Solubilization and Physical Characterization of Acceptors for Dendrotoxin and β-Bungarotoxin from Synaptic Membranes of Rat Brain†

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ABSTRACT: Dendrotoxin (DTX), an M_r 7000 convulsant polypeptide from the venom of *Dendroaspis angusticeps*, or its facilitatory homologues act through blockade of certain voltage-sensitive K^+ currents in a variety of neurons. High-affinity acceptors for DTX have been demonstrated in synaptic plasma membranes of rat or chick brain, and a fraction of these avidly bind β-bungarotoxin (β-BuTX), a presynaptically active protein whose lighter B polypeptide is homologous to this toxin. Extraction of rat synaptic plasma membranes using Triton X-100 in K^+-containing buffer yielded binding sites with K_D values of ~0.5 and 0.7 nM for ^{125}I-labeled DTX and β-BuTX, respectively. The content of high-affinity sites obtained for β-BuTX, including the contribution of a lower affinity component, approximates to the B_{max} (~1.3 pmol/mg of protein) obtained for the apparent single set of DTX acceptors. On solubilization, the pharmacological specificity of the acceptor for neurotoxic DTX congeners was retained. ^{125}I-β-BuTX binding (2.1 nM) was blocked efficaciously by DTX (IC_{50} = 1.6 nM) while the binding of 2.1 nM ^{125}I-DTX was inhibited completely by β-BuTX (IC_{50} = 25 nM); the lower potency of the latter could relate to the noncompetitive nature of the mutual competition and to the presence of high- and low-affinity sites for β-BuTX. On gel filtration, or sedimentation analysis in H_2O/sucrose and ^2H_2O/sucrose gradients, one peak of DTX binding activity was observed, and this was inhibitable by β-BuTX. From the hydrodynamic properties of the acceptor/detergent/lipid complex ($s_{20,w}$ = 13.2 S; Stokes radius = 8.6 nm), a molecular weight of 405 000–465 000 was estimated. The agreement of these values with those reported for β-BuTX binding activity of chick brain, together with the acceptor binding parameters observed for both toxins, and published cross-linking experiments, suggests that they share common acceptors which are likely constituents of some voltage-dependent K^+ channels.

Dendrotoxin (DTX),[1] an M_r 7000 protease inhibitor homologue from the venom of *Dendroaspis angusticeps* (Harvey & Karlsson, 1980, 1982; Joubert & Taljaard, 1980), is one of a group of homologous neurotoxins which include toxins, I, K, B, and E from *Dendroaspis polylepis* and Dv.14 from *Dendroaspis viridis* (Black et al., 1986). DTX has been shown to facilitate neurotransmitter release at a variety of central (Docherty et al., 1983; Dolly et al., 1984, 1986) and peripheral (Harvey & Karlsson, 1980, 1982; Harvey et al., 1984) synapses. The initial electrophysiological studies performed on rodent hippocampal slices indicated that this action results from its inhibition of the A current, a transient voltage-dependent K^+ current which is intimately involved in the control of cell excitability (Dolly et al., 1984; Halliwell et al., 1986). More recently, DTX or toxin I has been shown to inhibit very effectively a slow-inactivating K^+-current variant in a variety of neurons (Stansfeld et al., 1986, 1987; Penner et al., 1986; Benoit & Dubois, 1986). In keeping with its potent toxicity in rat brain [minimum lethal dose = 2.5 ng/g body weight (Dolly et al., 1984)], saturable binding of DTX to proteinaceous acceptors has been demonstrated in both rat and chick brain (Black et al., 1986; Black & Dolly, 1986). Evidence that these acceptors appeared to be in some way associated with the above-mentioned K^+ channels is provided (Dolly et al., 1987) by (a) a correlation of the toxicities of DTX homologues, and effectiveness in blocking K^+ conductances, with the acceptor affinities, (b) their neuronal location at sites equivalent to those where the toxin has been shown to block K^+ conductances, and (c) an apparent direct inhibitory action of DTX on a fast-activating, slowly inactivating, aminopyridine-sensitive K^+ conductance in isolated patches of neuronal membrane [cf. Dolly (1988)].

On the basis of biochemical and autoradiographic studies in rat and chick brain (Black et al., 1986; Black & Dolly, 1986; Dolly et al., 1986), it appears that DTX acceptors can be divided into at least two subtypes according to their differential interaction with the inhibitory neurotoxin β-bungarotoxin (β-BuTX). A species that binds both toxins occurs predominantly in rat brain areas rich in nerve terminals (Othman et al., 1983; Halliwell et al., 1986; Pelchen-Matthews & Dolly, 1988) whereas other DTX sites are also found on axonal and, to a lesser extent, on somatic membranes (Dolly et al., 1986; Pelchen-Matthews & Dolly, 1988). In fact, the common binding protein was first detected in rat brain (Othman et al., 1982) where the saturable binding of ^{3}H-labeled β-BuTX (K_D = 0.6 nM) to synaptosomes was shown to be blocked efficaciously by the DTX homologue toxin I. In addition to an M_r 7000 chain that is homologous to DTX congeners, β-BuTX from *Bungarus multicinctus* also contains a disulfide-linked polypeptide (M_r 13 500) which is responsible for its Ca^{2+}-dependent phospholipase A_2 activity (Kondo et al., 1978). Although the latter complicates the pharmacological activity of β-BuTX, saturable interaction with the acceptor identified

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[1] Abbreviations: β-BuTX, β-bungarotoxin; DTX, dendrotoxin; ^{125}I-DTX and ^{125}I-β-BuTX, radioiodinated DTX and β-BuTX, respectively; M_r, relative molecular weight; $\bar{v}$, partial specific volume; STI, soybean trypsin inhibitor; PMSF, phenylmethanesulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

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in brain synaptic membranes is involved in directing β-BuTX to a functional target (Rugulo et al., 1986); indeed, there is some recent information to suggest β-BuTX can attenuate certain K^+ conductances in peripheral neurons (Harvey & Anderson, 1986; Petersen et al., 1986).

