Identification by cross-linking of a neuronal acceptor protein for dendrotoxin, a convulsant polypeptide

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Dendrotoxin, a lijow molecular weight protein from the venom of *Dendroaspis angusticeps*, is known to be a potent convulsant that attenuates one type of voltage-sensitive K^+ channel in guinea-pig hippocampus. A biologically active preparation of ^{125}I-labelled dendrotoxin has been cross-linked to its high-affinity protein acceptor in synaptic plasma membranes from rat cerebral cortex. On SDS gel electrophoresis, a complex with a M_r of 72,000 was observed which, assuming one toxin molecule is attached, yields an apparent size of 65,000 for this subunit of the acceptor. Unlike dendrotoxin, low concentrations of β-bungarotoxin, another pre-synaptically acitve toxin, do not inhibit its labelling.

Dendrotoxin *Neuronal acceptor* *Neurotransmitter release* *Cross-linking*

1. INTRODUCTION

Neurotransmission in the nervous system is mediated by movement of ions through a network of channels, a process which governs the excitability of nerve cells. With the exception of voltage-sensitive channels for Na^+ [1] and to a lesser extent those for Ca^{2+} [2], little information is available on the molecular properties of the several other varieties known to exist. Fortunately, a number of neurotoxins have been identified that selectively perturb the operation of certain types of ion channels [1,3,4] and which are proving to be useful probes for these functional macromolecules. In this study, dendrotoxin (DTX), a protein isolated from the venom of the green mamba *Dendroaspis angusticeps*, which can modulate one type of voltage-sensitive K^+ channel [4] was used to identify the neuronal membrane component with which it interacts.

DTX is a single-chain polypeptide with a molecular mass of 7 kDa [5] and has extensive sequence homology with the B-chain of another presynaptically-active toxin, β-bungarotoxin (β-BuTX) [6]. Although DTX has relatively low toxicity peripherally (mouse lethal dose $>500\ \mu g$), at the neuromuscular junction of chick biventer cervicis muscle it facilitates the release of acetylcholine and is antagonistic to the action of β-BuTX thereon [5]; likewise, evidence for pharmacological antagonism between β-BuTX and toxin I, a homologue of DTX from *D. polylepis*, was obtained from toxicity tests in mice [7]. DTX is much more lethal when administered centrally [8], having a profound convulsant effect owing to its potentiation of the release of transmitter, as shown electrophysiologically in hippocampal slices in vitro [4,9]. From recent voltage-clamp experiments, it appears that this facilitatory action is mediated through inhibition of the A-current [4], an outward K^+ conductance contributed by a voltage-sensitive K^+ channel [10]. Consistent with this specific and novel action, saturable binding sites for ^{125}I-labelled DTX have been detected in discrete areas of rat hippocampus and other brain regions [4].

Here, we report that a protein component of M_r ~65000 on synaptosomal membranes, exhibiting high affinity for DTX, can be chemically cross-linked to ^{125}I-labelled DTX. This facilitatory neurotoxin is thus shown for the first time to be an invaluable neurochemical probe for a protein that

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is likely to comprise, or at least regulate, one type of K^+ channel.

2. MATERIALS AND METHODS

2.1. *Preparation of DTX*

The venom of *D. angusticeps* was obtained from Sigma and DTX was prepared as in [11], its homogeneity being ascertained by SDS-polyacrylamide gel electrophoresis (SDS–PAGE), on slab gels of 15% acrylamide, and by isoelectric focussing (pH 9–11) in gels of 5% acrylamide. The biological activity of the preparation was demonstrated by electrophysiological recording of its facilitatory action on neurotransmission in hippocampal slices [9]; in addition, its toxicity was checked by intraventricular injection into rat brain (lethal dose of 2.5 ng/g body wt) [8].

2.2. *Radioiodination of DTX*

DTX was radiolabelled with ^{125}I by a modification of the chloramine-T method [4] and was stored in the presence of 0.2% ethanol at 4°C. The specific activity of the ^{125}I-iodinated DTX (^{125}I-DTX) was determined by accurate measurement of protein content [7], using unlabelled DTX as standard, and incorporated radioactivity; it was found to be 60–120 Ci/mmol for different batches. ^{125}I-DTX exhibited a level of neurotoxicity comparable [4] to that of native DTX.

