Dendrotoxin acceptor from bovine synaptic plasma membranes

Binding properties, purification and subunit composition of a putative constituent of certain voltage-activated K^+ channels

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Dendrotoxin is a snake polypeptide that blocks selectively and potently certain voltage-sensitive, fast-activating K^+ channels in the nervous system, where it binds with high affinity to to membranous acceptors. Herein, the acceptor protein for dendrotoxin in bovine synaptic membranes is solubilized in active form and its complete purification achieved by affinity chromatography, involving a novel elution procedure. This putative K^+ -channel constituent is shown to be a large oligomeric glycoprotein containing two major subunits, with M_r values of 75000 and 37000.

INTRODUCTION

Dendrotoxin (DTX) is a 7000- M_r polypeptide from the venom of the Eastern green mamba, *Dendroaspis angusticeps*, which inhibits selectively a family of voltage-sensitive, fast-activating, aminopyridine-sensitive K^+ channels in mammalian (Dolly *et al.*, 1984; Halliwell *et al.*, 1986; Penner *et al.*, 1986; Stansfeld *et al.*, 1987; Anderson & Harvey, 1988) and amphibian (Benoit & Dubois, 1986) neuronal preparations. This makes it a new and valuable tool for the characterization of these important K^+ channels. High-affinity binding sites for ^{125}I -labelled DTX (^{125}I -DTX) or its congener, toxin I, have been demonstrated in the central nervous system of rat (Black *et al.*, 1986; Pelchen-Matthews & Dolly, 1987; Rehm *et al.*, 1988) and chick (Black & Dolly, 1986). The K_D for an apparent single set of sites observed in rat brain synaptosomes seems to correlate most closely with its effect on a fast-activating, slow-inactivating, K^+ conductance studied in rat nodose A cells (Stansfeld *et al.*, 1987). The transient K^+ current (I_A) recorded in CA_1 neurones of rat and guinea pig hippocampus is attenuated by higher concentrations of DTX; such variation in sensitivity to DTX may reflect subtle molecular differences in the channels which are responsible for their different kinetics.

Several lines of evidence (Dolly, 1988) suggest a direct action of DTX on the channel protein(s): (i) it appears to bind to brain regions equivalent to sites where the K^+ channels are known to be inhibited; (ii) the correlation between the toxicity of various DTX congeners and their affinities for the DTX binding site; (iii) the M_r of the acceptor subunit cross-linked to the toxin in brain synaptosomes corresponds to the size of proteins predicted from cloned genes for A current K^+ channels; (iv) the close correlation between the K_D for ^{125}I -DTX binding to synaptosomal acceptors and its enhancement of transmitter release (Tibbs *et al.*, 1989); (v) patch-clamp studies consistent with a direct action of DTX on a K^+ channel in dorsal root ganglion cells (Stansfeld & Feltz, 1988).

In this study, the DTX acceptor from bovine brain is characterized for the first time and its properties, together

with those of DTX, are exploited in devising a successful purification protocol for this putative K^+ channel constituent. It is shown to be a hetero-oligomeric glycoprotein comprised of two polypeptides; moreover, the novel procedure described will allow routine isolation of the protein for structure/function studies.

EXPERIMENTAL

Materials

Sephacrose 4B and CNBr-activated Sepharose were purchased from Pharmacia and WGA was from Vector Laboratories. All other chemicals were of analytical grade. Acid-cleaned glassware and Milli-Q water (Millipore Corp.) were used for all experiments using purified DTX acceptor. DTX and toxin I from *D. polylepsis* were purified as described previously (Black *et al.*, 1986).

Solubilization of brain membranes

Synaptic plasma membranes were prepared (Bennett *et al.*, 1986) from bovine cortex, obtained immediately after slaughter, and resuspended (10 mg of protein $\cdot$ ml $^{-1}$) in extraction buffer [62.5 mM-imidazole/HCl (pH 7.4)/250 mM-KCl/2.5 mM-EDTA/soybean trypsin inhibitor (25 μ g $\cdot$ ml $^{-1}$)/bacitracin (50 μ g $\cdot$ ml $^{-1}$)/0.25 mM-benzamide/0.5 mM-PMSF], containing 4% (w/v) detergent. After gentle rocking at 4 °C for 1 h, the mixture was diluted 2.5-fold with cold de-ionized water and centrifuged at 30000 g for 20 min and then at 100000 g for 45 min at 4 °C; the supernatant from the latter (crude extract) was used.