In view of these acceptors being ascribed as putative constituents of K^+ channels, further information on their subtypes was sought by a comparative examination of the binding of both DTX and β-BuTX in detergent extracts of rat brain membranes. Also, physical properties of the binding component(s) were investigated and discussed in relation to the predicted structure of proteins derived from analysis of a related K^+ channel in *Drosophila* (Tempel et al., 1987).

Experimental Procedures

Materials

D. angusticeps venom was obtained from Sigma Chemical Co., Poole, Dorset, U.K. DTX was purified and radioiodinated (300–400 Ci/mmol) as described previously (Black et al., 1986). β-BuTX and toxin I were purified to homogeneity in this laboratory by A. Breeze and K. Awan. β-BuTX was radioiodinated to a specific activity of 200–600 Ci/mmol; its characterization is detailed elsewhere (Breeze & Dolly, 1988). Other proteins were obtained from Sigma as were ^2H_2O (99%), imidazole (grade I), and protease inhibitors. Silanized Eppendorf tubes and pipet tips (Black et al., 1986) were employed for all assays involving toxins, in order to minimize adsorption. All other reagents were of analytical grade.

Methods

Assay of Toxin Binding in Solubilized Extracts. Rat cerebrocortical synaptosomes were prepared by the method of Dodd et al. (1981); following hypotonic lysis, synaptic plasma membranes were separated by centrifugation onto a cushion of 1.2 M sucrose. Except where otherwise stated, soluble extract was prepared from these membranes (~10 mg of protein/mL) by shaking for 1 h at 4 °C with 4% (w/v) Triton X-100 in 50 mM imidazole hydrochloride, pH 7.4, containing 0.3 mM benzamidine, 0.02% (w/v) soybean trypsin inhibitor (STI), 0.01% (w/v) bacitracin, 1 mM phenylmethanesulfonyl fluoride (PMSF), 500 mM NaCl, and 250 mM KCl. Insoluble material was sedimented at 34000*g* for 20 min, followed by 100000*g* for 45 min. For assay of toxin binding activity, the extract was diluted 25-fold to a volume of 250 μL containing 25 mM imidazole hydrochloride, pH 7.4, 110 mM KCl, 20 mM NaCl, and 0.2% Triton X-100 (final concentrations); for measurement of β-BuTX binding, 1.5 mM $SrCl_2$ and 0.2% (w/v) phosphatidylcholine were included. ^{125}I-DTX, ^{125}I-β-BuTX, or competing ligands were included at the stated levels; nonspecific binding was determined by inclusion of a 100-fold molar excess of unlabeled DTX or β-BuTX. After 20 min at 23 °C, 200 μL of the incubation mixture was subjected to a modification of the rapid gel filtration assay outlined by Rehm and Betz (1984); the eluate was collected, and the level of bound radioactivity was determined by γ counting at 77% efficiency. For measurement of ^{125}I-DTX and ^{125}I-β-BuTX binding to membranes, the aforementioned assay buffer was used, but Triton and phosphatidylcholine were omitted; bound toxin and free toxin were separated by centrifugation through an oil mixture (Black et al., 1986) and quantified as above.

For Scatchard analysis (Scatchard, 1949), the concentration of free ^{125}I-labeled toxins was taken as the difference between the total amount loaded and that present in the eluate. To measure the dissociation rate of the ^{125}I-DTX/acceptor complex, the extract was labeled as above with 1.2 nM ^{125}I-DTX, and 120 nM DTX was added at the stated times before the incubation mixture was applied to the columns. Nondisplaceable binding was determined by inclusion of 120 nM DTX in the original incubation mixture, with addition of buffer at the appropriate times. Nonlinear Scatchard plots were graphically analyzed by the method of Rosenthal (1967). For protease treatments, extracts were prepared as described above, except that the NaCl and protease inhibitors were omitted; the extract was incubated with 1 mg/mL trypsin or chymotrypsin at 37 °C for 40 min. Prior to assaying ^{125}I-DTX binding activity, STI (to 5 mg/mL for trypsin) or PMSF (to 5 mM for chymotrypsin) was added. Controls were prepared by addition of the appropriate inhibitors prior to the proteases. For heat and acid treatments, rat synaptic membranes were incubated at 95 °C (30 min) or with 1 M HCl at 37 °C (40 min): control samples were incubated at 37 °C for 30 or 40 min, respectively, in 50 mM imidazole hydrochloride, pH 7.4. This was followed by washing with 2 × 1 mL of the latter buffer; the membranes were then solubilized, as outlined previously, and assayed using 1.2 nM ^{125}I-DTX. Protein was measured by the methods of Bradford (1976) or Lowry et al. (1951), using bovine serum albumin as a standard.

