Involvement of neuronal acceptors for dendrotoxin in its convulsive action in rat brain

Adrian R. BLACK, Alexander L. BREEZE, Iekhsan B. OTHMAN* and J. Oliver DOLLY†

Department of Biochemistry, Imperial College, London SW7 2AZ, U.K.

1. Dendrotoxin, a snake-venom polypeptide, is a potent convulsant that facilitates transmitter release apparently by inhibition of voltage-sensitive K^+ channels responsible for A-currents. A biologically active ^{125}I -iodinated derivative of this toxin was prepared and used to characterize kinetically homogeneous non-interacting high-affinity acceptors in synaptic membranes from rat cerebral cortex and hippocampus.
2. Binding of radiolabelled toxin from *Dendroaspis angusticeps* to its membrane acceptor protein was inhibitable by homologous polypeptides from other mamba snakes; most importantly, their rank order of potency was identical with that for their central neurotoxicities in rats, furnishing evidence for involvement of this binding component in the convulsive symptoms observed.
3. β -Bungarotoxin, a presynaptically acting neurotoxin whose action on neurotransmitter release at the neuromuscular junction and effects on brain synaptosomes are antagonized by dendrotoxin, was only able to inhibit the binding of the ^{125}I -labelled toxin with low efficacy, although dendrotoxin apparently interacts avidly with the acceptor sites for β -bungarotoxin. This weak interaction of β -bungarotoxin with the acceptor was not attributable to its phospholipolytic action.
4. Other neurotoxins and ion-channel antagonists failed to affect the binding of dendrotoxin.
5. The findings presented here, together with recent electrophysiological data, favour the interpretation that dendrotoxin binds to a membrane protein comprising, or closely associated with, this one group of voltage-dependent K^+ channels.

INTRODUCTION

Dendrotoxin (DTX) from the venom of *Dendroaspis angusticeps* and its congeners from other mamba snakes are single-chain polypeptides ($M_r \sim 7000$) that show sequence homology with Kunitz-type trypsin inhibitors (Joubert & Taljaard, 1980; Harvey & Karlsson, 1982). Interestingly, some of these homologues, including DTX and toxin I from *D. polylepis*, facilitate neurotransmitter release at peripheral (Harvey & Karlsson, 1982; Harvey *et al.*, 1984) and central (Docherty *et al.*, 1983; Dolly *et al.*, 1984) synapses; in fact, DTX is highly neurotoxic in rat brain, where it exhibits pronounced convulsive symptoms (Dolly *et al.*, 1984). Recently, voltage-clamp studies on hippocampal slices (Dolly *et al.*, 1984; Halliwell *et al.*, 1986) have demonstrated that this action results from a selective inhibition by DTX of the A-current, a fast transient K^+ conductance mediated by voltage-sensitive channels concerned with the regulation of cell excitability (Gustafsson *et al.*, 1982). DTX is also homologous with the lighter B-chain of β -bungarotoxin (β -BuTX), an inhibitory presynaptic neurotoxin exhibiting Ca^{2+} -dependent phospholipase A_2 activity (Strong *et al.*, 1976; Kondo *et al.*, 1982). In regard to their opposing pharmacological actions, it is notable that the interactions of these two toxins at chick neuromuscular junction (Harvey & Karlsson, 1982) and the ability of DTX to inhibit totally ($K_i \sim 0.5$ nM) the synaptosomal binding of 3H - β -BuTX (Othman, 1983) indicate that these toxins share, at least, some common acceptors.

In view of the novel action of DTX and its potential usefulness as a unique probe, it was pertinent in the present study to characterize the neuronal membrane acceptors for DTX and examine their interaction with a series of homologous toxins, including β -BuTX.

EXPERIMENTAL

Materials

Crude venom of *Dendroaspis angusticeps* was purchased from Sigma, Poole, Dorset, U.K., as were trypsin, bovine basic pancreatic trypsin inhibitor, soybean trypsin inhibitor and chymotrypsin. Dinonyl phthalate and Dow-Corning MS-550 silicone fluid were purchased from BDH, Poole, Dorset, U.K. Pure preparations of proteins were donated as follows: toxins I (Dr. A. Harvey and Dr. E. Karlsson), E and B (Dr. F. Joubert) from *D. polylepis*; taipoxin (Dr. D. Mebs); subunits of crotoxin (Dr. C. Bon); phospholipase A_2 from bee venom and *Naja melanoleuca*; a toxin fraction (Dv-14) from *D. viridis* and apamin (Dr. R. Shipolini). Homogeneous β -bungarotoxin was isolated and characterized by Mrs. A. Pelchen-Matthews of this laboratory, as described elsewhere (Spokes & Dolly, 1980). $Na^{125}I$ (carrier-free) was obtained from Amersham International, Amersham, Bucks., U.K. All other reagents were of analytical grade.