Binding of DTX to its acceptor

Membrane-bound acceptor was assayed using ^{125}I -DTX and an oil centrifugation method as described (Black *et al.* 1986). Routinely, a rapid gel filtration assay was used for measuring binding to solubilized acceptors (Black *et al.*, 1988) using Sephadex G-100; 2 ml columns in 2 ml syringes were washed with 1 bed volume of column wash buffer [25 mM-imidazole/HCl (pH 7.4)/1 mM-EDTA/30 mM-KCl/0.2% (w/v) Triton X-100]

Abbreviations used: DTX, dendrotoxin; ^{125}I -DTX, ^{125}I -labelled DTX; WGA, wheat germ agglutinin; DTT, dithiothreitol; SDS/PAGE, SDS/polyacrylamide-gel electrophoresis; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate; PMSF, phenylmethanesulphonyl fluoride.

and centrifuged for 2 min at 100 *g*. An aliquot (200 μ l) of the incubation mixture was then carefully applied to the columns before recentrifugation as above. The toxin-acceptor complex was collected in the void volume and quantified by γ -radiation counting. Where stated, the method of Bruns *et al.* (1983) was used, employing GF/C filter discs (Whatman) soaked in 0.3% (w/v) poly(ethyleneimine) in column wash buffer. In all cases, total incubation volume was 250 μ l with the final buffer consisting of 15 mM-imidazole/HCl (pH 7.4)/0.6 mM-EDTA/60 mM-KCl/0.1–1% detergent/3 nM- 125 I-DTX/cytochrome *c* (100 μ g·ml $^{-1}$). For measurement of non-displaceable binding, incubations also contained 500 nM unlabelled DTX. K_D and B_{max} values were determined using LIGAND, a non-linear curve fitting method (Munson & Rodbard, 1980) on an Apple IIe computer.

Coupling of toxin I to Sepharose

Sepharose 4B was activated by the method of Kohn & Wilchek (1982) using 0.8 mg of CNBr·(ml of settled resin) $^{-1}$; measurement of the active species on the resin gave values of 0.35–0.60 μ mol of cyanate esters·(ml of settled gel) $^{-1}$. After washing to remove excess CNBr and acetone, the resin was resuspended with 2 vol. of coupling buffer [50 mM-triethanolamine/HCl (pH 7.8)/0.5 M-NaCl] and reacted with 0.3 mg of toxin I·(ml of resin) $^{-1}$ for 2 h at room temperature; the resin was then briefly centrifuged and the A_{280} of the supernatant measured to determine the amount of toxin I coupled (generally 50–80%). Any free reactive groups remaining were blocked with 0.5 M-ethanolamine/HCl, pH 8.5, for 4 h at room temperature; the resin was washed sequentially on a sintered glass funnel with 1 litre of coupling buffer, 0.1 M-sodium acetate buffer (pH 5.0)/1 M-NaCl, water and 0.1 M-Tris/HCl (pH 8.5)/1 M-NaCl. The resin was resuspended in storage buffer [0.1 M-sodium acetate (pH 6.0)/0.5 M-NaCl/0.05% (w/v) NaN $_3$]. WGA was coupled to CNBr-activated Sepharose (Pharmacia) at 5 mg·(ml of resin) $^{-1}$ using the manufacturer's instructions.