Hydrodynamic Studies. Linear sucrose gradients in H_2O and ^2H_2O were poured by using a Buchler Auto-Densi-Flow II. Gradients (11.3 mL) of a 5–20% sucrose solution containing 50 mM imidazole hydrochloride, pH 7.4, 100 mM KCl, 0.5% Triton, 0.2% phosphatidylcholine, 0.02% STI, 0.01% bacitracin, 0.3 mM benzamidine, and 1 mM PMSF were poured on top of a 0.2-mL cushion of 60% sucrose in 12-mL Beckman centrifuge tubes. The gradients were then cooled on ice. In the preparation of solubilized acceptor sample for the H_2O gradients, NaCl had to be omitted from the extraction buffer in order to make the density of the sample lower than that of the top of the gradient. Samples (500 μL) containing standard protein markers were layered on top of both gradients and centrifuged at 190000*g* (39000 rpm) for 17 h in a Beckman SW40 Ti rotor. Gradients were fractionated using the Densi-Flow II, and an identical number of fractions (~0.3 mL) were collected for all gradients in any one experiment.

Gel filtration of membrane extract containing standard protein markers was carried out on a Sepharose-6B column equilibrated with 50 mM imidazole hydrochloride, pH 7.4, containing 100 mM KCl, 0.5% Triton X-100, and 0.2% phosphatidylcholine. ^{125}I-DTX binding in each of the fractions from gel filtration and sucrose density centrifugation was assayed as detailed above by adjusting the volume to 250 μL with the sucrose or column buffer containing ^{125}I-DTX (to a final concentration of 1.5 nM); a 200-fold excess of unlabeled DTX was included in the controls. β-Galactosidase and alcohol dehydrogenase were assayed by the methods of Lederberg (1950) and Vallee and Hoch (1955); cytochrome *c* levels were determined by its absorbance at 410 nm. For calculation of the density of the fractions from the gradients, the densities of the stock solutions were taken as being that of the first and penultimate fractions; other density values were interpolated from these.

Density gradients were analyzed by the method of Meunier et al. (1972) which utilizes the formula:

$$r = ks_{20,w}(1 - \bar{v}\rho_{1/2})$$

where r is the distance travelled by a particle down a density gradient, $\bar{v}$ and $s_{20,w}$ are the partial specific volume and sedimentation coefficient (in water at 20 °C) of the particle, respectively, k is a constant for a solvent of a given density, and $\rho_{1/2}$ is the density of the gradient at $r/2$. A plot of r against $1 - \bar{v}\rho_{1/2}$ for standard proteins will give a straight line,

Table I: Cation Dependence of the Solubilization by Triton X-100 of Binding Activities for DTX and β-BuTX

		fmol bound/mg of protein[a]		% of act. measured in membranes[a]	
cation	concn (mM)	DTX	β-BuTX	DTX	β-BuTX
K^+	250	373	124	51	50
	100	186	12	26	5
	50	0	0	0	0
Rb^+	250	240	20	34	9
Cs^+	250	20	0	3	0
Na^+	250[b]	0	0	0	0

[a] Assays of the solubilized and membrane-bound preparations were performed as detailed under Methods using 2 nM ^{125}I-DTX or ^{125}I-β-BuTX. In all experiments, ~80% of total membrane protein was extracted using 4% Triton X-100. When 250 mM KCl was used and detergent omitted, extraction was only 15% and gave a recovery of 8% for the binding activity of each toxin. Other conditions for the extraction of rat synaptic plasma membranes are given under Methods; in all cases, the total salt concentration was adjusted to 750 mM with NaCl. [b] K^+ included during the assay.

of slope k. By determination of k for two solvents in this manner, $s_{20,w}$ and $\bar{v}$ can be determined for any unknown. Gel filtrates were analyzed by the method of Porath (1963) which uses the formula:

$$K_d^{1/3} = \frac{(V_e - V_0)^{1/3}}{V_i - V_0} = \alpha - \beta R_s$$

where V_0 and V_i are the void and inclusion volumes of the column, respectively, and V_e is the elution volume of a particle of Stokes radius R_s. The symbols α and β are undefined constants which relate the partition coefficient ($K_d^{1/3}$) to the Stokes radius. A plot of $K_d^{1/3}$ against the Stokes radius for standard proteins gives a straight line from which the Stokes radius of an unknown can be extrapolated. The molecular weight (M_r) of the acceptor/detergent/lipid complex was calculated by using the derivation of the Svedberg equation:

$$M_r = \frac{6\pi N \eta_{20,w} R_s s_{20,w}}{1 - \bar{v}\rho_{20,w}}$$

where N is Avogadro's number and $\eta_{20,w}$ and $\rho_{20,w}$ are the viscosity and density of water at 20 °C, respectively. Finally, the weight fraction (X_a) of acceptor in the complex was calculated by

$$X_a = \frac{\bar{v}_c - \bar{v}_{d/l}}{\bar{v}_a - \bar{v}_{d/l}}$$

where $\bar{v}_c$, $\bar{v}_a$, and $\bar{v}_{d/l}$ are the partial specific volumes of the complex, acceptor, and bound detergent/lipid, respectively.