2.3. *Synaptosomal preparations*

Synaptosomes were prepared from the cerebral cortex of rats (200–250 g) by density gradient centrifugation [7] and freeze-thawed prior to resuspension in Krebs-phosphate medium at 5–6 mg protein/ml. Synaptic plasma membranes were made as in [12]; this involved lysing the crude mitochondrial pellet (P2) in 5 mM Tris–HCl buffer (pH 8.0) for 40 min at 5°C and isolating the membranes by flotation/sedimentation, using a 3-step sucrose gradient.

2.4. *Measurement of ^{125}I-DTX binding to synaptosomes*

Synaptosomal suspensions (0.7–0.8 mg protein/ml) in Krebs-phosphate medium (pH 7.4) were incubated for 45 min at 23°C in the presence of various concentrations (0.1–10 nM) of ^{125}I-DTX (250 μl final volume). Aliquots (200 μl) of the suspension were then removed and pipetted on top of 200 μl of a 45% (v/v) solution of dinonyl phthalate in Dow Corning MS-550 silicone fluid (oil). Bound ^{125}I-DTX was separated from the free toxin by centrifugation at 8500 × *g* for 2 min. Aliquots (100 μl) were removed from the aqueous phase above the oil for determination of the free ^{125}I-DTX concentration at equilibrium. The remainder of the aqueous layer was then aspirated and the tubes were washed with 0.5 ml Krebs-phosphate buffer; the radioactivity remaining in the pellet was measured by γ-counting to determine the quantity of bound ^{125}I-DTX. Non-specific binding was measured similarly in the presence of a 100-fold molar excess of DTX. For determination of protein concentrations [7] in these assays, triplicate synaptosomal suspensions were treated similarly to the test samples except for the omission of the oil in the centrifugation steps; the supernatant was aspirated and the pellet dissolved in 0.45 M NaOH.

2.5. *Cross-linking of ^{125}I-DTX to synaptic membranes*

Suspensions of synaptic plasma membranes (1.5–2 mg, 120 μl), in Krebs phosphate buffer (pH 7.4) containing 1 mg/ml bovine serum albumin, were incubated with ^{125}I-DTX (2.5–10 nM final concentration) for 1 h at 23°C. Control samples were preincubated similarly but with the inclusion of excess DTX (1 μM unless otherwise stated). The suspensions were centrifuged at 8500 × *g* for 2 min and the pellets washed at 4°C, once with phosphate-buffered saline (25 mM, pH 7.2) and twice with triethanolamine·HCl buffer (0.25 M, pH 8.5); finally, the samples were resuspended in 1 ml of the latter buffer containing a series of concentrations (0.05–5 mg/ml) of dimethyl suberimidate (DMS) and incubated at 23°C for 2 h. After centrifugation, the pellets were washed with triethanolamine buffer (1 ml, twice) and suspended in 50 mM sodium phosphate buffer (pH 8.0) containing protease inhibitors (1 mM EDTA, 0.1 mM benzamidine, 0.5 mM phenylmethylsulphonyl fluoride, 10 μg/ml soybean trypsin inhibitor, 5 μg/ml bacitracin, 2 μg/ml leupeptin) and 1% deoxycholate. The pellets were solubilised at 4°C overnight, centrifuged at 8500 × *g* for 5 min or 200000 × *g* for 30 min and the supernatant was subjected to gel electrophoresis; the residual par-

ticulate fraction was further solubilised in 2% SDS/5% β-mercaptoethanol and analysed similarly.

2.6. *Other methods*

SDS-polyacrylamide gel electrophoresis (SDS–PAGE) was performed [4] on slab gels of 8% acrylamide. Protein content of membrane samples was determined by the Folin Lowry method, with bovine serum albumin as standard [7].