Affinity chromatography

The typical protocol used was as follows: 750 mg of membrane protein was solubilized in 4% (w/v) Lubrol PX as described above. After dilution and centrifugation, ~215 ml (~140 pmol of 125 I-DTX binding) was loaded at a flow rate of 30–40 ml·h $^{-1}$ onto a 9.5 ml column (1.5 cm $\times$ 5.5 cm) of toxin I-Sepharose equilibrated in 25 mM-imidazole/HCl (pH 8.2)/100 mM-KCl/0.2 mM-benzamidine/0.2% (w/v) Lubrol PX/0.5 mM-PMSF (buffer A). The column was then washed with 200 ml of buffer A at 100 ml·h $^{-1}$ and eluted at 5 ml·h $^{-1}$ by the addition of 10 mM-DTT. An aliquot of each fraction (2.5 ml) collected was diluted once with buffer A, and DTT was removed by the centrifugation/gel filtration method prior to assay of 125 I-DTX binding. A portion (0.2 ml) of each fraction was also precipitated for SDS/PAGE analysis by the method of Wessel & Flügge (1984). The peak fractions of acceptor activity were pooled and loaded at 5 ml·h $^{-1}$ onto a 5 ml column (1.5 cm $\times$ 3 cm) of WGA-Sepharose equilibrated in buffer A. This column was washed at 100 ml·h $^{-1}$ with 100 ml of buffer A, followed by 100 ml of buffer A/0.5 M-NaCl and finally 100 ml of buffer A before eluting with 10 mM-*N*-acetylglucosamine in buffer A at 20 ml·h $^{-1}$; 2 ml fractions were collected and, after aliquots were taken for 125 I-DTX binding assay, each

fraction was lyophilized and resuspended in 100 μ l of water before precipitation as above.

Other procedures

SDS/PAGE was carried out (Laemmli, 1970) in 8% slab gels (12 cm $\times$ 12 cm, 2 mm thick) under reducing conditions; the gels were stained by the method of Bürk *et al.* (1983). Protein was determined (Markwell *et al.* (1978), using bovine serum albumin as standard.

RESULTS

Detergent extraction of DTX acceptor from bovine synaptic plasma membranes

As the acceptor is an integral membrane protein (Black *et al.*, 1988), its solubilization in biologically active form required extraction of the plasma membranes with non-ionic or zwitterionic detergents. Fig. 1 shows a saturation curve constructed using the data derived from an equilibrium binding experiment with 125 I-DTX using a Lubrol PX membrane extract. The 'LIGAND' program fits the data to a single set of sites with a K_D of 0.08 nM and B_{max} of 0.31 pmol·(mg of protein) $^{-1}$. 125 I-DTX binding to the membranes, measured under the same conditions, gave similar values of K_D (0.1 nM) and B_{max} [(0.45 pmol·(mg of protein) $^{-1}$]. In initial studies, Triton X-100 was used at an optimal concentration of 4% (w/v) (equivalent to a detergent:phospholipid molar ratio of 9). However, the half-life of the acceptor activity in this medium is only 20–30 h; as this is impractical for lengthy procedures (e.g. purification and reconstitution), several other detergents were evaluated. Nonidet P40, CHAPS and Lubrol PX all extracted the acceptor with similar efficacy to Triton X-100, whereas

Fig. 1. Binding of 125 I-DTX to solubilized bovine synaptic plasma membranes

A 4% (w/v) Lubrol PX extract was incubated with varying amounts of 125 I-DTX and the bound ligand measured using the centrifugation/column assay (see the Experimental section). Data were fitted to the best line using 'LIGAND', a non-linear regression program. ● and ○ are data points for specific and non-displaceable binding (measured in the presence of 500 nM unlabelled DTX), respectively.

Tween 80 or 20 proved ineffective. Most importantly, CHAPS and Lubrol PX increased significantly the stability of the activity to a satisfactory level (half-life at 4 °C > 7 days). As observed for a Triton X-100 extract of rat brain membranes (Black *et al.*, 1988) recovery of solubilized acceptor is critically dependent on K⁺ or Rb⁺; Cs⁺ is less effective whilst Na⁺ or Li⁺ are unable to serve this role (Table 1). Interestingly, activity is not regained by exchange of Na⁺ with K⁺ after extraction in the former. Despite being a blocker of DTX-sensitive K⁺ channels (see the Introduction), 4-aminopyridine was unable to substitute for K⁺ in stabilizing the toxin binding site (Table 1); furthermore, it has no effect on ¹²⁵I-DTX binding to rat synaptosomes (Black *et al.*, 1986) or bovine solubilized or membrane-bound acceptors. It is noteworthy that when the gel filtration columns used for the assay are equilibrated with buffer containing 1 M-KCl or -NaCl, DTX binding is reduced by > 90%; this finding was confirmed using a filtration disc assay (see the Experimental section) and establishes that the inhibition is not specific to K⁺, as indicated for the binding of ¹²⁵I-labelled toxin I (Rehm & Lazdunski, 1988).

