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EXISTENCE OF DIFFERENT POPULATIONS OF THE DENDROTOXIN I BINDING PROTEIN ASSOCIATED WITH NEURONAL K^+ CHANNELS

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SUMMARY. The binding sites of dendrotoxin I, mast cell degranulating peptide, and β-bungarotoxin are thought to be associated with neuronal K^+ channels. The different binding sites seem to reside on the same molecular assembly as each toxin can allosterically inhibit the binding of the others. Affinity chromatography on a β-BTX Aca 22 affinity column has shown that there is an heterogeneous population of dendrotoxin I binding proteins. Two subtypes were separated : DTX_I binding proteins with low affinity for β-BTX (60-70% of total) and DTX_I binding proteins with high affinity for β-BTX (30-40% of total). Binding of ^{125}I-DTX_I and ^{125}I-MCD to the former subtype is inhibited by β-BTX with a low affinity (IC_{50} = 560 nM), while inhibition at the latter subtype occurs with a high affinity (IC_{50} = 10-16 nM). The DTX_I binding subtype with low affinity for β-BTX contains most (85-90%) of the binding sites for ^{125}I-MCD.

INTRODUCTION. Dendrotoxin I (DTX_I) (M_r = 7 kDA) from the venom of *Dendroaspis polylepis* snakes shows sequence homology with Kunitz-type trypsin inhibitors. β-Bungarotoxin (β-BTX) (M_r = 21 KDa) is also a snake venom toxin which consists of two subunits. The A-subunit (M_r = 13.5 KDa) carries a phospholipase A_2 activity. The B-subunit (M_r = 7.5 KDa) has sequence homology to protease inhibitors (1-4).

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The **abbreviations** used are : DTX_I, dendrotoxin I, β-BTX, β-bungarotoxin, MCD, mast cell degranulating peptide.

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The mast cell degranulating peptide (MCD) is a basic 22 amino acid peptide toxin from bee venom (5).

DTX_I and β-BTX affect neurotransmission at the neuromuscular junction by an interaction with pre-synaptic nerve terminals (2, 3, 6). MCD has been shown to induce hippocampal theta rhythm in the motionless animal (7) associated to long-term potentiation in hippocampal slices (8). Both MCD and DTX_I have been shown to induce epileptiform activity and paroxystic seizures when injected in rat brain at high doses (7, 9). The target of these toxins in neuronal membranes is a family of neuronal K^+ channels (10-13).

Binding sites for all three types of toxins have been identified in brain (14-18). These binding sites seem to reside on the same protein complex, as : **(i)** each toxin can inhibit the binding of the others (17-20), **(ii)** the solubilized binding proteins corresponding to receptors of the three different toxins have similar stabilization requirements (15, 18, 19). **(iii)** MCD, β-BTX and DTX_I binding sites copurify. Binding activity for MCD and β-BTX is still present after a 4000-5000-fold purification of DTX_I binding sites thought to lead to the pure or nearly pure DTX_I binding protein (21).

It has been clearly shown that the three different types of toxins associate to different categories of binding sites (17, 20). However the mutual relationship of these different binding sites for the different toxins is still far from being completely understood : **(i)** Binding experiments consistently indicate less MCD and β-BTX binding sites than DTX_I binding sites (14, 17-19, 22). **(ii)** Histological distributions of β-BTX and DTX_I binding sites in brain do not seem to coincide (23). **(iii)** Although DTX_I potently and completely inhibits the binding of ^{125}I-MCD and ^{125}I-β-BTX, β-BTX inhibits the binding of ^{125}I-DTX_I, and MCD that of ^{125}I-β-BTX, only at high toxin concentrations in the μM range (17, 18, 20-22).

Black and Dolly (22) have previously suggested that there may be iso-receptors for dendrotoxin. The experiments described in this paper use affinity chromatography to provide direct evidence for the existence of different populations of DTX_I binding proteins.

MATERIALS AND METHODS. β-BTX, horse heart cytochrome C, compound 48/80 and soybean phospholipids were from Sigma. Activated Aca 22 and SP-Trisacryl were from LKB, and DEAE-Affigel blue from Biorad. DTX_I and MCD were purified from *Dendroaspis polylepis* venom and bee venom respectively as described previously (18, 21).

Iodinated DTX_I and MCD and Triton X-100 extracts from rat brain membranes were prepared as described (18). Binding of the iodinated toxins to these extracts was determined using a GF/C filter assay (18). Binding of ^{125}I-MCD was determined in the presence of 25 μg/ml of compound 48/80 (16, 18).

