Cloning and Functional Expression of Dendrotoxin K from Black Mamba, a K^+ Channel Blocker†

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ABSTRACT: Mamba dendrotoxins, 7K M_r polypeptides with three disulfide bonds, selectively inhibit certain fast-activating, voltage-sensitive neuronal K^+ channels and have been instrumental in their identification, localization, and purification. However, derivatives with more refined specificity are essential to define the structural and functional properties of the multiple subtypes known to reside in the nervous system. Hence, utilizing a constructed cDNA library from the venom glands of the black mamba (*Dendroaspis polylepis*), the gene encoding dendrotoxin K was isolated, amplified, and expressed as a maltose-binding fusion protein in the periplasmic space of *Escherichia coli*. After cleavage of the chaperone from the affinity-purified product, a recombinant protein was isolated and shown to be identical to native dendrotoxin K in its N-terminal sequence, chromatographic behavior, convulsive-inducing activity, and binding to voltage-activated K^+ channels in bovine synaptic membranes. This successful expression of refolded active toxin, in adequate yield, makes possible for the first time the preparation of mutants with specificity tailored for each K^+ channel subtype, based both on the recently derived three-dimensional structure of α-dendrotoxin and the identified binding site on cloned K^+ channels.

Dendrotoxins K and I from the venom of *Dendroaspis polylepis*, together with α and δ from *Dendroaspis angusticeps*, are homologous low molecular weight (~7K) proteins that structurally resemble Kunitz protease inhibitors [reviewed by Harvey et al. (1990)]. These single-chain, basic polypeptides (57–60 residues), containing three conserved and essential disulfide bonds, induce convulsive activity due to their facilitation of transmitter release (Dolly, 1992a). The symptoms caused by α-dendrotoxin (α-DTX)[1] have been attributed to a selective inhibition of certain fast-activating, voltage-sensitive K^+ channels that are concerned with regulating neuronal excitability and synaptic transmission (Dolly et al., 1984, 1987; Halliwell et al., 1986; Dreyer, 1990).

Recently, dendrotoxin K (DTX_K) was found to be a potent inhibitor of a voltage-dependent, noninactivating K^+ current in rat ganglionic neurons, with a slowly inactivating variant being less susceptible (Hall et al., 1992). Several K^+ channel subtypes have been distinguished and localized in the central nervous system, using dendrotoxins as probes (Pelchen-Matthews & Dolly, 1989; Bidard et al., 1989). These toxins have allowed the purification of a mixture of K^+ channels from mammalian brain (Rehm & Lazdunski, 1988; Parcej & Dolly, 1989); while the varying specificity of the DTX homologues has facilitated the characterization of neuronal K^+ channel proteins (Stuhmer et al., 1989). Importantly, the binding site for α-DTX has been located on the external mouth of the ion filter on a rat brain K^+ channel expressed in *Xenopus* oocytes (Hurst et al., 1991).

In view of the subtle heterogeneity of this important K^+ channel family, there is a necessity for additional probes with more discriminatory specificities in order to achieve the fundamental goal of relating the molecular structures of each subtype to the biophysical characteristics of the channel variants. As an essential first step in producing a panel of mutant dendrotoxins, a cDNA library was prepared from mRNA isolated from the glands of *D. polylepis*. A cDNA encoding DTX_K was isolated, characterized, and expressed in *Escherichia coli*. The biological activity of the recombinant toxin was established from its ability to bind to voltage-sensitive K^+ channels and by its central toxicity in an *in vivo* assay.

MATERIALS AND METHODS

Adult specimens of *D. polylepis* were obtained from Leakey (Nakuru, Kenya, East Africa). A lambda ZAP II/*Eco*RI CIAP treated vector kit and Gigapack II Gold packaging extract were purchased from Stratagene (LaJolla, CA). A cDNA synthesis kit, *Eco*RI/*Not*I adaptors, T7 sequencing kit, Deaza T7 sequencing mixes, and restriction enzymes were provided by Pharmacia (Piscataway, NJ). The GeneAmp PCR reagent kit was purchased from Perkin-Elmer Cetus (Norwalk, CT). A protein fusion and purification System was supplied by New England Biolabs (Beverly, MA). Oligonucleotides used for screening cDNA libraries and as primers for polymerase chain reactions (PCR) and dideoxynucleotide sequencing were synthesized on a Biosearch 8700 DNA synthesizer by β-cyanoethyl phosphoramidite chemistry and purified on Oligo-Pak columns (MilliGen/Biosearch, Burlington, MA).