Effect of reducing and oxidizing agents on ¹²⁵I-DTX binding to its acceptor

Addition of low concentrations (< 10 mM) of DTT to the incubation mixture abolished the binding of ¹²⁵I-DTX to both membrane-bound and solubilized acceptor, consistent (see below) with the lability of the disulphides present in toxin I (Hollecker & Creighton, 1983) and DTX. Also, inclusion of 30 mM-GSSG inhibited completely ¹²⁵I-DTX binding in the membrane-bound and solubilized states. However, if the DTT or GSSG were removed by dialysis from the solubilized acceptor, activity was restored to the control value. This shows that any loss of activity due to modification of the acceptor itself is reversible, a property to be used in developing a biospecific elution of acceptor from immobilized toxin I.

Purification of the DTX acceptor by affinity chromatography on toxin I- and WGA-containing gels

Evaluation of a number of purification methods including chromatography on Sepharose 6B, DEAE-Sepharose or phenyl-Sepharose gave, at best, 5–10-fold enrichment of the acceptor. Thus, affinity chromatography was seen as the best prospect. Toxin I was selected as the ligand of choice due to its higher affinity for the acceptor (Black *et al.*, 1986). Initial experiments using AH-Sepharose, with coupling through carboxyl groups, were unsuccessful; CNBr-activation of Sepharose 4B was, therefore, performed. Coupling of toxin I to resin with a low extent of activation (detailed in the Experimental section) proved most successful for acceptor purification.

Despite using such an improved affinity resin, elution with high concentrations of K⁺ yielded a complex polypeptide pattern on SDS/PAGE (results not shown). To improve elution, it occurred to us that the total inhibition of ¹²⁵I-DTX binding produced by DTT could be exploited. The optimized conditions for such biospecific elution are summarized in the Experimental section. The binding capacity of the toxin I-Sepharose used routinely was 70 pmol of acceptor per mg of toxin I on the resin; > 80% of toxin binding normally became adsorbed

Table 1. Effect of cations and 4-aminopyridine on the recovery of ¹²⁵I-DTX binding in detergent extracts of bovine synaptic plasma membranes

Extraction of membranes with 4% (w/v) Triton X-100 and measurement of ¹²⁵I-DTX binding were carried out as detailed in the Experimental section, except cations (as chloride salts) or 4-aminopyridine were included as indicated.

Addition	Concentration (mM)	Relative specific binding of ¹²⁵ I-DTX (% ± S.D.)
K ⁺	250	100* ± 2.9
K ⁺	100	89 ± 5.0
4-Aminopyridine	100	12 ± 4.2
Rb ⁺	250	105 ± 9.6
Cs ⁺	250	35 ± 14
Na ⁺	250	0
Li ⁺	250	0

* All the data are expressed relative to this value.

while > 95% of the protein passed through. Biospecific adsorption of acceptor to this gel was demonstrated because binding activity was not retained on a column coupled similarly to toxin B, a biologically inactive but structurally very similar homologue of toxin I (Black *et al.*, 1986). The presence of DTT and detergent precluded the direct measurement of the low amounts of protein present in the eluate fractions; therefore, purification was monitored by SDS/PAGE and silver staining. Results of three typical experiments with toxin I-Sepharose are shown in Fig. 2. In contrast to the numerous bands observed in the crude extract, only two major polypeptides were seen in the DTT eluate, with *M_r* values of 75000 and 37000. When SDS/PAGE was carried out under non-reducing conditions, the same pattern was seen (not shown). In addition to background bands seen in the buffer blank, some artefactual material is normally revealed near the ion front (Fig. 2b) by the high sensitivity of the silver staining method, particularly when concentrated samples are used. Some minor contaminants were also visible; however, the relative intensity of only the two major bands correlated with the amounts of ¹²⁵I-DTX binding detected therein. Recovery in the experiments shown was ~ 10%, which may be an underestimate (see below). As some extraneous bands present did not correspond to acceptor activity, an additional affinity purification step on a lectin resin was employed. All of the material applied adsorbed to the WGA-Sepharose column, showing the protein to be glycosylated and to contain neuraminic acid or *N*-acetylglucosamine residues. Neither *Ricinus communis* agglutinin I-Sepharose or concanavalin A-Sepharose bound the acceptor; this shows that it is devoid of galactose and methyl D-mannopyranoside residues, respectively (see Lotan & Nicholson, 1979). The eluate from the WGA column (Fig. 2b) shows essentially the same pattern as that seen in the toxin I eluate (including similar artefacts at top and bottom of the gel) except for the removal of minor contaminants, particularly a band of *M_r* 72000, which appeared in the breakthrough of this column. The recovery from the WGA-Sepharose