Purification of DTX

The venom of *D. angusticeps* was gel-filtered on Sephadex G-50 (superfine grade) as detailed by Harvey

Abbreviations used: DTX, dendrotoxin; ^{125}I -DTX, ^{125}I -labelled dendrotoxin preparation; β -BuTX, β -bungarotoxin; 3H - β -BuTX, di[3H]propionylated β -bungarotoxin; MLD, minimum lethal dose.

* Present address: Department of Biochemistry, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia.

† To whom correspondence and reprint requests should be addressed.

& Karlsson (1980); the resultant DTX-containing peak was fractionated on Bio-Rex 70 or CM-Sepharose CL-6B by using a two-step (0.10–0.35 M- and 0.35–1.40 M-ammonium acetate) or a linear (0.1–1.4 M-ammonium acetate) salt gradient respectively. The DTX peak was desalted on Sephadex G-10 and freeze-dried. As an additional purification step, some samples were subjected to h.p.l.c. on molecular-sizing columns (two Waters I-125 in series) in 0.1% trifluoroacetic acid. Purity of the DTX preparations was assessed by electrophoresis on gradient (15–22%, w/v) polyacrylamide gels in the presence of SDS by a method adapted from that of Laemmli (1970) and on native cathodic discontinuous gels [modified, from the method of Reisfeld *et al.* (1962), for slabs]. The biological activity of DTX was assessed from the MLD obtained after intraventricular injection into rats (Othman *et al.*, 1982) and from its ability to facilitate transmitter release in hippocampal slices and in chick nerve-muscle preparations; the electrophysiological measurements were kindly performed by Dr. J. Halliwell and Dr. A. Harvey respectively.

¹²⁵I-iodination of DTX

This was achieved by using a modification of the chloramine-T method; Na¹²⁵I (~2 mCi; 20 µl) was added to DTX (20 µg) in 100 mM-sodium phosphate buffer, pH 7.4, containing 0.02% Triton X-100; the reaction was initiated by adding chloramine-T (final concn. 0.21 mM) in a maximum final volume of 50 µl. After 30 s, the reaction mixture was diluted to 1 ml with 20 mM-sodium phosphate buffer, pH 7.4. Duplicate aliquots (10 µl) of the solution were removed for measurement of the specific radioactivity of the ¹²⁵I-DTX; this was determined by precipitation of the protein with ice-cold 10% (w/v) trichloroacetic acid in the presence of 1% bovine serum albumin, followed by γ -radiation counting of the precipitated material. The remainder of the solution was applied to a CM-Sepharose CL-6B ion-exchange column (2 ml) equilibrated in 20 mM-sodium phosphate, pH 7.4. Radioactive iodide that had not reacted was washed from the column with 2 ml of the latter buffer, followed by elution of ¹²⁵I-DTX with a linear gradient of NaCl (0–1 M) in the same buffer. Samples were analysed by electrophoresis/autoradiography (Mehraban *et al.*, 1984). ¹²⁵I-DTX was stored at 4 °C and diluted before use.

Membrane preparations

Rat cerebrocortical synaptosomes were prepared as described previously (Othman *et al.*, 1982), except that latterly the sucrose solutions were buffered to pH 7.5 with 2 mM-Hepes. P₂ fractions from rat cortex and hippocampus were prepared similarly, except for the omission of the gradient-centrifugation step. Synaptic plasma membranes were isolated from rat cerebral cortex by the method of Jones & Matus (1974). All tissue preparations were stored at –80 °C until required.

Binding of ¹²⁵I-DTX to membrane preparations

This was measured initially in Krebs–Ringer solution, pH 7.4, using a conventional centrifugation/washing assay as described previously (Othman *et al.*, 1982) for ³H- β -BuTX; silane-treated tubes and pipette tips were used in all the assays. Latterly, a binding assay which entailed rapid centrifugation of the membranes through a silicone-based oil mixture was employed; this method

effected separation of bound from free ligand in ~20 s (Mehraban *et al.*, 1984). In this case, membrane suspensions in 20 mM-Tris/HCl/100 mM-NaCl/5 mM-KCl/1 mM-CaCl₂, pH 7.4 (assay buffer) or, where stated, the latter minus Ca²⁺ and with the addition of 2 mM-EGTA, were incubated in the presence of 1.5 nM-¹²⁵I-DTX (unless otherwise stated). After 45 min at 22 °C, portions were centrifuged through the oil mixture; after washing of the top of the oil layer with 0.5 ml of assay buffer, the bound radioactivity associated with the pellet was measured by γ -radiation counting. With this oil-based system, less than 10% dissociation of ¹²⁵I-DTX was shown to occur during the centrifugation/washing assay, confirming the latter's utility in the present study. Measurement of the kinetics of the association between ¹²⁵I-DTX and its acceptor in rat cortex P₂ fraction was performed at 22 °C by using the oil-separation method. Likewise, dissociation rates were measured after preincubation of synaptosomes (0.35–0.4 mg of protein/ml) with 2 nM-¹²⁵I-DTX for 30 min at 22 °C; dissociation was monitored after the addition of a 100-fold molar excess of unlabelled DTX. Non-specific binding was monitored simultaneously in parallel samples in which a 100-fold excess of DTX had been included during the preincubation. Membrane protein concentrations were routinely determined by the methods of Lowry *et al.* (1951) or Bradford (1976), with bovine serum albumin as a standard.