The affinity column was prepared as follows : β-BTX (10 mg) or cytochrome C (102 mg) were coupled to activated Aca 22 (5 and 6 ml respectively) in 0.5 M K^+ phosphate buffer at pH 7.6 for 24 h at ambient temperature according to the protocol of the manufacturer. The capacity of the β-BTX Aca 22 affinity column (750 fmol of DTX_I binding sites/ml of gel) is much lower than what would be expected from the amount of β-BTX (100 nmol/ml of gel) which was coupled. Most of the β-BTX seems to be inactivated by the coupling procedure. This inactivation was also observed with other affinity supports such as Affigel 10 and 15 and CNBr activated Sepharose (H. Rehm, unpublished observations).

Columns of β-BTX Aca 22 (0.9 x 6.5 cm) or cytochrome C Aca 22 (0.9 x 9.5 cm) were equilibrated with a 20 mM Na^+-Hepes buffer at pH 7.4 containing 120 mM KCl, 1 mM EDTA, 2 mM $MnCl_2$, 0.05% (w/v) Triton X-100, 10% (w/v) glycerol and 0.01% (w/v) of soybean phospholipid (buffer I). The detergent extract was loaded on the column at a flow rate of 5 ml/h. The column was washed with buffer I containing 1% (w/v) of Triton X-100 and 0.2% (w/v) of soybean phospholipid (flow rate 10 ml/h) and eluted with the same buffer but containing 220 mM KCl instead of 120 mM and no $MnCl_2$ or 2 μM of DTX_I. When the column was eluted with 2 μM DTX_I, the eluted fractions (2 ml) were treated twice with 100 μl of SP-Trisacryl in buffer I in order to adsorb DTX_I. For rechromatography of the breakthrough of the β-BTX Aca 22 column, the column was first washed with 3-5 column volumes of buffer I.

Miscellaneous. The DTX_I binding protein was purified with ion-exchange chromatography on DEAE-Affigel blue, affinity chromatography on DTX_I-Aca 22 and affinity chromatography on wheat-germ agglutinin-Affigel 10 as described elsewhere (21).

Protein concentrations were determined with the BCA assay (Pierce) after protein precipitation according to Wessel and Flügge (24). SDS-PAGE on exponential gradient gels (6.5-17%) was done according to Laemmli (25). The Coomassie stained gels were scanned with a LKB ultroscan.

RESULTS

Affinity chromatography of ^{125}I-DTX$_I$ binding sites using a β-BTX Aca 22 affinity column. When a Triton X-100 extract from rat brain membranes was chromatographed on β-BTX Aca 22, the column retained ^{125}I-DTX$_I$ binding sites with a capacity of 750 fmol of binding sites/ml of gel (Fig. 1). Retained ^{125}I-DTX$_I$ binding sites could be eluted by increasing the KCl concentration of the buffer from 120 to 220 mM. The capacity and the elution properties of the β-BTX column were different from those previously observed for the DTX$_I$ Aca 22 column which we used for the purification of the DTX$_I$-binding protein (21). This DTX$_I$ affinity column had a capacity of at least 16 000 fmol of binding sites/ml of gel and the KCl concentration of the buffer had to be increased much more than for the β-BTX Aca 22 column (from 120 to 620-720 mM) in order to obtain the elution of

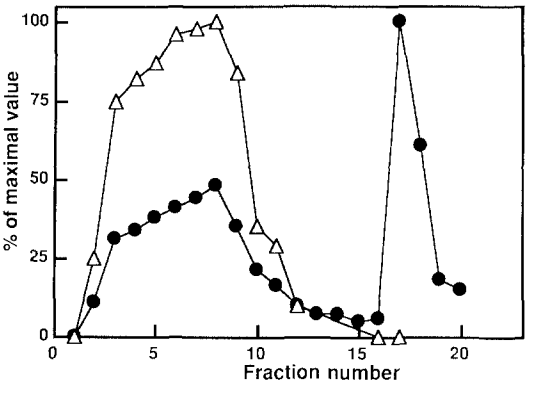

Fig. 1. Chromatography of detergent extract on β-BTX Aca 22. Detergent extract (37 mg of protein, 12 000 fmol of ^{125}I-DTX$_I$ binding sites) was prepared and chromatographed on β-BTX Aca 22 as described in Materials and Methods. Protein (Δ) and ^{125}I-DTX$_I$ binding (●) in the indicated fractions are shown. The values are given in % of the maximal value of the respective parameter of the fractions.

the binding sites. Although the same protocol was used for the preparation of the two types of affinity columns, the DTX_I column was prepared with 25 times more toxin (in moles) than the β-BTX column. Also the affinity of DTX_I for its receptor (K_d = 50-150 pM (18, 21)) is 10 times higher than the affinity of β-BTX for its binding site (K_d = 1-2 nM (19)).