Fig. 2. Purification of DTX acceptor from bovine synaptic membranes using toxin I-Sepharose and WGA-Sepharose

(a) SDS/PAGE of two separate preparations of purified acceptor using toxin I-Sepharose only (see the Experimental section): c, crude extract; bt, breakthrough of toxin I-Sepharose column; st, Pharmacia low- M_r standards (thyroglobulin, M_r 94000; bovine serum albumin, 67000; ovalbumin, 43000); F1₁–F4₁, fractions of the DTT eluate of toxin I-Sepharose column (one experiment); F1₂–F2₂ (right-hand side) toxin I-Sepharose eluate fractions (second experiments). Note the artefact bands present in the buffer blank (b). The ^{125}I -DTX binding in each fraction, relative to that in the peak of each of two separate preparations, is shown underneath the gels. (b) A typical experiment in which the DTT eluate from the toxin I column was further purified by lectin affinity chromatography as in the Experimental section; b, buffer blank; c, crude extract; txl (e), DTT eluate from toxin I column (pooled peak); wga(bt), breakthrough from the WGA-Sepharose column; wga(e), sugar eluate from the WGA-Sepharose column (pooled fraction).

column was consistently $> 90\%$, which indicates some loss of activity when removing DTT from the toxin I-Sepharose eluate prior to assay. The overall recovery of acceptor activity was $\sim 10\%$ relative to the crude extract.

DISCUSSION

The DTX acceptor has been characterized in bovine cerebral cortex, which is a rich and readily available tissue source for large scale purification. When the bovine membranes were assayed in the buffer used for rat synaptic plasma membranes (Black *et al.*, 1986), a similar K_D (0.4 nM) and $B_{\max}$ (1.0 pmol $\cdot$ mg $^{-1}$) were obtained. Although the specific activity (~ 0.3 pmol $\cdot$ mg $^{-1}$) and the recovery (40%) of the acceptor in the Lubrol PX extract were at reasonable levels and similar to the values reported recently for toxin I binding to a detergent extract of rat cortex (Rehm & Lazdunski, 1988), some inactivation could have occurred or another acceptor form may exist that was not extracted under the conditions used. The acceptor is very stable in Lubrol PX, unlike the case for Triton X-100; although acceptor stability in the latter can be improved by inclusion of 10% (w/v) glycerol, propylene glycol or sucrose, the viscosity of the mixture is increased. There is an absolute requirement for K^+ during solubilization for maintenance of acceptor activity, indicating the presence of a binding site for K^+ on the acceptor. The contrasting non-specific inhibitory effect of high salt concentrations on DTX binding is probably due to interference with electrostatic interaction between the basic toxin and acidic acceptor protein. The basis of the abolition of toxin binding by reduction is not clear, as experiments to date have not distinguished between underlying effects on the acceptor

protein, DTX or both. Oxidized glutathione, though, must act on the acceptor, since the toxin has no free SH groups (Hollecker & Creighton, 1983); formation of a mixed disulphide in the toxin binding site of the acceptor is, therefore, implicated.