RESULTS

Preparation and properties of ¹²⁵I-DTX

DTX, purified by gel filtration and ion-exchange chromatography, migrated as a single band on electrophoresis under denaturing (Fig. 1a) or native (Fig. 1b) conditions. However, minor contaminants (<10%) could be separated by h.p.l.c.; as their removal did not alter (Mehraban *et al.*, 1986) the neurotoxicity (MLD = 2.5 ng/g body wt.; Table 1), acceptor-binding properties (see below) or the facilitatory action of DTX on neuromuscular transmission (A. Harvey, personal communication) or hippocampal slices (Halliwell *et al.*, 1986), toxin from the ion-exchange step was normally used. Samples of DTX were radioiodinated by using a modification of the chloramine-T procedure, which minimizes possible denaturation of the toxin. ¹²⁵I-DTX was eluted with salt as a single peak from the ion-exchange column; more than 95% of the radioactivity was acid-precipitable. Upon analysis by SDS/polyacrylamide-gradient-gel electrophoresis, ¹²⁵I-DTX displayed the same mobility as native toxin (Fig. 1a). Electrophoresis on native cathodic gels (Fig. 1b) separated a major radioactive derivative, together with small amounts of an even less basic species, from native DTX; presumably these represent mono- and di-iodinated derivatives.

The standard iodination conditions yielded labelled toxin with relatively high levels (300–400 Ci/mmol) of specific radioactivity. Moreover, the central toxicity of the labelled toxin (MLD = 2.8 ng/g body wt., *n* = 3) was not decreased relative to that of the native toxin. Since it proved impractical to separate preparatively the major species of radioiodinated DTX from the unlabelled toxin, the contribution of the latter to the measured neurotoxicity of ¹²⁵I-DTX could not be established directly. However, when other samples were iodinated by a similar

Fig. 1. Electrophoretic analysis of DTX and ^{125}I -DTX

(a) Samples of DTX and ^{125}I -DTX, prepared as described in the Experimental section, were subjected to electrophoresis in a 15–22% (w/v) polyacrylamide gel containing 0.1% SDS and 5% (v/v) β -mercaptoethanol. The gel was stained with Coomassie Brilliant Blue R-250, dried and autoradiographed. Tracks contained: A, DTX; B, ^{125}I -DTX revealed by autoradiography; C, standard polypeptides (intact myoglobin and its CNBr cleavage products): myoglobin, M_r 17200; myoglobin I+II, M_r 14400; myoglobin I, M_r 8200; myoglobin II, M_r 6400; myoglobin III, M_r 2600. (b) DTX and ^{125}I -DTX were subjected to discontinuous cathodic electrophoresis under non-denaturing conditions in gels of 15% (w/v) polyacrylamide; the gels were stained and subjected to autoradiography. Tracks were as follows: A, DTX; B, ^{125}I -DTX (DTX was added as a carrier) revealed by autoradiography.

procedure to low specific radioactivity, using an isotope mixture of ^{125}I and ^{127}I under conditions (0.42 mM-chloramine-T; 240 μM -NaI) that resulted in greater-than-equimolar incorporation of iodine into the protein, again no loss of neurotoxicity was detectable. Furthermore, measurement of the binding of ^{125}I -DTX to brain membranes showed that iodination did not alter the affinity of the toxin for its acceptors (see below).

Demonstration of proteinaceous acceptors for DTX in synaptic membrane preparations

^{125}I -DTX bound saturably to purified synaptic plasma membranes from rat cerebral cortex; a Scatchard plot of the data was linear (Fig. 2), indicating the presence of kinetically homogeneous non-interacting sites. The calculated K_D of 0.3 nM accords with the value of 0.4 nM reported for freeze-thawed synaptosomes (Mehraban *et al.*, 1984), whereas the higher site content (1.4–1.7 pmol/mg of protein) obtained relative to that for synaptosomes (1.0–1.2 pmol/mg of protein) highlights

Table 1. Correlation of the central neurotoxicities of DTX congeners with their relative affinities for the acceptor in rat synaptic membranes

The K_I for the inhibition of the binding of 1.5 nM- ^{125}I -DTX to cerebrocortical synaptosomal preparations was determined for duplicate samples by using a range of concentrations of each homologue in the oil assay method, as detailed in the Experimental section. The resultant IC_{50} values were used to calculate the K_I for each toxin using the equation:

$$K_I = \text{IC}_{50} / \left(1 + \frac{[^{125}\text{I-DTX}]}{K_D} \right)$$

Central-neurotoxicity toxicity after injection into cerebroventricular spaces of rat brain; the MLD values shown are derived from three determinations for each of a series of toxin concentrations.