Since β-BTX is a basic protein (pI = 9.5 (26)), the possibility existed that the retention of the acidic (18, 21) ^{125}I-DTX_I binding protein was solely due to ionic interactions. The following experiments strongly suggest that β-BTX Aca 22 truly behaves as an affinity column: **(i)** ^{125}I-DTX_I binding sites retained by the column were eluted not only by high concentrations of KCl but also with 2 μM DTX_I. **(ii)** Virtually no ^{125}I-DTX_I binding sites were retained on an Aca 22 column prepared with cytochrome C (150 nmol/ml lof gel) (instead of β-BTX). Cytochrome C is a protein which is of similar or higher basicity than β-BTX. **(iii)** The purification obtained with the β-BTX Aca 22 column (≈ 100-fold) was significantly higher than that obtained by ion-exchange chromatography (10-17-fold) on different ion exchangers (18, 21).

^{125}I-DTX_I binding sites are separated into two populations by β-BTX Aca 22 chromatography. Only a fraction (37 ± 4%) of the ^{125}I-DTX_I binding sites applied to the β-BTX Aca 22 column (in amounts within the capacity of the column) was retained. Rechromatography of ^{125}I-DTX_I binding sites which were eluted in the breakthrough of the column led to no significant additional retention of these binding sites (Fig. 2).

These results suggest that there are two populations of ^{125}I-DTX_I binding sites, one with a high affinity for β-BTX which is retained by the β-BTX Aca 22 column, the other one with a lower affinity for β-BTX which is not retained under the experimental conditions used in this work. Fig. 3A confirms this view in showing that β-BTX in-