3. RESULTS AND DISCUSSION

A biologically active preparation [4,8] of ^{125}I-DTX, labelled to high levels of specific radioactivity, was found to bind saturably to a single set of non-interacting sites on freeze-thawed synaptosomes (fig.1 inset) or synaptic membranes from rat cerebral cortex. Scatchard plots of the binding data (fig.1) revealed a site content of 1–1.2 pmol/mg protein and a K_d of 0.43 nM (SD ± 0.07; $n = 4$); a Hill plot of the same data yielded a slope of unity. Heat treatment of synaptosomes or digestion with proteolytic enzymes abolished the binding of ^{125}I-DTX, indicating that its acceptor contains a proteinaceous component [4].

Further characterization of this acceptor was achieved by covalently cross-linking it to ^{125}I-DTX. Under the conditions of labelling, the extent of specific DTX binding was high (70–90%); after treatment of these labelled membranes with DMS, practically all the bound radioactivity remained associated with the membrane pellet following overnight washing at 4°C. Extraction of the labelled, washed membranes with deoxycholate follow-

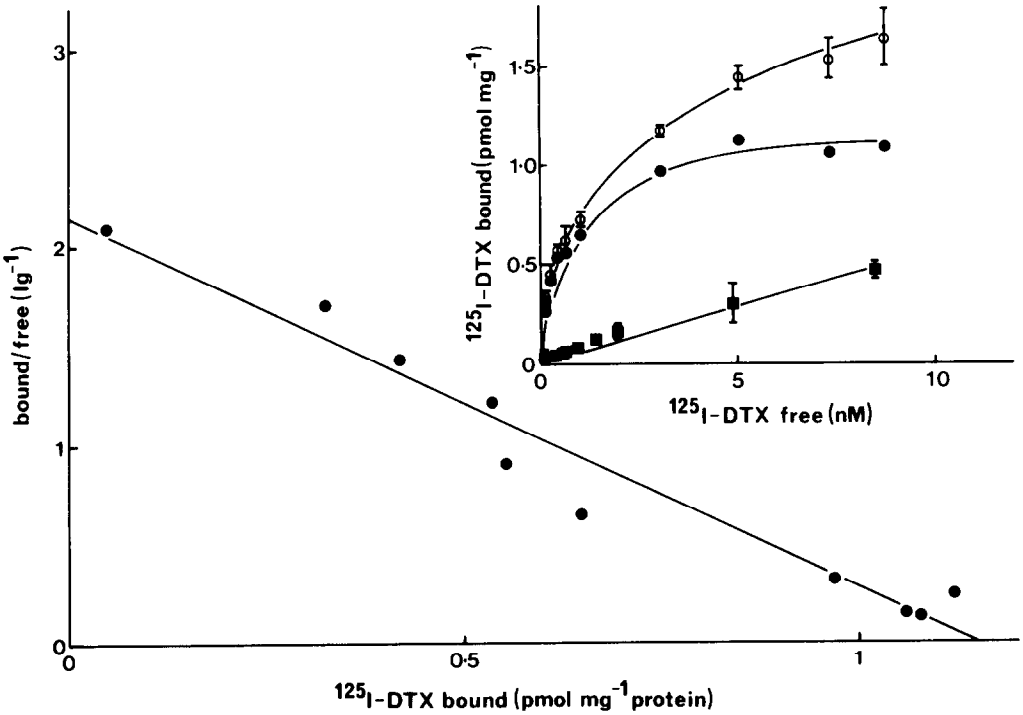
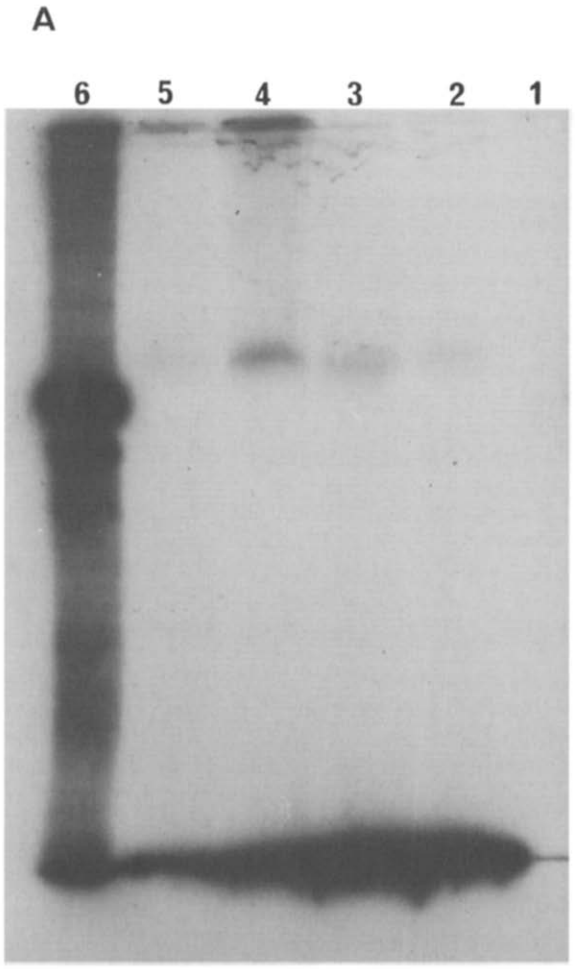
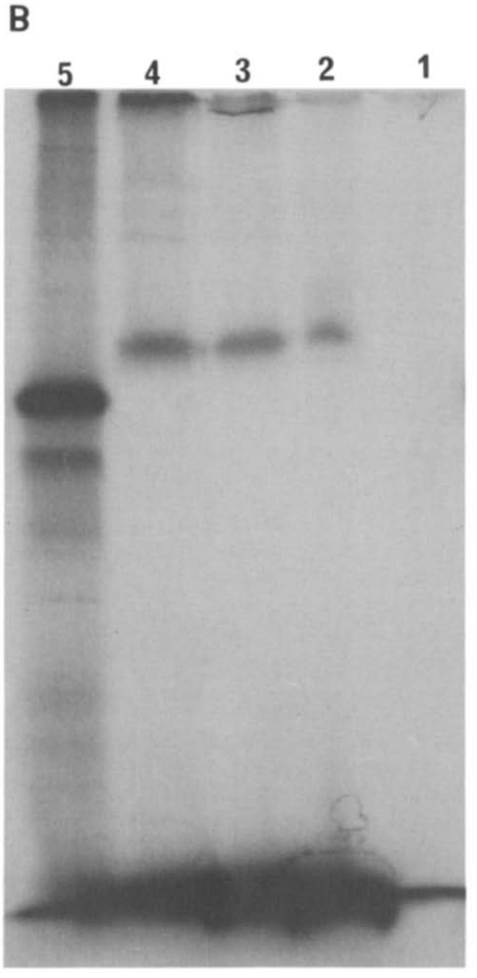