Toxin	Species	K_I (nM)	Central neurotoxicity (MLD; ng/g body wt.)
Toxin I	<i>D. polylepis</i>	0.05	0.5
DTX	<i>D. angusticeps</i>	0.4	2.5
Dv-14	<i>D. viridis</i>	0.4	2.5
Toxin E*	<i>D. polylepis</i>	1.5	25
Toxin B	<i>D. polylepis</i>	> 2000	> 150

* See 'Note added in proof'

the enrichment of these acceptors in the plasma-membrane fraction. Non-specific binding comprised an acceptably low proportion of the total (e.g. less than 20% at 0.5 nM), was relatively invariant with different batches of ^{125}I -DTX and increased only slightly over 3–5 weeks.

Since the electrophysiological effects of DTX were measured in hippocampus, its acceptors were investigated therein by using the P_2 fraction to maximize the yield of membranes. A Scatchard plot of the saturable binding obtained demonstrated again the presence of kinetically homogeneous non-interacting sites with a K_D of 0.3 nM and B_{max} of 1.2 pmol/mg of protein (Fig. 3); this indicated the occurrence of DTX acceptors with similar properties in both these brain regions.

The proteinaceous nature of the acceptors was ascertained by subjecting cerebrocortical synaptosomes to various treatments. Proteolysis of the membranes with either trypsin or chymotrypsin significantly decreased the specific binding ($\sim 70\%$), with little effect on non-specific binding of the toxin (Table 2); acidification or heating resulted in complete inactivation of the acceptors. Omission of Ca^{2+} from the binding medium and addition of 2 mM-EGTA did not affect the subsequent binding of ^{125}I -DTX (Fig. 4b); this contrasts with the high-affinity binding of ^{125}I -iodinated (Rehm & Betz, 1982) or tritiated (A. L. Breeze & D. Parcej, unpublished work) β -BuTX to chick or rat brain membranes, which shows an absolute requirement for bivalent cations.

Measurement of the rate of dissociation of the ^{125}I -DTX acceptor complex was monitored after the addition of excess unlabelled DTX to synaptosomes that had been preincubated with ^{125}I -DTX, as described in the Experimental section. At 22 $^{\circ}\text{C}$, the process was found to have a half-time ($t_{1/2}$) of approx. 11 min (Fig. 5); a first-order plot (Fig. 5, inset) was linear with a slope corresponding to a dissociation rate constant (k_{-1}) of

Fig. 2. Concentration dependence of ^{125}I -DTX binding to synaptic plasma membranes from rat cerebral cortex

A suspension of the purified synaptic membranes (0.5 mg/ml of protein) was incubated in Krebs–Ringer solution at 22 °C for 45 min with various concentrations of ^{125}I -DTX (final vol. 250 μl). Bound and free toxin were separated by centrifugation; the supernatant was removed and the pellet washed twice with ice-cold Krebs–Ringer solution (2×1 ml). The radioactivity associated with the washed membranes and in the original supernatant was quantified by γ -radiation counting. Non-specific binding was determined likewise in the presence of a 100-fold molar excess of unlabelled DTX. A Scatchard plot of the specific binding is shown; the inset shows the data from which the plot was derived. Specific binding (●) was calculated as the difference between the total (○) and non-specific (■) binding.

Fig. 3. Saturable binding of ^{125}I -DTX to hippocampal membranes

P_2 membrane fraction of hippocampus was incubated in assay buffer at a protein concentration of 0.7–0.8 mg/ml in the presence of various concentrations of ^{125}I -DTX (final vol. 250 μl). After 45 min at 22 °C the concentrations of bound and free ^{125}I -DTX were determined by the oil-separation method described in the Experimental section. Non-specific binding was determined similarly in the presence of a 100-fold molar excess of unlabelled DTX. A Scatchard plot of the specific binding is shown. Concentration-dependence of the total (○), non-specific (■) and specific (●) binding is given in the inset.

Table 2. Effects of various treatments on the DTX acceptors in synaptosomes from rat cerebral cortex

Synaptosomal suspensions in Krebs–Ringer solution were subjected to the specified conditions; at the end of the indicated times, proteolysis was stopped by addition of 5 mM-phenylmethanesulphonyl fluoride (to chymotrypsin) or 5 mg of soybean trypsin inhibitor/ml (to trypsin) and acidification was terminated by washing (2×1 ml) with Krebs–Ringer solution. The binding of 1.2 nM - ^{125}I -DTX to treated synaptosomes was then assayed by using the conventional centrifugation assay. Non-saturable binding, measured likewise in the presence of 100-fold excess of unlabelled DTX, was subtracted from the total binding observed for quadruplicate samples ($\pm \text{s.e.m.}$). The resultant specific binding is expressed relative to that obtained for respective control samples in which appropriate inhibitors were present throughout the incubation (for proteolysis), or where membranes were incubated at 37°C in Krebs–Ringer solution for either 30 min (for heat treatment) or 40 min (for acidified samples). Specific binding in untreated and control samples was 700 fmol/mg of protein.