mRNA Isolation and Preparation of a cDNA Library. Glands were removed from the snakes 2 days after venom evacuation (Vandenplas et al., 1985), immediately frozen in liquid nitrogen, and stored at −70 °C until used. Total RNA

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[1] Abbreviations: α-DTX, α-dendrotoxin; DTX_K, dendrotoxin K; PCR, polymerase chain reaction; IPTG, isopropyl β-D-thiogalactoside; PMSF, phenylmethanesulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid; SDS–PAGE, sodium dodecylsulfate–polyacrylamide gel electrophoresis; M_r, relative molecular mass; FPLC, fast protein liquid chromatography; ^{125}I-α-DTX, ^{125}I-labeled α-DTX; RER, rough endoplasmic reticulum.

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was isolated by the guanidinium isothiocyanate–hot phenol extraction method (Feramisco et al., 1982), and poly(A)$^+$ RNA was enriched by passage over an oligo(dT)–cellulose column (Aviv & Leder, 1972), yielding 10 μg of poly(A)$^+$ RNA per g of gland. DNA was prepared from 5 μg of mRNA (Gubler & Hoffman, 1983) using a cDNA synthesis kit. Hemiphosphorylated adaptors containing *Not*I and *Eco*RI restriction enzyme sites were ligated to the termini of the synthesized, double-stranded cDNA. The modified cDNA was inserted into *Eco*RI-predigested, phosphatased lambda ZAP II arms and packaged into viable phage particles by using packaging extracts. The recombinant bacteriophage was used to infect *E. coli* host strain, XL1-Blue, which generated the primary cDNA library. The primary library contained approximately 1.35×10^5 pfu/μg of mRNA.

Analysis of the cDNA Library and Subcloning of cDNA Inserts from Lambda ZAP II Clones. Approximately 100 000 plaques from an amplified cDNA library were analyzed for sequences encoding DTX_K by using a nick-translated 393 base pair (bp) cDNA encoding DTX_I (TCTTCTTCTCCTGCTGGGACTCCTCACCCCTCTGGGCAGGAACTGACCCCTGTCTCCGGACAGCCCCTTCGAAAGTTGTGTATACTGCATCGTAACCCCGGACGATGTTATCAGAAAATACCAGCCTTCTACTATAACCAGAAAAAAAAACAATGCGAAGGGTTTACTTGGAGTGGCTGTGGGGGCAATTCCAACAGATTTAAGACCATAGAGGAATGCCGCCGCACCTGTATTCGTAAGTAGGATGACCAATGAGGAGACCCACCCAGAATGGATCCAGTGTTCCAATTTGAGCCAAAGACCCTGCTTTTGCCCCGGACCACCCTGGAGACCCTCCCCAGAAACCCCACCCTGGGCTCATTCCTTTTTCTCTGCAATAAAGCTTTGGTTCCA). DTX_K shares a 58% amino acid sequence homology with DTX_I (Strydom, 1973). The library was screened for DTX_K on nitrocellulose filters according to standard procedures (Sambrook et al., 1989). Filters were prehybridized for 4 h at 42 °C in 6× SSC (90 mM sodium citrate containing 0.9 M NaCl, pH 7.0), containing 1× Denhardt's and 100 μg/mL sonicated and denatured salmon sperm DNA. Filters were then hybridized in 4× SSC, pH 7.0, containing 1× Denhardt's and the nick-translated DTX_I cDNA probe for 16 h at 42 °C. Sucessive washes were performed in 2× SSC, pH 7.0, at 37 °C for 30 min before autoradiography for 16 h at −70 °C using X-AR film with intensifying screens. Double-stranded cDNA inserted into the multiple cloning site (MCS) of pBluescript SK$^-$ contained within lambda ZAP II were removed as phagemids by an *in vivo* excision process designed by Stratagene (LaJolla, CA). Colonies from the *in vivo* excision were selected by ampicillin resistance and propagated, and the phagemids were isolated by alkaline extraction. The size of the inserts from the recombinant phagemids was measured on agarose gel electrophoresis after digestion with the restriction enzyme *Eco*RI.

Asymmetric PCR and DNA Sequencing. The template for asymmetric PCR was double-stranded pBluescript SK$^-$ containing cDNA inserts of approximatively 400 bp. Oligonucleotides designated as LS4 (5′-GAGTTAGCTCACTCATTAGGC-3′) and LS5 (5′-ATTTTCATTCGCCATTCAGGC-3′) were used as primers in asymmetric PCR. Sanger dideoxynucleotide sequencing employed T7 DNA polymerase according to the manufacturer's protocol. Single-stranded plasmid DNA was used as template. Programs for sequence analysis from Intelligenetics, Inc. (Mountain View, CA), including GENED, SEQ, and IFIND, were used on a VAX from Digital Equipment Corp. (Maynard, MA).

