins can block both classes of voltage-gated K channels (3) and can completely displace one another from their specific binding sites implies that we should have observed a similar number of binding sites for the two toxins. The apparent discrepancy may be an artifact that could be explained by an error in estimating the specific activity and/or the radiochemical purity of either or both toxins.

Displacement of ^{125}I-α-DaTX binding by unlabeled β-DaTX revealed the competitive type inhibition expected if the two DaTXs compete for the same binding sites (Fig. 4A). In contrast, displacement of ^{125}I-β-DaTX by unlabeled α-DaTX was more complex (Fig. 4B). This raises the possibility that β-DaTX binds, with similar apparent affinities, to two different sites, one of which may be identical to the α-DaTX binding site.

Other characteristics of ^{125}I-α-DaTX and ^{125}I-β-DaTX binding to synaptic membranes were found to be very similar. They exhibited comparable pH dependence and cation dependence, and the binding of the two iodinated peptides was inhibited in similar fashion by a series of four homologous unlabeled DaTXs. Furthermore, the two iodinated toxins covalently labeled polypeptide receptors of approximately the same molecular weight (M_r = 65,000). These data could be explained if α-DaTX and β-DaTX preferentially bind, respectively, to distinct sites on different but structurally homologous K channels.

The binding of α-DaTX and β-DaTX to the native synaptic membranes was inhibited by K and Rb, which permeate through K channels. This raises the possibility that the inhibition may be due, in part, to displacement of the toxin by the ions penetrating from the inside of the channel (16). However, the potent block by other impermeant ions such as Li, Cs, Ca, and Ba requires a different explanation. The fact that Cs and Ba are noncompetitive inhibitors of α-DaTX binding (Fig. 5) suggests that these effects involve an allosteric interaction. Perhaps these ions can bind in the channel mouth and thereby interfere sterically with the binding of the DaTXs.

Solubilized α-DaTX and β-DaTX receptors. Most properties of the solubilized α-DaTX and β-DaTX receptors were similar to those observed in the native membranes, although the affinity for α-DaTX was decreased 10-fold and that for β-DaTX was decreased 3.5 fold. ^{125}I-α- and ^{125}I-β-DaTX were displaced from the native membrane and from the solubilized receptors by unlabeled α-, β, and γ-DaTX with similar sequences of inhibition, but the inhibition by δ-DaTX was more complex (compare Figs. 3 and 8).

The properties of the solubilized α-DaTX receptor were studied further. Whereas K inhibited α-DaTX binding to the native membranes (Table 1), this ion was required for maximum stabilization of the solubilized receptor (Table 2), as observed by others (17, 18). Rb, which is also permeant through K channels, was about half as effective as K in maintaining the stability of the solubilized receptor, whereas relatively impermeant monovalent cations (Cs, Li, and Na) were less effective (Table 2). This raises the possibility that K (and Rb) ions exert their primary effect on the solubilized toxin receptor (putative K channel) by binding in the channel selectivity filter (19) and helping to maintain the protein in its native conformation (i.e., as a channel). The toxin binding may depend upon the retention of the polymeric structure (i.e., an intact channel), which, in the solubilized form, is promoted by phospholipids and permeant cations. The permeant cation may not be necessary if surrounding structures in the native membranes help to retain the normal configuration of the channel protein. It seems possible that steric interference and/or electrostatic repulsive effects of the cations that enter the mouth of the channel may then prevail; this could contribute to both the altered cation dependence and the reduced affinity of the toxin for the solubilized receptor.

Inferences regarding potassium channel structure. Much evidence indicates that voltage-gated channels are members of a family of homologous proteins (20–23). The Na and Ca channels appear to be formed by four similar peptide units or domains strung together as single large polypeptide chains. Each of the units contains multiple membrane-spanning peptide sequences and is analogous to one of the independent subunits of ligand-gated channels such as the acetylcholine receptor (23).

Less structural information is available about K channels. However, the structure of a polypeptide that apparently represents a component of an A-type K channel has been deduced from complementary DNA cloned from the *Shaker* locus of *Drosophila* (24). This polypeptide, which has structural homology to the Na and Ca channel proteins, has a molecular weight of about 72,200 (24). Families of such peptides (M_r = 65,000–75,000) have recently been identified by molecular genetic methods in vertebrates as well as in invertebrates (24–27). A K channel may be formed by the parallel stacking of four of these subunits around the channel pore (23), as either a homo- or heterotetramer (28). Thus, the overall structural arrangement of K channels may be similar to that of Na and Ca channels, except that the four major domains that constitute the Na and Ca channels are linked to form a single large polypeptide.

Our observations are consistent with this model of the A-type K channel. The M_r 65,000 polypeptide that we identified from cross-linking studies with ^{125}I-α-DaTX (Fig. 6A) is comparable in size to the receptor subunit in rat brain synaptic membranes identified by Mehraban *et al.* (29), who used similar methodology. It is comparable to the polypeptide (M_r = 76,000–80,000) labeled by cross-linking rat brain membranes with the homologous toxin $DaTX_I$ (17, 30). The recent purifications of the $DaTX_I$ receptor from rat brain (17) and the α-DaTX receptor from bovine brain (31) also indicate that a polypeptide of M_r = 75,000–80,000 is a component of the receptor. However, additional low molecular weight polypeptides (M_r = 35,000–38,000) copurify with the larger polypeptides (17, 31). The significance of these smaller polypeptides is not known.

The molecular weight of the rat brain α-DaTX receptor that we estimated from sucrose density gradient measurements is 270,000 (Fig. 8). This is virtually identical to that obtained by Rehm *et al.* (30), who used similar methods. A similar size was estimated from radiation inactivation measurements of the α-DaTX receptor (1, 32). However, a much larger size (M_r = 405,000–465,000) has been estimated from studies of the hydro-

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dynamic properties of the α-DaTX receptor solubilized with Triton X-100 (18).

Our data on the subunit and solubilized receptor size complement the information obtained from molecular genetics studies. They fit with the idea (23) that the α-DaTX receptor/A-type K channel in rat brain synaptic membranes may have a molecular weight of about 250,000–270,000 and may consist of four similar or identical subunits with molecular weights of about 65,000.

A Ca-independent, noninactivating, voltage-gated ("delayed rectifier-type") K channel that is sensitive to α-DaTX has been expressed from a rat brain cDNA (33) that was obtained by hybridization with the *Drosophila Shaker* cDNA using a low stringency protocol. The protein encoded by this cDNA shows substantial homology to the *Shaker* proteins; the major differences are observed at the (cytoplasmic) C-terminal end (26), which is postulated to be the region responsible for channel inactivation (28, 34).

In rat brain synaptosomes, β-DaTX preferentially blocks delayed rectifier-type K channels, although at slightly higher concentrations this toxin also blocks the inactivating voltage-gated (A-type) K channels (3). In the present study, we found that ^{125}I-β-DaTX, like ^{125}I-α-DaTX, cross-links to a M_r 65,000 peptide, but the size of the solubilized β-DaTX receptor has not yet been determined. In light of the increasing evidence that polypeptide K channel toxins exhibit cross-reactivity with different classes of K channels (30, 35–38) and the evidence for homologies in channel structure (23), our limited data on the β-DaTX receptor are consistent with other evidence (e.g., Ref. 33) that the noninactivating (delayed rectifier) K channels may have subunit structures very similar to those of the inactivating (A-type) K channels.

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