Fig.1. Saturable binding of ^{125}I-DTX to synaptosomes from rat brain. Synaptosome suspensions (0.7–0.8 mg protein/ml) in Krebs phosphate medium were incubated with ^{125}I-DTX (0.1–10 nM) for 45 min at 23°C. Aliquots of the suspension were then removed for the determination of free and bound radioactivity as described in section 2. Non-specific binding was determined in the presence of a 100-fold molar excess of DTX. A Scatchard plot of the specific binding of ^{125}I-DTX is presented; the insert shows the saturation curve for the same experiment. Specific binding (●) was calculated as the difference between total binding (○) and non-specific binding (■) at equilibrium. Average values are plotted for duplicate determinations. Similar binding data were obtained with purified synaptic plasma membranes.

ed by electrophoresis and autoradiography resulted in the appearance of a polypeptide band of M_r ~72000 (fig.2A, tracks 2–5; B, tracks 2–4). This band was absent from control samples labelled in the presence of excess DTX (fig.2A,B, track 1), indicating that the cross-linked protein is involved in the saturable binding of ^{125}I-DTX. The same polypeptide was labelled using a range of concentrations of ^{125}I-DTX (2.5–10 nM) and DMS (0.05–5 mg/ml). Analysis of the residual radioactivity in the detergent-extracted membranes (the extent of this varied with the DMS concentration used; with up to 1 mg/ml, it represented ~50% of the total) following dissolution of the pellet in SDS sample buffer containing β-mercaptoethanol, gave a similar electrophoretic pattern. Likewise, when a different bifunctional reagent, dimethyl pimilimidate, was used under the same conditions a polypeptide of identical M_r was labelled specifically (not shown).