Reconstruction of Recombinant DTX_K Gene and Its Expression as a Fusion Protein. A PCR-derived gene segment encoding DTX_K was cloned into the expression vectors pMALc and pMALp to produce a DTX_K fusion protein; this was directed into the cytosol and periplasmic space of *E. coli* by varying the leader sequence of the MBP. PCR primers were designed from cDNA sequence data to incorporate unique restriction enzyme sites flanking the structural gene region of DTX_K. The 3′ PCR primer provided a unique *Hin*dIII site, while the 5′ primer duplicated the entire MCS, as well as the factor Xa protease recognition sequence represented in the vectors. The reconstructed gene was cleaved with restriction endonucleases *Sac*I and *Hin*dIII, gel purified, and ligated to similarly prepared vector DNA. After transformation into competent *E. coli* DH5α cells, individual clones were grown at 37 °C in Super Broth containing 100 μg/mL ampicillin and 2 μg/mL dextrose to a cell density of (2–4) × 10^8 cells/mL ($A_{600} \sim 0.4$). Cell induction was then initiated by the addition of isopropyl β-D-thiogalactoside (IPTG) (final concentration, 0.3 mM). Cells were harvested 2–2.5 h after the addition of IPTG. Cell pellets used for preparing cytoplasmic extracts were frozen at −70 °C and subsequently thawed, while those used for the periplasmic extracts were processed without freeze-thawing.

Purification and Characterization of Recombinant DTX_K. Frozen cells harboring the pMALc construct were resuspended in lysis buffer (10 mM sodium phosphate, 30 mM NaCl, 0.25% Tween 20, 10 mM EDTA, pH 7.0), containing 15 μM pepstatin A, 15 μM leupeptin, and 0.5 mM phenylmethanesulfonyl fluoride (PMSF) and lysed by sonication. Cells containing the pMALp construct were lysed by osmotic shock (Neu & Heppel, 1965). After centrifugation, crude extracts were applied to an amylose affinity resin. Unbound proteins were eluted from the amylose resin by buffer A (10 mM sodium phosphate, pH 7.0, and 0.5 M NaCl) containing 0.25% Tween 20. The bound MBP-DTX_K fusion protein was eluted by buffer A containing 10 mM maltose. Fusion proteins were cleaved at 26 °C for 24–48 h by using factor Xa in buffer B (20 mM Tris-HCl, 100 mM NaCl, and 2 mM $CaCl_2$, pH 8.0) at a ratio of 0.1%–0.5% (w/w) of factor Xa (1000 units/mg) to the fusion protein. Recombinant DTX_K was then purified by FPLC Superose-12 10/30 (molecular sieve) column chromatography. Fractions from FPLC were analyzed by sodium dodecyl sulfate–polyacylamide gel electrophoresis (SDS–PAGE) in an Amersham PhorCast Gel System (Amersham Life Sciences, Arlington Heights, IL). For amino acid sequence analysis, selected fractions from FPLC were further purified on HPLC reverse-phase chromatography and pyridylethylated (Cavins & Friedman, 1970; Schmidt & Middlebrook, 1989), and automated Edman degradation was performed by a model 470A amino acid sequencer from Applied Biosystems (Foster City, CA).

Purification, Characterization, and Iodination of Native Dendrotoxins. DTX_K and α-dendrotoxin were purified from the venom of *D. polylepis* and *D. angusticeps*, respectively, as previously described (Dolly, 1992b). Both toxins were radioiodinated by a modification of the chloramine-T method, detailed elsewhere (Black et al., 1986); the central toxicities of native and recombinant DTX_K were quantified in rats (Othman et al., 1982).

Measurement of Saturable Binding of ^{125}I-Labeled Toxins to Membrane-Bound K^+ Channels. Synaptic plasma membranes purified from bovine cerebral cortex were employed to measure the binding of radiolabeled toxins (Black et al., 1986). Analysis of the data from triplicate determinations

was carried out using the Grafit program (Erithacus software). For monitoring the ability of DTX_K to antagonize the saturable binding of 1 nM ^{125}I-toxins, varying concentrations of DTX (native or recombinant) were included in the reaction. Prior to use, DTX_K was absorbed on a reverse-phase SEP-PAK C-18 column (Millipore Corp) in 0.1% TFA and eluted with 60% acetonitrile in 0.1% TFA. The peak was freeze-dried and redissolved in water.

