Denmotoxin, a Three-finger Toxin from the Colubrid Snake *Boiga dendrophila* (Mangrove Catsnake) with Bird-specific Activity*

Received for publication, June 19, 2006, and in revised form, July 21, 2006 Published, JBC Papers in Press, July 24, 2006, DOI 10.1074/jbc.M605850200

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colubrid snake venom toxin (α-colubritoxin from -
), a reversible antagonist of chick muscle nAChRs (18). The large polyphyletic family Colubridae consists of approximately two-thirds of the described species of the advanced snakes and includes >700 different venomous species (19, 20). Colubrid snakes possess a venom gland (the Duvernoy's gland) with a common duct extending to the posterior lingual region of the maxilla, commonly associated with grooved or enlarged posterior maxillary teeth. Relatively little is known of the chemistry, toxicology, and immunology of colubrid venoms, and a more thorough investigation of colubrid venom components holds great promise for discovering new bioactive compounds (21, 22).

In this work, we report the isolation, purification, and pharmacological and structural characterization of a novel

drophila1

H_2O). BioMultiView software was used to analyze the mass spectra.

— pyroglutamate aminopeptidase digestion was performed to remove the pyroglutamic acid residue that was blocking the N terminus of the protein. Enzymatic digestion was carried out in 50 mM sodium phosphate (pH 7.0) containing 10 mM dithiothreitol and 1 mM EDTA at a ratio of 2 milliunits of enzyme to 1 nmol of protein. The reaction was incubated for 10 h at 50 °C, followed by N-terminal sequencing by automated Edman degradation using an ABI Procise 494 pulsed-liquid phase protein sequencer (PerkinElmer Life Sciences) with an on-line 785A phenylthiohydantoin-derivative analyzer.

—Lyophilized protein (500 g) was dissolved in 500 l of 0.13 M Tris-HCl, 1 mM EDTA, and 6 M guanidine HCl (pH 8.5) and reduced with 10 l of β-mercaptoethanol. This mixture was incubated at 37 °C for 2 h under N_2. Subsequently, 100 l of alkylating reagent (4-vinylpyridine) was added, and the mixture was incubated for another 2 h under N_2 at room temperature in the dark. The alkylated toxin mixture was immediately loaded onto a RPC C2/C18 column attached to a Smart workstation (Amersham Biosciences AB). The column was washed for 30 min with solvent A (0.1% trifluoroacetic acid in H_2O), followed by protein elution with a linear gradient of 0–50% solvent B (80% acetonitrile and 0.1% trifluoroacetic acid in H_2O). Lyophilized pyridylethylated protein was then subjected to enzymatic digestion with Lys-C endopeptidase or trypsin. Protein (250 g) was dissolved in 250 l of enzymatic digestion buffer (50 mM Tris-HCl, 4 M urea, and 5 mM EDTA (pH 7.5)), and proteases were added at a ratio of 1:50 (w/w). Cleavages were performed at 37 °C for 16 h. The peptides generated were separated on a RPC C2/C18 column attached to the same chromatography system using a linear gradient of 15–40% solvent B over 1 h (solvent A = 0.1% trifluoroacetic acid in H_2O and solvent B = 80% acetonitrile and 0.1% trifluoroacetic acid in H_2O). The flow rate was 200 l/min. Eluted peptides were monitored at 215, 254, and 280 nm. The amino acid sequences of the peptides were obtained by automated Edman degradation using an ABI Procise sequencer.