Treatment	Relative specific binding of ^{125}I -DTX (%)
Chymotrypsin (1 mg/ml; 37°C for 40 min)	36 ± 16
Trypsin (1 mg/ml; 37°C for 40 min)	34 ± 13
Heat (95°C for 30 min)	2 ± 2
Acidification (1 M-HCl; 37°C for 40 min)	3 ± 1

$1.03 \times 10^{-3} \text{ s}^{-1}$. The monophasic dissociation observed constitutes further evidence for the existence of a kinetically homogeneous population of sites for DTX. The K_D and k_{-1} for ^{125}I -DTX binding predict an association rate constant (k_{+1}) of $3.4 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ and a $t_{1/2}$ calculated by using the second-order rate equation, of 3.8 min under the conditions employed (0.3 nM - ^{125}I -DTX; 0.3 mg of synaptosomal protein/ml; 22°C). However, it should be noted (see the Experimental section) that the oil-separation assay does not effect instantaneous separation of bound from free ligand. Therefore the association rate measured, especially at early time points, is more rapid than predicted from theory; indeed, the observed $t_{1/2}$ was ~ 1.5 min. When the time taken for the membranes to sediment through the oil ($\sim 20 \text{ s}$) was taken into account, the second-order plot appeared linear and gave a k_{+1} of $\sim 8 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$. This value, together with the measured k_{-1} of $1.03 \times 10^{-3} \text{ s}^{-1}$, indicates a K_D of 0.13 nM for ^{125}I -DTX binding, a value close to that obtained from equilibrium data.

Selectivity of the synaptic DTX acceptor

Unlabelled DTX (with and without final purification by h.p.l.c.) inhibited the binding of the labelled toxin with a K_I , calculated from the IC_{50} (concn. causing 50% inhibition) value, of 0.4 nM (Fig. 4); this indicates that the radioiodination procedure employed did not significantly alter its affinity. In contrast, crotoxin, an inhibitory presynaptic toxin that contains Ca^{2+} -dependent phospholipase A_2 activity (reviewed by Chang, 1985), failed to affect ^{125}I -DTX binding in either the absence or presence

Fig. 4. Effect of presynaptically active toxins and phospholipases A_2 on ^{125}I -DTX binding to synaptosomes

(a) Synaptosomes (0.5 mg of protein/ml) were incubated at 37°C for 30 min in Krebs–Ringer solution in the presence of 2.5 nM - ^{125}I -DTX and various concentrations of unlabelled DTX ($\bullet$), toxin I ($\circ$), taipoxin ($\blacktriangle$) or phospholipases A_2 from *Naja melanoleuca* ($\square$) and bee venom ($\blacksquare$). At equilibrium, bound ^{125}I -DTX was separated from free labelled toxin by the centrifugation/washing technique and quantified by γ -radiation counting. Non-specific binding was determined similarly in the presence of 250 nM -unlabelled DTX. (b) As in (a), except Ca^{2+} -free medium containing 2 mM -EGTA was used. (c) Synaptosomes (0.35 – 0.4 mg of protein/ml) in assay buffer were incubated for 45 min at 22°C in the presence of 1.5 nM -DTX and various concentrations of unlabelled DTX ($\bullet$), Dv-14 ($\blacksquare$), toxin E ($\blacktriangle$, but see 'Note added in proof') or β -BuTX ($\circ$). Bound ^{125}I -DTX was quantified by the oil-separation method. Non-specific binding was determined similarly in the presence of 150 nM -unlabelled DTX. B_0 and B represent the amount of ^{125}I -DTX bound in the absence and presence respectively of competing ligand. Specific binding in the absence of competing ligand was 750 fmol/mg of protein.

Fig. 5. Dissociation of ^{125}I -DTX-acceptor complex

^{125}I -DTX (2 nM) was incubated at 22 °C for 30 min with a suspension of synaptosomes (0.35–0.4 mg of protein/ml) in assay buffer. Unlabelled DTX (200 nM) was added and the amount of specifically-bound radioactivity remaining after various times was quantified (●) by centrifugation through an oil mixture as detailed in the Experimental section. Non-specific binding (■) was measured simultaneously in separate tubes by including 200 nM-DTX in the initial incubation mixture. Inset: first-order plot of the dissociation of acceptor-toxin complex where B and B_t represent the amounts of specific binding at zero time and after time t respectively. B_0 is defined in the legend to Fig. 4.