RESULTS

Isolation and Sequencing of the cDNAs Encoding DTX_K. From a low-density screening of an amplified black mamba cDNA library, we isolated 50 positive clones using a radiolabeled DTX_1 cDNA probe (see Materials and Methods). Many of these clones had inserts of 400–500 bp, shown by location and size on agarose gel electrophoresis after cleavage of the cloned DNA with the restriction enzyme *Eco*RI. A number of cDNAs from positive clones were sequenced with asymmetric PCR-amplified templates (Gyllensten & Erlich, 1988). Single-stranded DNA obtained with an excess of LS4 primer (see Materials and Methods) was sequenced with T7 sequencing primer. The complementary single-stranded DNA obtained with an excess of LS5 primer was sequenced with T3 primer. The use of amplification primers located about 200 bp upstream to the site of the sequencing primer annealing removed the need for extensive purification of the asymmetric PCR-products (Wilson et al., 1990).

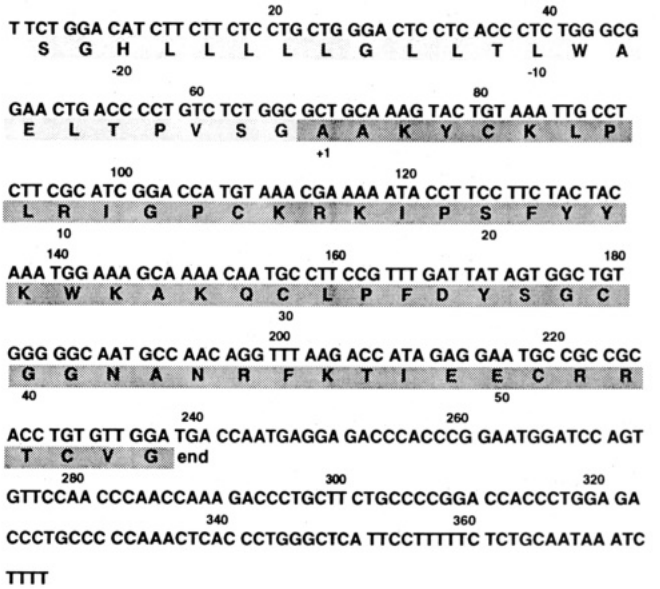
Of the cloned cDNA sequenced, three encoded DTX_K gene products. All had truncated 5′ termini lacking an initiation codon and a 5′-untranslated region. All cDNA sequences displayed one major open reading frame (ORF) of about 236 nucleotides. The ORF demonstrated a coding capacity for a toxin precursor because it was larger than the toxin previously purified and characterized from the venom (Strydom, 1973). Figure 1A shows the nucleotide sequence of the cDNA from the clone designated DPD 32.3. The sequence infers a signal peptide of at least 22 amino acids followed by a 57 amino acid protein with a sequence identical to that obtained from direct amino acid sequence analysis of DTX_K (Strydom, 1977). At the 3′ end of the cDNA, a putative polyadenylation signal (AATAAA), usually found 10–16 nucleotides upstream of the poly(A^+) tail (Proudfoot & Brownlee, 1976), was present.

Subcloning the Structural Gene of DTX_K in E. coli Expression Vectors. A cDNA encoding DTX_K was amplified by PCR employing nondegenerate sense and antisense primers to anneal to the beginning and end of the desired sequence. We used this technique to remove the leader and untranslated sequences from the cDNA and to add specific restriction enzyme sites for subcloning into the pMAL(c) and pMAL(p) expression vectors. After subcloning into the vectors, colonies resistant to ampicillin were grown, and the plasmid DNA was analyzed by restriction digests and DNA sequence analysis (data not shown). Digestion with *Sac*I and *Hin*dIII, and subsequent electrophoresis in an agarose gel, showed the presence of a fragment of about 220 bp. Also revealed was the nucleotide sequence at the junction where the 5′ end of the DTX_K gene was fused to the multiple cloning site (MCS) and factor Xa cleavage site. We used an antisense sequencing primer corresponding to amino acid residues 27–31 located in the center of the protein. The sequence at the junction of the DTX_K gene and the MCS–factor Xa cleavage site showed the DTX_K gene to be in a proper reading frame with the maltose-binding protein (MBP) gene.