Results

Solubilization and Characterization of Acceptor Proteins for DTX and β-BuTX. Optimized conditions for extraction of rat synaptic membrane with Triton X-100 yielded 50% of the binding activity for ^{125}I-DTX and ^{125}I-β-BuTX, when measured using 2 nM toxin in each case, provided K^+ (maximal at 250 mM) was present during the solubilization (Table I). For DTX binding, K^+ could be replaced in the buffer by Rb^+ but not by Na^+ or Cs^+; β-BuTX binding, in contrast, showed greater dependency on K^+, with ~90% of the activity being lost in Rb^+. The cation dependence was due to a stabilizing effect on the acceptor, as extraction in K^+-free, Na^+-containing medium followed by addition of K^+ resulted in negligible specific binding of either toxin in the extract. Additionally, in the case of β-BuTX binding the presence of 100 mM K^+ was required during the incubation whereas interaction with DTX was unaffected by the total salt, or K^+ concentration over the range 10–100 mM. In contrast to solubilization with detergent where ~80% of the membrane protein was recovered, extraction with high salt concentrations (500 mM NaCl/250 mM KCl) proved ineffective, yielding 10–20% of the total protein and 6–10% of the toxins' binding activities. However, inclusion of 500 mM NaCl in buffer containing 4% Triton gave the highest yield; also, protease inhibitors were added to minimize proteolysis. Initially, a single centrifugation step was used to pellet insoluble material in the extract; inclusion of an additional low-speed spin increased the yield of both membrane protein and binding activities for β-BuTX (Rehm & Betz, 1984) and DTX.

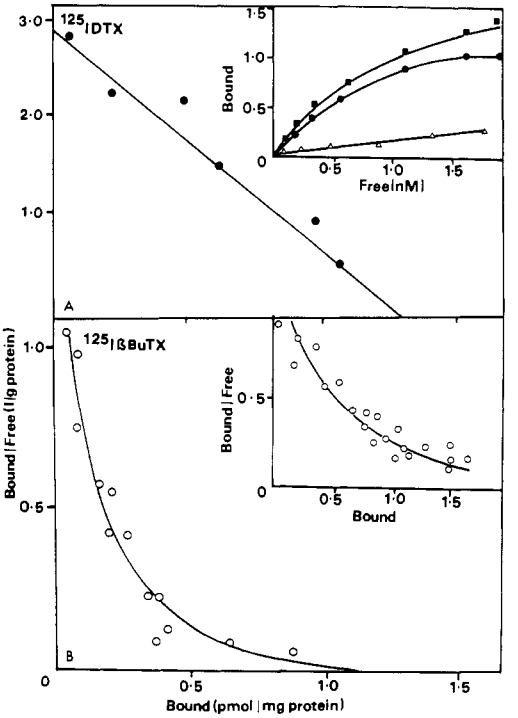

FIGURE 1: Equilibrium binding of ^{125}I-labeled DTX and β-BuTX to solubilized or membrane-bound acceptor preparations. (A) Binding of various concentrations of ^{125}I-DTX to a Triton X-100 extract of rat synaptic plasma membranes was measured in imidazole buffer as described under Methods. Nonspecific binding (△), determined in the presence of 200 nM DTX, was subtracted from the total (■) to give the amounts of specifically bound (●) toxin (inset). A Scatchard plot is shown of the specific binding obtained in a typical experiment; the average values for duplicate determination at each DTX concentration are presented. The average values calculated from four experiments for K_D and B_{max} were 0.5 ± 0.1 nM and 1.0 ± 0.3 pmol/mg of protein. (B) A representative Scatchard plot of the specific binding of ^{125}I-β-BuTX to the solubilized extract, measured as in (A). For comparison, the inset shows a similar plot of ^{125}I-β-BuTX binding to the membrane preparation used for acceptor solubilization; the specific binding to membrane-bound acceptor was quantified by an oil centrifugation assay (see Methods). When the curvilinear Scatchard plots were analyzed graphically, in each case two sets of sites could be derived; approximate values (±SEM) thus obtained for the solubilized preparation, in three experiments performed in duplicate, were K_d = 0.7 ± 0.2 and 16.0 ± 3.2 nM and B_{max} = 0.3 ± 0.03 and 1.0 ± 0.02 pmol/mg of protein. For membranes, the corresponding values were 0.6 ± 0.2 and 10.5 ± 8.6 nM and 0.6 ± 0.1 and 1.8 ± 0.2 pmol/mg of protein.

Saturable, high-affinity binding of ^{125}I-DTX was observed in the solubilized extract (Figure 1A inset) with a reasonably low nonspecific value (15–20% at 1.2 nM). Scatchard plots revealed an apparently homogeneous set of acceptor sites with a K_D of ~0.5 nM and a B_{max} of ~1.3 pmol/mg of protein (Figure 1A). A comparison with the values obtained in a parallel study on synaptic membranes (K_D ~ 0.3 nM; B_{max} ~ 2.5 pmol/mg of protein) revealed that, although solubili-

zation did not alter significantly the affinity for DTX, the difference in specific activities is indicative of some inactivation having occurred; incomplete extraction of acceptor was excluded by the absence of detectable activity in the residual membrane. A linear first-order dissociation plot was obtained for the acceptor/^{125}I-DTX complex that gave a k_{-1} of 2×10^{-3} s^{-1} and $t_{1/2} \sim 6$ min at 23 °C, as reported for the membrane-bound acceptor (Black et al., 1986). It proved difficult to measure the association rate, but a k_1 of 4×10^6 M^{-1} could be calculated from the values of k_{-1} and K_D, showing that association was rapid.