of Ca^{2+} ; nor was any inhibition effected by the individual subunits of crotoxin (results not shown). Another snake protein, taipoxin, which also exhibits phospholipase A_2 activity (Chang, 1985), was ineffective towards the binding of ^{125}I -DTX when Ca^{2+} was removed from the medium (Fig. 4b) or replaced with Sr^{2+} . In the presence of Ca^{2+} , however, this toxin displayed feeble inhibitory activity (Fig. 4a), as did bee-venom phospholipase A_2 (which was also ineffective in the absence of Ca^{2+}) (Figs. 4a and 4b); the latter had a K_i (9.3 nM) about 20-fold higher than the K_D for DTX binding. Interestingly, phospholipase A_2 from the venom of *Naja melanoleuca*, despite having similar specific activity to the bee enzyme, was unable to inhibit the binding (Fig. 4a); this, and the similar result noted with crotoxin, indicate a certain substrate specificity for phospholipolytic action on the DTX-binding macromolecule.

Surprisingly, β -BuTX only reduced the binding of ^{125}I -DTX when very high concentrations were used, and the inhibition was incomplete even with the highest dose (10 μM) tested (Fig. 4c). As this feeble antagonism was obtained in both the presence and absence of Ca^{2+} , it could not have arisen from the phospholipolytic A_2 action

of β -BuTX. The same pattern of inhibition by β -BuTX of ^{125}I -DTX binding was obtained in hippocampal membranes (results not shown). Thus the apparent lack of significant perturbation of the binding of ^{125}I -DTX by low concentrations of β -BuTX indicates that, under the conditions used, only a small proportion of the observed acceptor sites in cerebral cortex and hippocampus exhibit high affinity for both β -BuTX and DTX.

Effect of ion-channel blockers on acceptor binding of ^{125}I -DTX

Tetraethylammonium chloride, 4-aminopyridine and an analogue of phencyclidine, thienylcyclohexylpiperidine, all failed to exert any inhibition on the binding of 1.5 nM- ^{125}I -DTX to cerebrocortical synaptosomes (Table 3). Although these agents are all selective blockers of various classes of K^+ channels, these results do not exclude the possibility that DTX binds to distinct sites on the macromolecular complex that may comprise the voltage-gated K^+ channel; alternatively, DTX may exert an indirect action on the channel. As the bee-venom toxin apamin has been shown to block a different variety of K^+ channel (Ca^{2+} -dependent) in muscle cells (Romey & Lazdunski, 1984), its lack of inhibition of ^{125}I -DTX binding is not surprising (Table 3). Finally, the specific binding of ^{125}I -DTX was unaffected by a range of con-

Table 3. Effects of ion-channel antagonists on the binding of ^{125}I -DTX to synaptosomes

Binding was determined by using 1.5 nM- ^{125}I -DTX as described in the Experimental section; 100% represents ^{125}I -DTX bound specifically in the absence of competing ligand. Results are means of three duplicate determinations ($\pm$ S.D.) except for TCP (thienylcyclohexylpiperidine, D-600, nifedipine and nimodipine (duplicate determination). Specific binding in the absence of competing ligand was 750 fmol/mg of protein.

Antagonist	Concn. (M)	Relative binding (%)
Tetraethylammonium chloride	1×10^{-6}	99 ± 3.8
	1×10^{-4}	102 ± 5.4
	1×10^{-3}	107 ± 5.5
	1×10^{-2}	98 ± 2.4
4-Aminopyridine	1×10^{-6}	106 ± 6.3
	5×10^{-4}	93 ± 2.5
	5×10^{-3}	99 ± 3.4
	1×10^{-2}	100 ± 4.3
TCP	1×10^{-8}	101
	1×10^{-6}	116
	1×10^{-4}	116
Apamin	1×10^{-9}	110 ± 1.6
	1×10^{-7}	97 ± 2.2
	1×10^{-6}	97 ± 4.6
	1×10^{-5}	101 ± 3.8
D-600	1×10^{-10}	96 ± 7
	1×10^{-8}	96 ± 3
	1×10^{-6}	104 ± 2
	1×10^{-4}	99 ± 0
Nifedipine	1×10^{-10}	102 ± 4
	1×10^{-8}	99 ± 3
	1×10^{-6}	96 ± 5
	1×10^{-4}	97 ± 2
Nimodipine	1×10^{-10}	100 ± 3
	1×10^{-8}	95 ± 2
	1×10^{-6}	103 ± 3
	1×10^{-4}	100 ± 1

centrations of nifedipine, nimodipine or D-600 (Table 3), classical antagonists of voltage-sensitive Ca^{2+} channels.