In the affinity chromatography, specific adsorption of acceptor to a toxin I-Sepharose column was optimal when the degree of activation is kept low (~ 25 -fold lower than that on a CNBr-activated resin from Pharmacia); this presumably minimizes multi-point attachments between resin and toxin. Elution of the gel with high concentrations of K^+ was not satisfactory, giving numerous bands on SDS/PAGE, as observed by Rehm & Lazdunski (1988). An improved procedure was, thus, essential for routine and reproducible purification. Fortunately, the DTT susceptibility of ^{125}I -DTX binding offered the necessary improvement, with near complete purification (in six experiments) being achieved in one simple step. This contrasts with the recently reported protocol for the rat brain protein (Rehm & Lazdunski, 1988) where suboptimal affinity chromatography necessitated the inclusion of two additional, laborious steps (ion-exchange chromatography and sucrose density gradient centrifugation).

Notwithstanding these difficulties, the subunit pattern obtained by this method for the rat acceptor is very similar to that reported herein for bovine cortex. The diffuseness of the 75000- M_r band reveals micro-heterogeneity that could result from variable degrees of glycosylation or, possibly, proteolysis. Alternatively, multiple protein species with M_r in the region of 75000 might have arisen from subtypes of the acceptor known to exist in brain (Dolly, 1988). Interestingly, the relative mobility of the broad band approximates to that of the

polypeptide identified by cross-linking of toxin to its acceptor in rat (Mehraban *et al.*, 1984; Rehm *et al.*, 1988), chick (Black & Dolly, 1986), and bovine (results not shown) membranes; moreover, it corresponds in size to that of the protein predicted from gene cloning of the A-type K⁺ channel from *Drosophila* (Tempel *et al.*, 1987), and mammalian brain (Tempel *et al.*, 1988; Baumann *et al.*, 1988). Indeed, it is tempting to relate the several 'isoforms' of the sequences from *Drosophila* to the heterogeneity of the 75000-*M_r* protein alluded to above, and to the multiple and distinct binding sites (Schmidt *et al.*, 1988; Bidard *et al.*, 1987; Breeze & Dolly, 1988) on the acceptor(s) for DTX, β -bungarotoxin and mast cell degranulating peptide. In this context, it must be emphasized that there is no evidence for the presence of a subunit larger than *M_r* 75000 in the acceptor isolated from bovine (Fig. 2) or rat (Rehm & Lazdunski, 1988) cortex; this is curious because β -bungarotoxin binds to a subpopulation of DTX acceptors (Black & Dolly, 1986; Breeze & Dolly, 1988) and has been cross-linked to a 95000-*M_r* protein in chick brain (Rehm & Betz, 1983).

It is difficult to exclude that the 37000-*M_r* polypeptide arises from proteolysis of the 75000-*M_r* protein or from fragmentation of a 42000-*M_r* band which appeared in variable and trace amounts in the purified acceptor (Fig. 2). However, both the sharpness and intensity of the 37000-*M_r* band on SDS/PAGE, together with the use of various protease inhibitors throughout, argues against proteolysis. On the reasonable assumption, therefore, that this low-*M_r* protein is part of the acceptor/putative K⁺ channel, interesting parallels can be drawn with the Na⁺ channel which also has distinct sites for binding several toxins (reviewed by Catterall, 1988). Its two β -subunits are of similar size to the 37000-*M_r* polypeptide, whilst the large glycosylated α chain contains four repeat domains which could be equivalent to multiple 75000-*M_r* subunits in the DTX binding protein. Such a postulated model (Dolly, 1988) is supported by the large oligomeric size of the bovine acceptor, as demonstrated by its similar behaviour to the rat brain protein (Black *et al.*, 1988) on sedimentation analysis and gel filtration. It is also noteworthy that expression of mRNA for one form of the large chain of either protein (Catterall, 1988; Timpe *et al.*, 1988) produces the requisite membrane conductance, though creation of currents identical to those produced by native channels (Iversen *et al.*, 1988) will be likely to involve other chains.

Experiments are under way to show directly that the DTX acceptor oligomer(s), containing the two identified non-covalently linked subunits, can function as fast-activating, voltage-sensitive K⁺ channel(s). As these are likely to be successful, it will then be imperative to determine the combinations of 75000-*M_r* species and the smaller subunit that give rise to the variants of this family of K⁺ currents found in neuronal preparations.

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