Under all the experimental conditions employed, a considerable quantity of specifically bound ^{125}I-DTX was seen to migrate near the ion front (fig.2A,B). This low molecular mass band could not be attributed to free ^{125}I-DTX alone since insignificant amounts of radioactivity (less than 5% of the total) were removed on extensive washing (2 h at 23°C) of the cross-linked membranes, indicating that virtually all the toxin became irreversibly attached; dissociation from untreated membranes is, in contrast, relatively fast ($t_{1/2}$ ~8 min at 23°C). Rather, this band is believed to represent ^{125}I-DTX which has become specifically cross-linked to low molecular mass material present in





the membranes (e.g., lipid). Similar observations have been made in cross-linking studies on voltage-sensitive Na^+ [1,13,14] or Ca^{2+}-activated K^+ channels [15] and by authors in [16] who cross-linked ^{125}I-β-BuTX to its acceptor in chick brain membranes. Since the specific binding of ^{125}I-DTX is abolished completely by proteases and heating [4], the possibility of binding being solely to lipid moieties is unlikely; however, it is envisaged that when ^{125}I-DTX is bound specifically to its protein acceptor, neighbouring lipid could become linked covalently to it. Consistent with this proposal, such labelling would be inhibited by excess DTX, as found experimentally (fig.2A,B). In view of the sensitivity of ^{125}I-DTX binding to phospholipolysis of the membrane (e.g., by bee venom phospholipase A_2) [4], it is plausible that lipid interaction is important in maintaining the acceptor in an active conformation. Such a role for lipid has been implicated also in the specific binding to neuronal membranes of β-BuTX [4,7] and botulinum neurotoxin [17].

An appreciable amount of specifically bound radioactivity failed to enter the gel, particularly when high DMS concentrations were used (fig.2A,B); hence, this high molecular mass material may have arisen from extensive cross-linking of the DTX oligomer. Radiation inactivation of the DTX binding component in synaptosomal membranes [4] has indicated that in the membrane-bound state this acceptor has a large molecular size ($M_r > 200000$); a similar conclusion was reached when deoxycholate extracts of synaptosomal membranes, cross-linked to ^{125}I-DTX, were subjected to gel filtration (unpublished).