Functionality of the acceptors for DTX

In general, only those toxins that are active on neuromuscular transmission inhibited ^{125}I -DTX binding; the inhibition curves obtained for toxin I, DTX and Dv-14 [a protein from *D. viridis* that is 61% homologous with DTX (cf. Mehraban *et al.*, 1986)] are all consistent with competitive interaction at one set of acceptor sites (Fig. 4), whereas toxin B was ineffective (Table 1). However, toxin E was an effective inhibitor and displayed appreciable central neurotoxicity (Table 1), even though no facilitatory activity was detected at motor endplates (Harvey *et al.*, 1984). The possibility that this discrepancy is attributable to contamination with pre- and post-synaptically active toxins requires further investigation (see 'Note added in proof'). Importantly, the central-neurotoxicities of these toxins (toxin I > DTX = Dv-14 > toxin E $\gg$ toxin B) were found to correlate with their abilities to inhibit ^{125}I -DTX binding to synaptosomal membranes (Table 1). It should be noted that Dv-14 showed the same central toxicity and acceptor affinity as DTX (Table 1); furthermore, these two homologues have recently been found to have similar levels of facilitatory activity at motor nerve terminals (A. Harvey, personal communication).

DISCUSSION

The preparation of ^{125}I -DTX described here exhibits unaltered neurotoxicity and affinity for its neuronal acceptors; this, together with its high specific radioactivity and relatively low extent of non-saturable binding, makes it both a valid and practicable derivative for studies on the molecular action of this novel toxin. In fact, it has facilitated the autoradiographic localization of the acceptors in various brain regions (Halliwell *et al.*, 1986) and permitted the identification by cross-linking of polypeptides comprising the DTX acceptors in both rat (Mehraban *et al.*, 1984) and chick (Black & Dolly, 1986) brain membranes. More recently, the activity of solubilized DTX acceptors was demonstrated with this ^{125}I -DTX preparation (Mehraban *et al.*, 1985).

Analysis of the binding of ^{125}I -DTX to both cerebrocortical and hippocampal membranes by Scatchard (Figs. 2 and 3) and Hill plots (not shown) revealed the existence of high-affinity ($K_D = 0.3 \text{ nM}$) non-interacting acceptors that are homogeneous with respect to DTX binding. These neuronal acceptors appear to be located on the plasma membrane, since the ^{125}I -DTX binding is enriched 1.5-fold in synaptic membranes compared with that in synaptosomes (Mehraban *et al.*, 1984). The proteinaceous nature of the DTX acceptors was established by their sensitivity to protein-denaturing agents and proteolytic enzymes (Table 2). Note that incomplete inactivation of the acceptors in the membrane-bound state was produced by trypsin or chymotrypsin, whereas the same treatments abolished the DTX binding activity of solubilized acceptor (Mehraban *et al.*, 1985); this is indicative of the acceptor proteins being protected in the membrane. Although the inhibitory action of certain proteins exhibiting phospholipase A_2 activity (Figs. 4a and 4b) may reflect the occurrence of lipid moieties in

the DTX-binding component, this has been observed with acceptors for botulinum neurotoxin (Williams *et al.*, 1983) and probably reflects a conformational dependence of these binding components on their membrane environment.

The acceptors studied herein were found to be highly specific for DTX and its neurotoxic homologues (Table 1); no inhibition of DTX binding was seen with other neurotoxins or various ion-channel blockers (Figs. 4a and 4b; Table 3). It is unclear at present how these findings (Figs. 4a and 4b) relate to the pharmacological interactions of the facilitatory toxins with crotoxin or its subunit, seen at avian neuromuscular junction (Harvey & Karlsson, 1982). In view of the ability of toxin I (Othman *et al.*, 1982) and DTX (Othman, 1983) to inhibit the binding of ^3H - β -BuTX to rat brain synaptosomes, it is surprising that β -BuTX binds very weakly to the DTX-binding component ($K_I \sim 1 \mu\text{M}$ compared with K_D of 0.5 nM for ^3H - β -BuTX binding); apparently, the bulk of the DTX acceptors exhibit low affinity for β -BuTX under the ionic conditions used here. As the content of acceptors measured for ^{125}I -DTX is severalfold higher than that for ^3H - β -BuTX in rat brain (Othman *et al.*, 1982), the expected level of high-affinity inhibition by β -BuTX of DTX binding would be close to the resolution limit of the assay used; moreover, it could be partially masked by the inhibition seen with high β -BuTX concentrations (Fig. 4c). Although the predicted high-affinity interaction of both toxins with a small proportion of common acceptors was not detected in the present study, results obtained with solubilized rat brain acceptors (Mehraban *et al.*, 1985; Black, 1986) and chick synaptic membranes (Black & Dolly, 1986) indicate that this does occur. Heterogeneity of these acceptors is also supported by the somewhat different distributions observed for DTX and β -BuTX acceptors in rat brain regions (Dolly *et al.*, 1984; Othman *et al.*, 1983). Binding of β -BuTX to a class of sites common to DTX could explain facets of their functional interactions at peripheral (Harvey & Karlsson, 1982) and central (Nicholls *et al.*, 1985) nerve terminals; however, the existence (at least in brain) of another acceptor form that exhibits high affinity for DTX *only* remains to be reconciled with the interactions reported for the actions of these toxins at motor nerve endings of different species (cf. Chang, 1985).