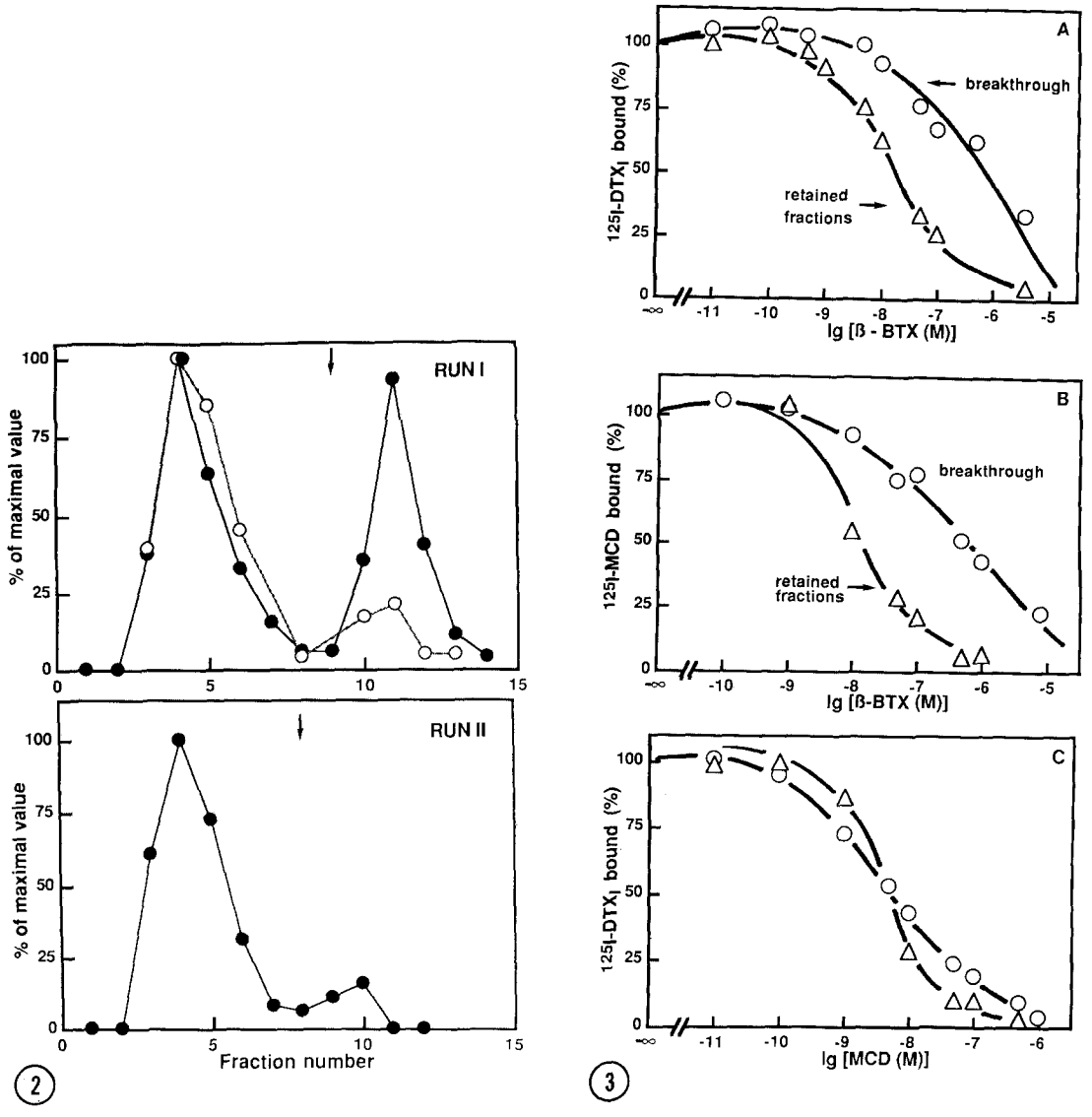

Fig. 2. Chromatography of detergent extract on β-BTX Aca 22 and rechromatography of the breakthrough. *Run I* : Detergent extract (4.4 mg of protein, 1600 fmol of ^{125}I-DTX$_I$ binding sites, 750 fmol of ^{125}I-MCD binding sites) was prepared and chromatographed on β-BTX Aca 22 as described in Materials and Methods. (●) ^{125}I-DTX$_I$ binding and (○) ^{125}I-MCD peptide binding. The values are given in % of the maximal value of the respective parameter of the fractions. *Run II* : After Run I the column was washed with 20 ml (5 column volumes) of buffer I. The pooled fractions Nr. 3, 4 and 5 from Run I were then loaded on the column and rechromatographed as described in Materials and Methods. ^{125}I-DTX$_I$ binding in the indicated fractions is given as % of the maximal value of the fractions. The arrows point to the start of elution with buffer I containing 220 mM of KCl.

hibits ^{125}I-DTX$_I$ binding to the fraction retained by the affinity column with an IC_{50} of 16 nM while it inhibits ^{125}I-DTX$_I$ binding from the fraction eluted in the breakthrough with an IC_{50} of 560 nM. This difference in affinity for β-BTX is not accompanied by a difference in affinity for DTX$_I$. ^{125}I-DTX$_I$ binds with similar affinities to breakthrough (K_d = 76 pM) and retained (K_d = 42 pM) fractions (not shown). This finding is consistent with the previous observation that nearly all (<99%) of the ^{125}I-DTX$_I$ binding sites are retained on a DTX$_I$ Aca 22 affinity column (21).

Most of the ^{125}I-MCD binding sites are not retained by β-BTX Aca 22 chromatography. Most (85-90% of total) of the binding sites for ^{125}I-MCD were not retained by the β-BTX Aca 22 column (Fig. 2). The non-retained population of ^{125}I-MCD binding sites differed from the small (10-15% of total) population of ^{125}I-MCD binding sites which were retained in their sensitivity to β-BTX. ^{125}I-MCD binding to the breakthrough was inhibited by β-BTX with an IC_{50} of 560 nM while ^{125}I-MCD binding to the retained fraction of the affinity column was inhibited by β-BTX with an IC_{50} of 10 nM (Fig. 3B). ^{125}I-DTX$_I$ binding to breakthrough and retained fractions was inhibited completely and with a similar high affinity by MCD (IC_{50} = 3-4 nM) (Fig.

Fig. 3. Chromatography on β-BTX Aca 22 with detergent extract was performed as described in Materials and Methods. **A.** Inhibition of ^{125}I-DTX$_I$ binding by β-BTX in breakthrough and retained fractions of the β-BTX Aca 22 column. The binding of ^{125}I-DTX$_I$ (110 pM) to breakthrough (○) and retained fractions (Δ) of the chromatography was then determined in the presence of the indicated concentration of β-BTX. **B.** Inhibition of ^{125}I-MCD binding by β-BTX in breakthrough and retained fractions from the β-BTX Aca 22 column. The binding of ^{125}I-MCD (22 pM) to breakthrough (○) and retained fractions (Δ) of the chromatography was then determined in the presence of the indicated concentrations of β-BTX. **C.** Inhibition of ^{125}I-DTX$_I$ binding by MCD in breakthrough and retained fractions of the β-BTX Aca 22 column. The binding of ^{125}I-DTX$_I$ (110 pM) to breakthrough (○) and retained fractions (Δ) of the chromatography was determined in the presence of the indicated concentrations of MCD.