Figure 1B shows the nonlinear Scatchard plots obtained for the specific binding of ^{125}I-β-BuTX in the soluble and membrane-bound states. From graphical analysis (Rosenthal, 1967) of the data, it is possible to dissect two sets of sites in both cases; similar K_D values were extrapolated for the high- (~0.7 nM) and low-affinity (~16 nM) sites in each preparation (see legend to Figure 1). Moreover, the content of the high- and low-affinity sites (B_{max} = 0.3 and 1.0 pmol/mg of protein, respectively) represented ~50% of the corresponding acceptors detected in the membranes (respective B_{max} values of 0.6 and 1.8 pmol/mg of protein) (Figure 1 and Table I), though error in the graphical analysis does not allow accurate quantitation of the site contents (particularly in the membranes).

Pretreatment of the soluble extract with trypsin or chymotrypsin virtually abolished the binding of ^{125}I-DTX (to less than 5% of the original), demonstrating the proteinaceous nature of the acceptor as has been reported for β-BuTX binding activity (Rehm & Betz, 1984). Freezing the extract totally inactivated the binding of both toxins; as appreciable amounts of the acceptor activity were lost on storage at 4 °C for 14 h, experiments were conducted (where possible) within 3 h of preparation of the extract.

Specificity of the solubilized DTX acceptor for protease inhibitor homologues was the same as that in the membrane-bound state (Black et al., 1986). DTX and toxin I inhibited totally the specific binding of ^{125}I-DTX with average K_i values of 0.5 and 0.2 nM, respectively (Figure 2A); unlike the neurotoxic homologues, toxin B and bovine pancreatic trypsin inhibitor were ineffective (data not shown). β-BuTX also produced complete inhibition of the binding of 2.1 nM ^{125}I-DTX but with relatively low potency ($IC_{50} \sim 25$ nM) (Figure 2A). Notably, ^{125}I-β-BuTX (2.1 nM) binding to the solubilized extract was blocked efficiently by DTX ($IC_{50} \sim 1.6$ nM) (Figure 2B); β-BuTX gave an $IC_{50} \sim 0.5$ nM. As the mutual inhibition of the binding of these toxins involves a noncompetitive mechanism (Breeze & Dolly, 1988) and because the competition assays have limited sensitivity for detecting low-affinity binding, the existence of multiple sites for β-BuTX (as revealed by Scatchard plot) could not be clearly reaffirmed by analysis of these curves.

Physical Properties of DTX Acceptor Protein. A single peak of ^{125}I-DTX binding activity was seen following centrifugation of soluble extracts through H_2O or ^2H_2O gradients (Figure 3). The respective recoveries of ^{125}I-DTX binding activity following centrifugation were ~7% and ~30% in H_2O and ^2H_2O gradients, reflecting the unstable nature of the solubilized acceptor and the greater stability in ^2H_2O, as reported previously for β-BuTX binding (Rehm & Betz, 1984). By comparison with standard proteins, the apparent sedimentation coefficients of the DTX acceptor were 11.7 ± 0.2 S (n = 4) and 10.0 ± 0.2 S (n = 4) in H_2O and ^2H_2O gradients, respectively. Plotting these data as distance travelled against $s_{20,w}(1 - v\rho_{1/2})$ (Meunier et al., 1972) gave a straight

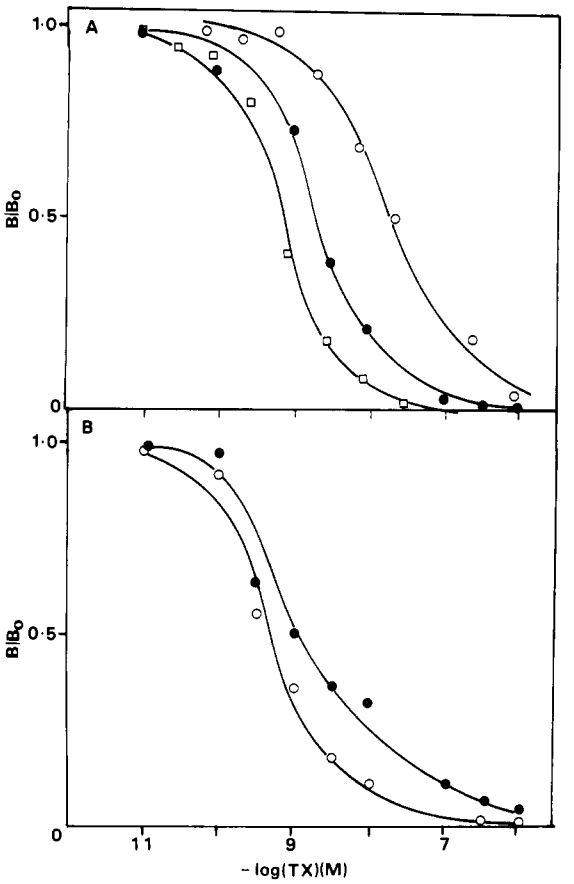

FIGURE 2: Mutual inhibition of ^{125}I-DTX and ^{125}I-β-BuTX binding to soluble extract by DTX and β-BuTX. (A) Binding of 1.2 nM ^{125}I-DTX to a Triton X-100 extract of synaptic plasma membranes from rat cerebral cortex was quantified by the filtration assay in the presence of varying concentrations of DTX (●), toxin I (□), or β-BuTX (○). Nonsaturable binding, measured in the presence of 200 nM DTX, has been subtracted from the total to give the specific binding. The values shown are means of duplicates (error <7%) obtained in the absence (B_0) and presence (B) of ligand. (B) Specific binding of 2.1 nM ^{125}I-β-BuTX was determined as above in the presence of DTX (●) or β-BuTX (○). The values presented are means of three experiments performed in duplicate; the standard error for each point was less than 10%.