Expression of Recombinant DTX_K in E. coli. Recombinant clones were initially grown as 10-mL cultures and induced

A.

Nucleotide and Inferred Amino Acid Sequence of DTXK from a *Dendroaspis polylepis* cDNA Library (Clone DPD 32.3)



B.

A A K Y C K L P L R I G P C K R K

FIGURE 1: Nucleotide and amino acid sequence of DTX_K. (A) The nucleotide and inferred amino acid sequence of DTX_K was determined from clone DPD 32.3 isolated from a *D. polylepis* cDNA library. The inferred amino acid sequence in the lighter shaded area (–22 to –1) represents the signal peptide sequence followed by a 57 amino acid DTX_K protein sequence in the darker shaded area. (B) The amino acid sequence of the first 17 residues of the purified recombinant DTX_K was determined by direct sequence analysis.

with IPTG, as described under Materials and Methods. Aliquots of cell extracts were analyzed under denaturing conditions by polyacrylamide gel electrophoresis. Extracts from induced cultures showed the presence of a protein band, with M_r ~47K, in relatively large quantity when compared with that in extracts from the uninduced cultures (Figure 2). Extracts from clones containing the DTX_K sequence in both the pMAL(c) and the pMAL(p) vectors had this band. The size of the protein fragment observed from the SDS–PAGE is in accordance with that expected from a fusion protein of MBP and DTX_K (M_r of 40 and 7K).

Large scale cultures were grown with the pMAL(c) and pMAL(p) constructs, induced with IPTG, and cell extracts prepared as described under Materials and Methods. Repeatedly, the product obtained from the cytoplasmic extract was predominantly MBP rather than fusion product. Protease degradation of the fusion protein was extensive (data not shown). The use of protease inhibitors as well as *E. coli* strains deficient in specific proteases did not alleviate the problem. The product obtained from the periplasmic space of *E. coli* experienced much less protease degradation than the cytoplasmic-derived fusion product. Thus, we performed all further work using only the periplasmic extract.

Purification of Recombinant and Native DTX_K. Periplasmic extracts from *E. coli* were subjected to chromatography on an amylose affinity column, and the resultant fractions containing fusion protein were digested with factor Xa. Treated samples were then applied to an FPLC Superose-12 molecular sieve column (Figure 3A), and DTX_K was purified from contaminating MBP and factor Xa (Figure 3B); the purified material was then concentrated by lyophilization and

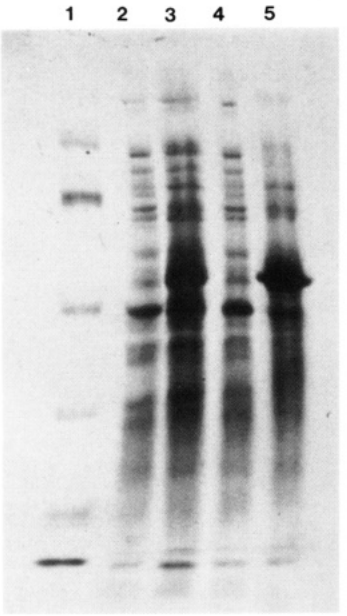


FIGURE 2: SDS–PAGE of extracts from uninduced and IPTG-induced *E. coli* cells harboring the structural gene encoding DTX_K in pMAL(c) and pMAL(p) vectors. (Lane 1) Molecular weight markers phosphorylase B (97 400), bovine serum albumin (66 200), ovalbumin (45 000), carbonic anhydrase (31 000), soybean trypsin inhibitor (21 500), and lysozyme (14 400). (Lanes 2 and 3) *E. coli* extracts of uninduced and induced cultures, respectively, in the pMAL(c) vector. (Lanes 4 and 5) *E. coli* extracts of uninduced and induced cultures, respectively, in the pMAL(p) vector.