—5 days after milking, the specimen was killed by an overdose of CO_2. The pair of venom glands was dissected and stored in RNA at −80 °C until used. Total RNA extraction was performed using a rotor homogenizer and an RNeasy mini kit. The amount of RNA was calculated according to the absorbance of the sample at 260 nm. ( )- —Based on the available amino acid sequence of the protein, the following degenerate primers were designed to obtain partial cDNA sequence: RT-forward, 5′-GCICCIGTRCARCAYTTIAC-3′; and RT-reverse, 5′-TGYTTIGCIGTIGGMCACAT-3′. One-step RT-PCR was carried out using 100 ng of total RNA under the following conditions: RT at 50 °C for 30 min, followed by activation of HotStartTaq polymerase at 95 °C for 15 min and 30 cycles of three-step PCR (94 °C for 1 min, 50 °C for 1 min, and 72 °C for 1 min) and a final extension at 72 °C for 10 min. PCR products were subjected to 1% agarose gel electrophoresis and visualized by ethidium bromide staining.

- —Amplification products were purified using a PCR purification kit, ligated with the pDrive vector, and transformed by a heat shock method into competent DH5α cells. Selection of the transformants (blue/white colony screening) was performed on LB-agar plates containing 100 g/ml ampicillin and supplemented with X-gal (isopropyl β-D-thiogalactopyranoside and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). The sizes of the inserts were estimated by EcoRI digestion, followed by 1% agarose gel electrophoresis and ethidium bromide visualization. All sequencing reactions were carried out with an ABI PRISM 3100 automated DNA sequencer using a BigDye terminator cycle sequencing ready reaction kit according to the manufacturer's instructions. All clones were sequenced in both directions using T7 and SP6 sequencing primers.

—Sequence analysis was carried out using the BLAST program at the NCBI Database (www.ncbi.nlm.nih.gov) and ExPASy proteomics tools (www.expasy.ch). Sequence alignments were carried out using the ClustalW program (www.ebi.ac.uk) or DNAMAN Version 4.15 (Lynnon Corp.).

—Chemical synthesis and assembly of protein were performed with an Applied Biosystems 431 peptide synthesizer using a modified version of the Applied Biosystems standard 0.1 mmol small-scale program (26) with -hydroxybenzotriazole as the coupling reagent and -methylpyrrolidone as the solvent. Fmoc-protected amino acids were used with the following side chain protections: -butyl ester (Glu and Asp), -butyl ether (Ser, Thr, and Tyr), -butylcarbonyl (Lys), trityl (Cys, His, Asn, and Gln), 2,2,5,7,8-pentamethylchromane-6-sulfonyl (Arg), and -butyloxycarbonyl (Trp). The toxin was assembled on Fmoc-Asp- -butoxy-Wang resin (loading, 0.55 mmol/g) (27). Synthesis was performed using the improved software armed with UV deprotection step monitoring, which, in the case of deprotection failure, automatically extends the deprotection and coupling time for the particular amino acid (28). After each coupling, the resin was acetylated with a mixture of 5% acetic anhydride and 6% 2,4,6-collidine in dimethylformamide. At the end of the synthesis, the peptide resin was treated with a mixture of trifluoroacetic acid (90%), triisopropylsilane (5%), and distilled water (5%) to cleave the peptide from the resin and to remove the protecting groups from amino acid side chains. After 2 h of incubation at room temperature with constant mixing, the crude material was filtered and precipitated in cold -butyl methyloxide. Precipitates were washed three times with -butyl methyloxide, followed by centrifugation at 1700 × and resuspension in 10% acetic acid. Lyophilized crude synthetic toxin was then

water to evaluate protein concentration based on absorbance at 278 nm. It was then diluted with refolding buffer (0.1 M Tris-HCl (pH 7.8) and 0.5 M guanidium chloride, GSH, and GSSH at a molar ratio of 10:1) to a concentration of 0.05 mg/ml and incubated for 2–4 days in 4 °C. The mixture was acidified with 20% trifluoroacetic acid and purified on a Discovery® BIO wide pore C5 column with a gradient of 30–50% solvent B over 40 min (solvent A = 0.1% trifluoroacetic acid in H_2O and solvent B = 60% acetonitrile and 0.1% trifluoroacetic acid in H_2O). The flow rate was 3 ml/min, the elution was followed at 215 and 280 nm. The synthetic and native toxins were compared by co-elution profiles and circular dichroism spectra.