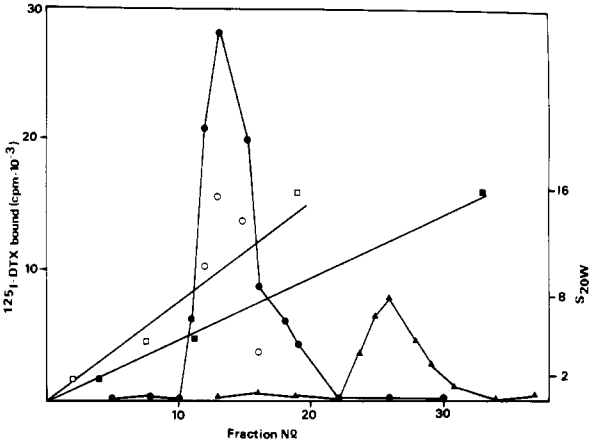

FIGURE 3: Sucrose gradient centrifugation analysis of DTX binding activity. Soluble extracts of rat cerebrocortical synaptosomes, containing standard proteins [10 units of *E. coli.* β-Galactosidase (16 S), 4 units of liver alcohol dehydrogenase (4.8 S), and 200 μg of cytochrome *c* (1.8 S)], were centrifuged (190000*g*; 19 h) simultaneously through 5–20% sucrose gradients made with H_2O (▲) or ^2H_2O (●, ○). Points shown on the linear plots for standard proteins [(□) ^2H_2O; (■) H_2O] are the peak positions estimated from their levels in each fraction. Saturable binding of ^{125}I-DTX (that displaceable by 300 nM DTX) measured in fractions in the absence (●, ▲) or presence (○) of 30 nM β-BuTX is also plotted.

Table II: Physical Properties of the DTX Acceptor in Synaptosomes from Rat Cerebral Cortex[a]

property	value
(A) Acceptor/Detergent/Lipid Complexes	
$s_{20}(H_2O)$ (S)	11.7 ± 0.2
$s_{20}(^2H_2O)$ (S)	10.0 ± 0.2
partial specific volume (mL/g)	0.750 ± 0.003
weight fraction of protein (g/g)	0.78–0.90
Stokes radius (nm)	8.6 ± 0.3
molecular weight	518 000 ± 30 000
(B) Solubilized Acceptor	
$s_{20,w}$ (S)	13.2 ± 0.2
molecular weight	405 000–465 000

[a] Data in (A) and (B) were obtained by gel filtration and sucrose gradient centrifugation of Triton X-100 extracts from rat synaptosomes. Values are means (±SEM) of at least four experiments or are derived from these.

line for each solvent. From this, a partial specific volume of 0.750 ± 0.003 mL/g and an $s_{20,w}$ of 13.2 ± 0.3 S for the DTX acceptor/lipid/detergent complex were calculated. In order to extrapolate the weight fraction of acceptor in the complex, it was assumed that (a) $\bar{v}$ of Triton X-100 = 0.91 mL/g (Pollet et al., 1981), (b) $\bar{v}$ of the lipid was that of phosphatidylcholine (0.981 mL/g; Huang & Charlton, 1971), (c) the ratio of detergent/lipid was 2.5/1 (as in the buffers used), and (d) the partial specific volume of the acceptor is between that of the *Electrophorus* Na^+ channel (0.70 mL/g) and that of the *Torpedo* acetylcholine receptor (0.73 mL/g) [as calculated from their amino acid compositions (Miller et al., 1983; Vandlen et al., 1979)]. This gave a weight fraction of acceptor in the complex of 0.78–0.90 g/g (Table II).

Following gel filtration of solubilized rat synaptosomes, ~25% of the saturable ^{125}I-DTX binding activity was recovered; as with density gradient centrifugation, this activity was seen in a single peak (Figure 4). From the straight line produced when the Stokes radii of standard proteins were plotted against their partition coefficient ($K_d^{1/3}$; Porath, 1963), a Stokes radius for the acceptor/lipid/detergent complex of 8.6 ± 0.3 nm (n = 4) was established. In conjunction with the data obtained from density gradient centrifugation (Table II), this gave a molecular weight of 518 000 ± 30 000 for the complex; hence, molecular weights of the solubilized acceptors can be estimated as 405 000–465 000. When the solubilized extract was either gel filtered (Figure 4) or subjected to ^2H_2O/sucrose density gradient centrifugation (Figure 3), binding of 1.5 nM ^{125}I-DTX was reduced by 30 nM β-BuTX (40% and 51%, respectively) and abolished by 200 nM β-BuTX; for these assays, the gradient and column buffers used were as described previously except 1.5 mM $SrCl_2$ was included. These observations, together with competition experiments (Figure 2) described earlier, indicate that the binding sites for DTX and β-BuTX may reside on the same oligomeric protein(s).

Discussion

The conditions developed for solubilization with Triton X-100 of the toxins' acceptor from synaptic plasma membranes maximize specific activity and yield. As found for the high-affinity β-BuTX sites in the membrane-bound and solubilized states (Othman et al., 1982; Rehm & Betz, 1984), ^{125}I-DTX binding in the extract was abolished by protein denaturing agents; this reaffirms the evidence obtained with membranes for its proteinaceous nature (Dolly et al., 1984; Black et al., 1986). The effectiveness of detergent but not high concentrations of salt in extracting the binding activity for both toxins shows that the sites reside on integral membrane protein(s).