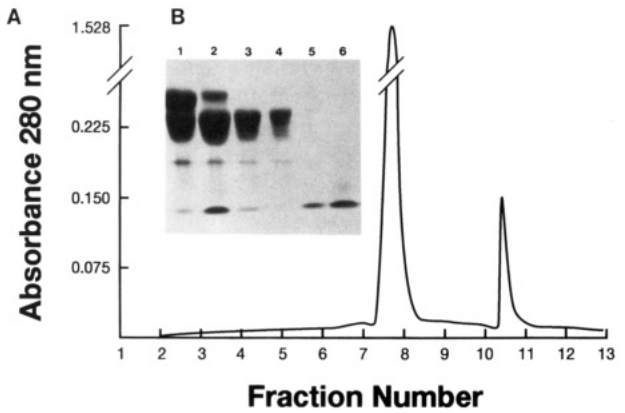


FIGURE 3: Purification of recombinant DTX_K. (A) Aliquots of factor Xa-cleaved MBP-DTX_K fusion protein obtained from the periplasmic space of *E. coli* were applied to an FPLC Superose-12 column as described under Materials and Methods. Fractions were analyzed by SDS–PAGE. (B) SDS–PAGE of affinity-purified fusion protein, factor Xa-cleaved fusion protein, and purified fractions of recombinant DTX_K from Superose-12. (Lane 1) Affinity-purified fusion protein (see Materials and Methods); (lane 2) affinity-purified fusion product partially digested with factor Xa; (lanes 3–6) fractions (7, 8, and two different concentrations of fraction 10, respectively) from Superose-12 chromatography.

used for further characterization. For its comparison with native toxin, we purified DTX_K from the venom of *D. polylepis* by conventional gel filtration and ion-exchange chromatography (Dolly, 1992b). A final cation-exchange HPLC step was used to remove trace contaminants; most significant, the resultant peak of DTX_K coeluted with the recombinant toxin in the latter system (data not shown).

N-Terminal Sequence of Recombinant DTX_K. Amino acid sequencing was performed on reduced and pyridylethylated samples by automated Edman degradation. This analysis allowed us to identify the first 17 residues of the purified recombinant DTX_K (Figure 1B). The end amino-terminal

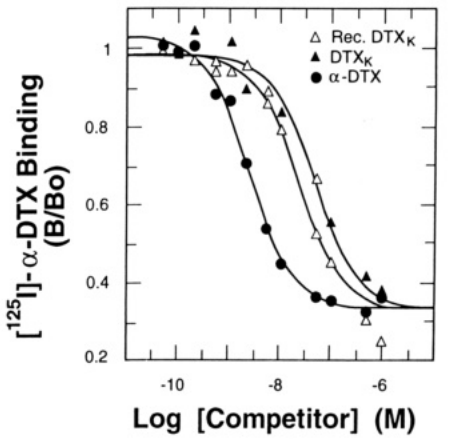


FIGURE 4: Inhibition of ^{125}I-α-DTX binding to synaptic membranes by native and recombinant DTX_K. Bovine cerebrocortical membranes were incubated at 22 °C for 45 min in imidazole buffer, pH 7.4, containing 0.1% bovine serum albumin, in the presence of 1 nM ^{125}I-α-DTX and various concentrations of unlabeled α-DTX (●), native (▲), or recombinant DTX_K (Δ). After separation of bound ^{125}I-α-DTX, the values of fractional binding in the presence (B) and absence (B_o) of competing toxin were plotted. The IC_{50} values obtained were used to calculate K_i by the equation $K_i = IC_{50}/1 + ([^{125}I\text{-}\alpha\text{-DTX}]/K_D$, where K_D is the measured association constant for ^{125}I-α-DTX binding to K^+ channels.

sequence observed for the recombinant DTX_K was identical to that previously determined for DTX_K (Strydom, 1977).

Neurotoxicity of Recombinant DTX_K and Its Binding to Voltage-Activated Neuronal K^+ Channels. The central toxicities of the purified preparations of recombinant and native DTX_K were assessed by intraventricular injection of rats. Both toxins were potent convulsants, and the apparent minimal lethal dose (MLD approximately 3 ng/g of body weight) obtained for each was identical; this finding established the biological activity of the recombinant protein. As α-DTX is a well-established probe for neuronal voltage-activated K^+ channels [reviewed by Dolly (1991)], inhibition of its binding was employed to evaluate the interaction of native and recombinant DTX_K with K^+ channels. We observed saturable binding of ^{125}I-α-DTX to synaptic plasma membranes from bovine cerebral cortex, and the K_i for its inhibition by α-DTX (0.4 nM) corresponded to the reported K_d (Parcej & Dolly, 1989). This binding was antagonized by both native and recombinant DTX_K (Figure 4). Notably, the resultant K_i values obtained for native and recombinant DTX_K, 7.8 and 7.1 nM, respectively, did not differ significantly, though they were appreciably larger than the K_i value for α-DTX. The difference in affinity can be reconciled with the level of homology (65%) between DTX_K and α-DTX. In view of the report that α- and δ-DTX bind to distinct sites on the K^+ channels and interact in a complex, noncompetitive manner (Muniz et al., 1990a,b), it was deemed necessary to radioiodinate DTX_K and to directly measure its interaction with K^+ channels. The ^{125}I-DTX_K preparation used had a specific radioactivity of 290 Ci/mmol. It showed saturable binding to bovine synaptic plasma membranes (Figure 5A), though the level of nonsaturable binding was somewhat high. Computer analysis of the data by the Grafit program yielded a K_d of 0.23 nM and a site content of 0.4 pmol/mg of protein ($n = 3$). Native and recombinant DTX_K were then compared for their ability to inhibit the saturable binding of ^{125}I-DTX_K (Figure 5B). The resultant K_i values obtained (0.3 and 1.0 nM, respectively) were not significantly different and approximated the K_d value obtained directly for ^{125}I-DTX_K.