The good correlation observed between the toxicities of several DTX homologues and their abilities to inhibit the binding of ^{125}I -DTX strongly suggests that binding to at least some of the acceptors identified here mediates their convulsant, facilitatory action. Further, the greater potency of toxin I relative to DTX in attenuating the A-current in hippocampal neurones (Halliwell *et al.*, 1986) accords with their respective affinities for the acceptors, suggesting that these acceptors are also implicated in K^+ -channel blockade. Studies on the purification and reconstitution of these acceptors are needed to establish the relationship of their different forms to voltage-sensitive K^+ channel(s).

Note added in proof (received 27 May 1986)

After removal of minor contaminants from the toxin E preparation by ion-exchange h.p.l.c., the resultant homogeneous protein exhibited low affinity ($K_I \sim 0.5 \mu\text{M}$) for DTX acceptors (see Table 1); its toxicity was not determined.

This work was supported by grants from the Wellcome Trust and the Medical Research Council (MRC); studentships were provided by the MRC (to A. R. B.), the Science and Engineering Research Council (to A. L. B.) and University of Malaya (to I. B. O.). We thank Mr. D. Green for performing the intracerebroventricular injections according to Home Office regulations.

REFERENCES

- Black, A. R. (1986) Ph.D. Thesis, University of London
- Black, A. R. & Dolly, J. O. (1986) *Eur. J. Biochem.* **156**, 609–617
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- Chang, C. C. (1985) *Procl. Natl. Sci. Coun. Repub. China, Part B* **9**, 126–142
- Docherty, R. J., Dolly, J. O., Halliwell, J. V. & Othman, I. B. (1983) *J. Physiol. (London)* **336**, 58P–59P
- Dolly, J. O., Halliwell, J. V., Black, J. D., Williams, R. S., Pelchen-Matthews, A., Breeze, A. L., Mehraban, F., Othman, I. B. & Black, A. R. (1984) *J. Physiol. (Paris)* **79**, 280–303
- Gustaffson, B., Galvan, M., Grafe, P. & Wigstrom, H. (1982) *Nature (London)* **229**, 252–254
- Halliwell, J. V., Othman, I. B., Pelchen-Matthews, A. & Dolly, J. O. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 493–497
- Harvey, A. L. & Karlsson, E. (1980) *Naunyn-Schmiedeberg's Arch. Pharmacol.* **312**, 1–6
- Harvey, A. L. & Karlsson, E. (1982) *Br. J. Pharmacol.* **77**, 153–161
- Harvey, A. L., Anderson, A. J. & Karlsson, E. (1984) *J. Physiol. (Paris)* **79**, 222–227
- Jones, D. H. & Matus, A. I. (1974) *Biochim. Biophys. Acta* **356**, 276–287
- Joubert, F. J. & Taljaard, N. (1980) *Hoppe-Seyler's Z. Physiol. Chem.* **361**, 661–674
- Kondo, K., Toda, H., Narita, K. & Lee, C.-Y. (1982) *J. Biochem. (Tokyo)* **91**, 1518–1530
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Mehraban, F., Breeze, A. L. & Dolly, J. O. (1984) *FEBS Lett.* **174**, 116–122
- Mehraban, F., Haines, A. & Dolly, J. O. (1986) *Neurochem. Int.* **9**, 613–623
- Mehraban, F., Black, A. R., Breeze, A. L., Green, D. G. & Dolly, J. O. (1985) *Biochem. Soc. Trans.* **13**, 507–508
- Nicholls, D. G., Snelling, R. & Dolly, J. O. (1985) *Biochem. J.* **229**, 653–662
- Othman, I. B. (1983) Ph.D. Thesis, University of London
- Othman, I. B., Spokes, J. W. & Dolly, J. O. (1982) *Eur. J. Biochem.* **128**, 267–276
- Othman, I. B., Wilkin, G. P. & Dolly, J. O. (1983) *Neurochem. Int.* **5**, 487–496
- Rehm, H. & Betz, H. (1982) *J. Biol. Chem.* **257**, 10015–10022
- Reisfeld, R. A., Lewis, U. J. & Williams, D. E. (1962) *Nature (London)* **195**, 281–283
- Romey, G. & Lazdunski, M. (1984) *Biochem. Biophys. Res. Commun.* **118**, 669–674
- Spokes, J. W. & Dolly, J. O. (1980) *Biochim. Biophys. Acta* **596**, 81–93
- Strong, P. N., Goerke, J., Oberg, S. G. & Kelly, R. B. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 178–182
- Williams, R. S., Tse, C. K., Dolly, J. O., Hambleton, P. & Melling, J. (1983) *Eur. J. Biochem.* **131**, 437–445

Received 28 August 1985/13 February 1986; accepted 19 February 1986