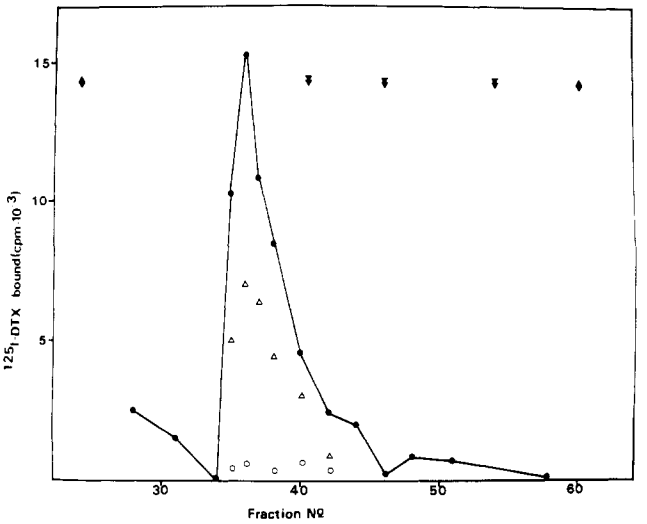

FIGURE 4: Gel filtration of solubilized DTX acceptor. A Triton X-100 extract (0.7 mL) of synaptosomes and standards with the indicated Stokes radii [20 units of β-galactosidase (6.4 nm), 40 units of yeast alcohol dehydrogenase (4.6 nm), and 200 μg of cytochrome c (1.9 nm); Stokes radii taken from Sigel and Monty (1966)] was chromatographed at 4 °C through a Sepharose-6B column (1 × 48 cm) at a flow rate of 10 mL/h; 0.45-mL fractions were collected. Dextran blue and ferricyanide were used to determine the exclusion and inclusion volumes of the column (♦). Arrowheads show the peak positions of standards estimated from their activities in the individual fractions. Saturable binding of 1.5 nM ^{125}I-DTX (that displaceable by 300 nM DTX) in the absence (○) or presence of 30 nM β-BuTX (△) or 200 nM β-BuTX (○) was quantified by a rapid gel filtration assay.

In view of the likelihood of the DTX binding component being associated with certain K^+ channels, it is interesting that K^+ is required to stabilize the solubilized acceptor; notably, Rb^+ was less effective than K^+, particularly in preserving β-BuTX activity. Indeed, the latter is less stable on storage and more sensitive to the composition of the medium (see below); also, unlike DTX binding, it is dependent on the presence of Ca^{2+} or Sr^{2+} in the assay buffer (Rehm & Betz, 1984).

Heterogeneity in the β-BuTX sites was apparent from the nonlinear Scatchard plot; in addition to the high-affinity population, graphical analysis revealed a larger content of low-affinity sites in the soluble extract. Two equivalent sets of β-BuTX acceptors were also detected in the membranes (Figure 2B), provided imidazole buffer is used (Dolly et al., 1987; Breeze & Dolly, 1988). In fact, when the latter conditions are used, the total content of high- and low-affinity acceptors for β-BuTX is similar to that seen for the single set of DTX sites, which remains constant in all buffers tested. In contrast, when Krebs–Ringer is used, the B_{max} obtained for high-affinity ^{125}I-β-BuTX binding in membranes or brain cryostat sections (Pelchen-Matthews & Dolly, 1988) represents only a fraction (~30%) of the value seen for DTX sites under the same conditions. Apparently, use of imidazole unmasks an additional low-affinity binding component.

In accord with the functional importance of the toxin's acceptor, neurotoxic congeners of DTX were effective in antagonizing ^{125}I-DTX binding to the soluble extract whereas nontoxic homologues were inactive (Black, 1986). Acceptor affinities for the homologues and the dissociation rate constants for the DTX/acceptor complex were similar in the soluble and membrane-bound states; thus, its properties were not altered by detergent solubilization. Further evidence for the association of the toxins' acceptor with K^+ channels was obtained from experiments using mast cell degranulating peptide. This was shown to block a DTX-sensitive K^+ conductance (Parcej

et al., 1987; Stansfeld et al., 1987), albeit with relatively low potency, and to inhibit the binding of both toxins to acceptors in membranes or solubilized preparations. Moreover, the complete inhibition produced by β-BuTX of DTX/acceptor interaction, and the corresponding DTX antagonism of ^{125}I-β-BuTX binding, is in accord with their pharmacological interaction at motor nerve terminals of some species (Harvey & Karlsson, 1982) or cerebrocortical synaptosomes (Rugulo et al., 1986) and abilities of both to attenuate certain K^+ conductances (see the introduction). Most importantly, all the numerous studies revealing mutual inhibition [which involves a complex noncompetitive mechanism (Breeze & Dolly, 1988)] indicate that the binding sites for DTX and β-BuTX reside on the same protein(s), a deduction supported by the hydrodynamic studies discussed below.