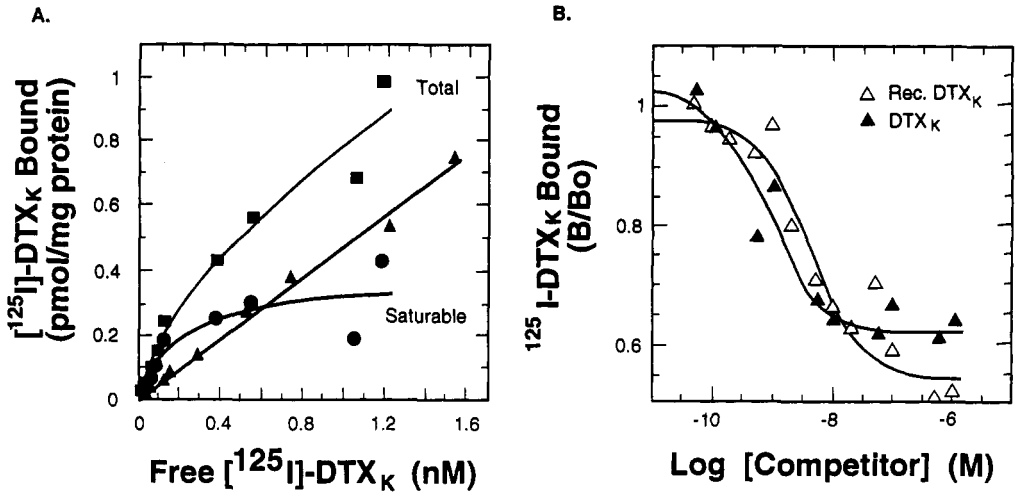


FIGURE 5: Concentration dependence of ^{125}I-DTX$_K$ binding to bovine synaptic membranes and antagonism by native and recombinant DTX$_K$ of ^{125}I-DTX$_K$ binding to cerebrocortical synaptic membranes. (A) A suspension of plasma membranes was incubated with various concentrations of ^{125}I-DTX$_K$ as detailed in Figure 4. To determine nondisplaceable binding, 1 μM unlabeled DTX$_K$ was included in the incubation. Specific binding (●) was calculated from the difference between total binding (■) and the corresponding values for nondisplaceable binding (▲), derived from the straight line for each concentration used, in triplicate. (B) Assays were performed and data plotted as detailed in the legend to Figure 4, except that 1 nM ^{125}I-DTX$_K$ was used in the presence of varying concentrations of native (▲) or recombinant (△) DTX$_K$.

DISCUSSION

Toxins isolated from the venoms of mamba snakes act on various components (acetylcholine receptor, acetylcholinesterase, or voltage-activated K^+ channels) of the neuromuscular junction involved with neuromuscular transmission. In an attempt to clone, characterize, and express these toxin genes, as well as other toxin genes encoding activities not completely elucidated as yet, we constructed a cDNA library from mRNA isolated from the glands of the black mamba, *D. polylepis*. Numerous cDNAs from this library were characterized by nucleotide sequence analysis. This work describes, for the first time, the expression of a recombinant DTX$_K$ in *E. coli* and a comparison of its activity to that of the native protein isolated from the venom. In total, sequences coding for four of the venom's proteins (DTX K, I, E, and B) were isolated; each had a leader sequence for 20–22 amino acids, but, in every case, initiation codons were absent. The reason why full-copy genes were not found is unclear as this was achieved with the same cDNA library for seven other proteins (L. Smith, unpublished data), implying that the mRNA used to construct the library was intact.