Treatment of unlabelled membranes with

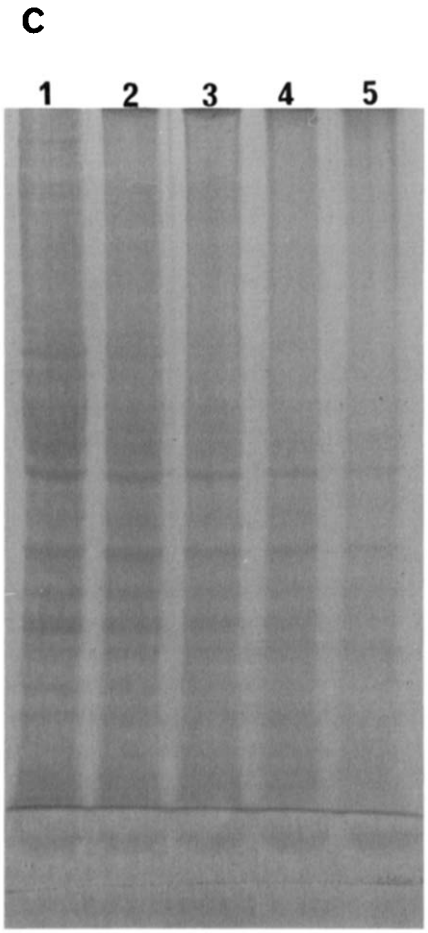


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Fig.2. Autoradiogram of SDS-polyacrylamide gels of membranes cross-linked to ^{125}I-DTX. (A) Synaptic membranes were reacted with 7 nM ^{125}I-DTX in the absence (tracks 2–5) and presence (track 1) of 1 μM DTX, as described in section 2. After washing, the samples were cross-linked with DMS, washed and solubilised in 50 mM phosphate buffer (pH 8.0) containing anti-proteases and 1% deoxycholate. They were centrifuged at 8500 × g for 5 min and the supernatants subjected to SDS–PAGE under reducing conditions on a polyacrylamide slab gel which was subsequently processed for autoradiography. The concentrations of DMS (mg/ml) used for cross-linking were: track 2, 0.05; 3, 0.1; 4, 2; 1 and 5, 5. (B) As in A except the membranes were labelled with 5 nM ^{125}I-DTX. The concentrations of DMS (mg/ml) used were: track 2, 0.25; 3, 0.5; 4, 1. As in A, a control sample was reacted with 1 mg/ml DMS (track 1). In both A (track 6) and B (track 5) standard proteins labelled with ^{125}I by the chloramine-T reaction were electrophoresed: these were thyroglobulin, M_r 330000; ferritin (half unit), M_r 220000; phosphorylase b, M_r 94000; bovine serum albumin, M_r 67000; catalase, M_r 60000; ovalbumin, M_r 43000; lactate dehydrogenase, M_r 36000. (C) SDS–PAGE of total protein (SDS extract) from brain membranes stained with Coomassie blue before (track 1) and after cross-linking as above with varying concentrations of DMS (mg/ml): track 2, 0.1; 3, 0.25; 4, 0.5; 5, 1.

relatively high concentrations (0.5–1.0 mg/ml) of DMS, followed by SDS–PAGE and staining with Coomassie blue, showed a change in the proportions of certain bands relative to those observed with non-crosslinked samples (fig.2C, tracks 1,4,5). However, at lower DMS concentrations (<0.5 mg/ml) no such changes were apparent (fig.2C, tracks 2,3) yet with this amount of cross-linking agent significant incorporation of ^{125}I-DTX into the 72-kDa band was obtained (fig.2A, tracks 2,3; B, track 2). In fact, this was the smallest protein band seen at any concentration of DMS tested, indicating that it is the minimal size of protein that can be cross-linked to DTX under the available experimental conditions. Therefore, it is unlikely that the 72-kDa component results from attachment of the DTX-acceptor subunit to other membrane polypeptides during the cross-linking reaction. Omission of reducing agent from the SDS gels (not shown) failed to produce an increase in the M_r of this labelled protein, demonstrating that it is not attached through disulphide bond(s) to other constituents of the acceptor oligomer (see above).

Assuming the incorporation of 1 mol ^{125}I-DTX per mol polypeptide labelled, an M_r of 65 000 is obtained for the free component. This is notably lighter than the 95-kDa polypeptide obtained by labelling chick brain membranes with ^{125}I-β-BuTX [16] and it is, therefore, unlikely that the two components are identical although the possibility of species differences cannot be ruled out. Furthermore, 50 nM β-BuTX was ineffective in reducing the binding [4] and/or cross-linking to the membranes of 2.5 nM ^{125}I-DTX; in contrast, 50 nM unlabelled DTX produced a large reduction in the extent of cross-linking seen. This is consistent with the interpretation that the DTX acceptor polypeptide cross-linked in this study is not directly responsible for the high-affinity binding of β-BuTX observed in rat synaptosomal membranes [7] and brain regions [18]. As expected, apamin, which inhibits the Ca^{2+}-activated K^+ channel in a number of tissues ([3], cf. [15]) and can be cross-linked to a protein of M_r 28000 [15], is unable to affect the binding of ^{125}I-DTX to synaptosomes.

In view of the reported attenuation by DTX of one type of voltage-sensitive K^+-channel in hippocampus and its notable neurotoxicity in brain, it can be deduced that the binding component reported here is likely to be associated with or part of this K^+-channel macromolecular complex. These cross-linking experiments represent the first direct identification of the DTX acceptor protein and are allowing the chemical nature of this functional component to be characterised further following its purification by immunoaffinity methods, using anti-DTX antibodies.

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