Further evidence that the acceptor contains binding sites for both DTX and β-BuTX was provided by sedimentation analysis and gel filtration; in each separation method, one peak of ^{125}I-DTX binding activity was obtained, and this could be abolished by β-BuTX. Moreover, the hydrodynamic properties determined for the DTX acceptor from rat brain bear striking resemblances to the values reported for the β-BuTX binding protein from chick brain (Rehm & Betz, 1984). In each case, the significant amount of detergent bound is consistent with the acceptor being an integral membrane protein. It must be noted that the weight fraction and the molecular weight derived from it are only estimates because of the variation in the partial specific volumes of the acetylcholine receptor and Na^+ channel proteins used to calculate the weight fraction of protein in the acceptor/detergent/lipid complex. Notwithstanding the difficulties in establishing the exact size of this acceptor, it is clearly a large glycoprotein (Dolly, 1988) that comprises several subunits. These could include one or more copies of a polypeptide with M_r ~65 000 demonstrated in rat synaptosomal membranes by chemical cross-linking of the acceptor/^{125}I-DTX complex (Mehraban et al., 1984). Additionally, it is likely to contain a subunit of M_r ~95 000 shown by photoaffinity labeling to bind β-BuTX in chick brain membranes (Rehm & Betz, 1983); this is based on the reasonable assumption [from mutual competition assays and hydrodynamic studies detailed herein and elsewhere, and cross-linking experiments (Black & Dolly, 1986)] that this same oligomer binds β-BuTX. Of course, due to the poor resolution of the separation methods used, the possibility has not been excluded that two oligomeric species of the common acceptor protein exist that are of similar size; in this case, both would bind DTX with high affinity while exhibiting heterogeneity in their affinity for β-BuTX. These possibilities can only be resolved by purification of the β-BuTX and DTX binding activities; the data presented herein facilitate this undertaking.

These findings on the oligomeric and subunit properties of the toxins' acceptor from mammalian brain should be useful in interpreting the extensive data obtained recently, by employing molecular genetic techniques, for related "A-type" K^+ channels from *Drosophila melanogaster* (Tempel et al., 1987; Schwarz et al., 1988) which are inhibitable by DTX (F. C. Wu, personal communication). With the use of "Shaker" mutant, the predicted structure has been obtained for "isoproteins" constituting this variety of K^+ channel (Timpe et al., 1988); notably, their sizes (64 000–74 000) approximate to those of the polypeptides identified in rat and chick membranes by cross-linking to ^{125}I-DTX (Mehraban et al., 1984; Black & Dolly, 1986). Although individual expression in *Xenopus* oocytes of two cDNAs for the fly channel protein each yield an A-type current, it seems possible from other data [reviewed by Dolly (1988)] that the channels assembled in vivo may be heterooligomers; at least some of these could be analogous to the structure postulated above for the oligomeric acceptor, observed experimentally. Further research, particularly at the protein level, is required to establish which combinations and stoichiometries of polypeptides create the multiplicity of K^+ channels observed electrophysiologically. Advances already made in this laboratory in the purification of the DTX acceptor, with a view to reconstitution and electrophysiological measurements, should, in parallel with molecular biology approaches, establish the relationship of the DTX acceptor subtypes found in discrete neuronal locations to the numerous members of this one family of important K^+ channels.

Registry No. DTX, 74811-93-1; β-BuTX, 12778-32-4.

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Structural Analysis of the Specific Capsular Polysaccharide of *Streptococcus pneumoniae* Type 45 (American Type 72)†

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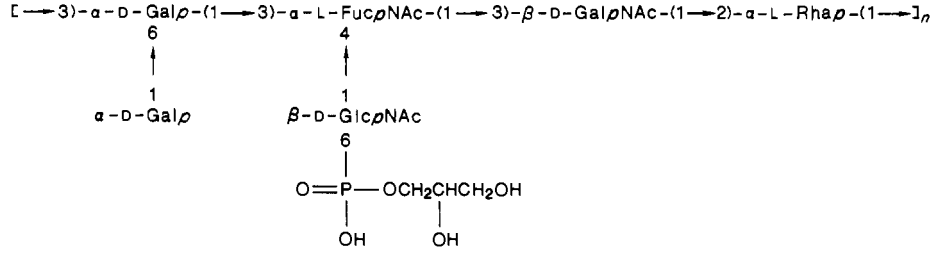
ABSTRACT: The specific capsular polysaccharide of *Streptococcus pneumoniae* type 45 (American type 72) was found to be a high molecular weight polymer composed of D-galactose, 2-acetamido-2-deoxy-D-galactose, 2-acetamido-2-deoxy-D-glucose, 2-acetamido-2-deoxy-L-fucose, L-rhamnose, glycerol, and phosphate (2:1:1:1:1:1:1). Partial hydrolysis, dephosphorylation, methylation analysis, periodate oxidation studies, and one- and two-dimensional ^1H and ^{13}C high-field nuclear magnetic resonance experiments showed the polysaccharide to be a branched polymer of a 1-phosphoglycerol-substituted hexasaccharide repeating unit having the structure:



Investigations on the specific capsular polysaccharides of *Streptococcus pneumoniae* serotypes have increased since the introduction of the wide-scale use of a pneumococcal vaccine composed of 23 of the 84 serologically defined pneumococcal polysaccharides (Lund & Henrichsen, 1978; Robbins et al., 1983). The formulation of the pneumococcal vaccine is based on surveys of the pneumococcal serotypes responsible for the major portion of human infections in a particular geographic region (Austrian, 1978; Austrian et al., 1976).

Serotypes 45 and 46 of *S. pneumoniae*, which appear persistent in infections found in Africa (Bureau of Biologics, 1979), are virtually absent in Europe and North America. It is not known whether this is due to racial or geographic restrictions. While types 45 and 46 pneumococcal poly-

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