3C). It has also been observed that ^{125}I-MCD binds to breakthrough (K_d = 76 pM) and retained fractions (K_d = 50 pM) with a similar affinity (not shown).

DISCUSSION

This paper provides direct evidence for an heterogeneity of the high affinity binding proteins for DTX_I and MCD which presumably correspond to neuronal K^+ channels. High affinity DTX_I and MCD binding molecules were separated by chromatography on β-BTX Aca 22 into two populations which differed by a factor of 40-50 in their affinity for β-BTX. Of course the possibility exists that each one of the two types of DTX_I binding proteins demonstrated in this paper may itself be heterogeneous. Also the inhibition of high affinity ^{125}I-β-BTX binding by MCD (IC_{50} = 1.6 μM (20)) cannot be mediated by the high affinity MCD binding sites discussed here, but rather by an additional population of low affinity sites for MCD.

Cross-linking studies have shown that, in rat brain, MCD and DTX_I bind to peptides of molecular weight 76-80 KDa (18, 22). Purification studies have also indicated that one of the two components of the DTX_I/MCD/β-BTX binding protein has a molecular weight of 76-80 KDa (21). The peptide pattern of a typical purification is indicated in Fig. 4. The heterogeneity of the DTX_I/MCD/β-BTX binding component is probably reflected by the broad and heterogeneous appearance of the 76-80 KDa band (Fig. 4) after Coomassie blue staining.

Recent analysis of the *Shaker* locus of *Drosophila* has given information about the structure of one class (A-type) of voltage-sensitive K^+ channels in this fly (27-30). Alternative splicing occurs and generates multiple *Shaker* products (M_r = 70 KDa) corresponding to subtypes of A channels with different kinetics and tissue distribution (31, 32). The heterogeneity found in this work for toxin receptor

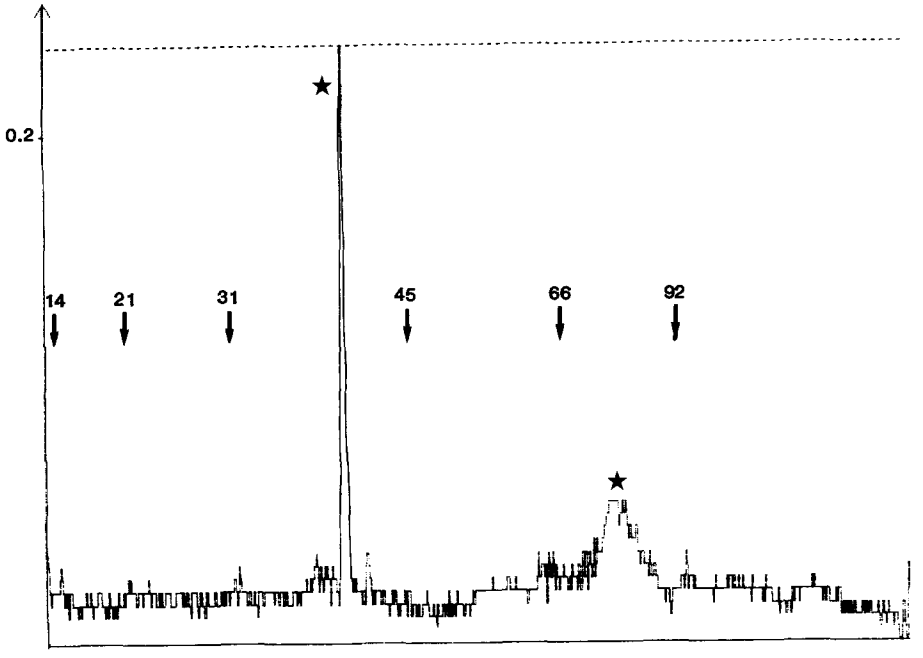

Fig. 4. Scan of the purified DTX_I binding protein components. DTX_I binding proteins were purified as described in Materials and Methods and run on 6.5-17% exponential SDS-PAGE. After staining with Coomassie blue the gel was scanned with a laser scanner. The peaks at 38 and 76-80 KDa corresponding to the protein components of the DTX_I receptor (21) are indicated by stars. Molecular weight markers are indicated by arrows.

proteins associated with voltage-dependent K^+ channels might be the reflection of a diversity of K^+ channels in mammalian brain.

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