Secreted proteins are generally synthesized with amino-terminal extensions, typically 15–60 residues in length, and aid in directing proteins to their final cellular or extracellular destination. Of the animal toxin genes previously cloned and characterized from venom glands [reviewed by Smith (1990)], all encode precursor proteins having signal peptides usually between 16 and 27 residues. Control mechanisms regulating the synthesis and secretion of venom components in these glands are not well understood. However, it is known that gland evacuation triggers morphologic and functional changes in secretory gland cells where most of the cell volume becomes occupied by rough endoplasmic reticulum (RER) and cisternae (Bdolah, 1979). Signal sequences from many venom precursor proteins including dendrotoxins K, I, E, and B contain an internal cluster of hydrophobic amino acid residues, which is to be expected for proteins destined for the ER. Precursor venom proteins are synthesized and transported into the ER lumen where protein disulfide isomerase accelerates the formation of disulfide bonds. Exportable proteins from the RER are transported into condensing vacuoles via a well-developed Golgi complex. The vacuoles are transformed into dense secretory granules which are the immediate source of secreted venom proteins. Venom proteins are secreted into the extended gland lumen where they are stored extracellularly (Sobol-Brown et al., 1975). Asynchronous synthesis of venom components has been reported following gland evacuation (Sobol-Brown et al., 1975; Taylor et al., 1986). Asynchronous transcription could explain why full-copy cDNA was not isolated for the dendrotoxins K, I, E, and B. It may be that the poly(A)$^+$ RNA encoding these proteins was not isolated at the ideal time after gland evacuation. In any case, the lack of a full-copy cDNA for dendrotoxin K was not a problem because our goal was to express the toxins in a heterologous host system; thus, the leader and nontranslated sequences were removed *in vitro* before subcloning them into an expression vector.

We attempted to express DTX$_K$ as a fusion protein by using two different constructs. As product yields are normally substantially higher for cytoplasmic expression, the first construct encoded a fusion protein that lacked a signal peptide for transport into the periplasmic space. The yields of cytoplasmic MBP-fusion protein were routinely 55 mg per liter of *E. coli* cells. However, recovery of recombinant DTX$_K$ from a factor Xa-cleaved cytoplasmic fusion protein was very low. On a weight basis, 55 mg of fusion protein should have yielded approximately 8 mg of the toxin. Our best recovery was 0.6 mg of recombinant DTX$_K$. Endogenous *E. coli* proteases made it difficult to obtain large quantities of recombinant DTX$_K$ even in the presence of protease inhibitors. In an attempt to reduce or eliminate the protease problem, we used a second construct encoding a fusion protein that included a MBP-signal peptide. The yields of fusion product isolated from the periplasmic space of *E. coli* by this recombinant plasmid have consistently been 15 mg per liter of culture and 1.5–2 mg of DTX$_K$ after cleavage from the fusion protein and purification on FPLC.

Like most animal toxins, DTX$_K$ possesses multiple disulfide bonds which are required for biological activity (Hollecker, 1983). It is known that in *E. coli* only the periplasmic space contains enzymes necessary to form disulfide bonds. With the fusion protein expressed into the periplasmic space, it was not only protected from protease degradation but also reached an environment where its biologically active conformation

could predominate. The purified recombinant DTX_K was characterized in four different ways: by its chromatographic behavior on an ion-exchange matrix relative to the native toxin, by end-terminal amino acid sequence, by an *in vivo* assay, and by receptor assay. In all cases, the recombinant DTX_K behaved like the native protein. The first 17 amino acids sequenced from the recombinant protein are also identical to those of the naturally occurring DTX_K. Therefore, we conclude that the recombinant toxin expressed and transported into the periplasmic space of *E. coli* as a fusion protein, and subsequently cleaved from its escort, represents authentic DTX_K.

Recently, the three-dimensional structure of a homolog of DTX_K has been determined crystallographically (Skarzynski, 1992). The refined tertiary structure of α-DTX shows two well-defined regions of secondary structure. In addition to a short length of α-helix, there is a region composed of a double-stranded, antiparallel β-sheet in which the β-sheet is twisted about 180°. There are three lysine residues between the β-sheets, which may be important for its inhibitory action on K^+ channels and its associated neurotoxicity. We have begun a detailed study of the structure–activity relationships of DTX_K and DTX_I, employing the technique of site-directed mutagenesis to alter the cloned genes. The mutant proteins generated by this method should yield conclusive evidence as to which residues of the toxin are necessary for binding to K^+ channels. Already, site-directed mutagenesis has been used to identify residues in an expressed neuronal K^+ channel that participate in the interaction with α-DTX (Hurst et al., 1991), and, interestingly, these are located at the external mouth of the ion filter.

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