—The native protein was injected intraperitoneally into male Swiss-Webster mice (19 ± 2 g) at 0.1, 1, 10, and 20 mg/kg (= 2). All doses were made up to a volume of 200 l in saline. The same volume of normal saline was injected intraperitoneally into control animals. Mice were observed for 6–8 h and killed after 24 h for autopsy. Mice (19 ± 2 g) were injected intracerebroventricularly with the protein at 0.1, 1, and 10 mg/kg in 5 l of saline (= 2 per dose) or with 5 l of control saline (= 2) using a fine capillary Hamilton microsyringe. Animals were killed for autopsy after 5 h of observation.

( ) —Pairs of CBCMs were isolated from 6–10-day-old chicks and mounted in an 8-ml organ bath (29) containing Krebs solution continuously aerated with 5% CO_2 in O_2. The temperature of the organ bath was maintained at 35 °C. The resting tension of the isolated tissue was adjusted to 1 g. Electrical field stimulation was carried out through platinum ring electrodes using an S88 stimulator (Grass Instruments, Quincey, MA). The data were transferred via a Model FT03 force displacement transducer (Grass Instruments) and recorded using a PowerLab 6 system (ADInstruments, Bella Vista, New South Wales, Australia). Maximal twitch responses of the muscle were elicited indirectly via stimulation of the motor nerve by applying an electrical field of 7–10-V potential difference at a frequency of 0.2 Hz in supramaximal rectangular pulses of 0.1-ms duration. The preparation was equilibrated for 30–50 min before the beginning of an experiment, with changes of Krebs solution at 10-min intervals. To ensure selective stimulation of the motor nerve, -tubocurarine (10 M) was added, with subsequent abolition of twitches, which were then re-established after a thorough wash. At appropriate intervals, submaximal control contractures to exogenously applied 300 M acetylcholine, 8 M carbachol, or 30 mM KCl were obtained in the absence of an electrical field. The contact times for these agonistic drugs were 30, 90, and 60 s, respectively, followed by a wash with 100 ml of fresh Krebs solution. The electrical stimulation was then recommenced; and after stabilization of twitch height, the effects of various concentrations of denmotoxin (0.1–100

RP-HPLC, and the individual fractions from RP-HPLC were assessed by electrospray ionization mass spectrometry (ESI/MS) (data not shown). Peak 2 from the cation exchange column (Fig. 1) contained the protein of interest (Fig. 1

RESULTS

—A liquid chromatography/mass spectrometry profile revealed that the most abundant proteins in the crude venom have molecular masses 8–10 kDa (25). Among them, we have identified one major component with a mass of 8508 Da (Fig. 1). This protein was purified to homogeneity by consecutive cation exchange chromatography (Fig. 1) and RP-HPLC (Fig. 1). venom was separated into 10 fractions on a UNO S6 column (Fig. 1). Each peak was then subjected to

class of non-conventional 3FTXs (3). Denmotoxin differs structurally from elapid 3FTXs in having seven additional residues at its N terminus, which is blocked by a pyroglutamic acid residue (Fig. 2). In this respect, it is similar to 3FTXs isolated from colubrid venoms such as α-colubritoxin (18) and boigatoxin-A (25). Denmotoxin shares ~ 50% identity with α-colubritoxin, but it shows <30% identity to elapid 3FTXs (Fig. 2) and only ~ 15–20% identity if the five disulfide bridges (which contribute significantly to the percentage of identity) are excluded.

The investigation of colubrid venom components is severely limited because of the tedious extraction procedure and low venom yields (23). Therefore, we chemically assembled denmotoxin by solid-phase peptide synthesis. The yield from peptide synthesis was ~ 30% based on deprotection monitoring at 301 nm. Reduced crude peptide was purified from contaminating side products by RP-HPLC (Fig. 3). The major peak contained a homogeneous peptide with a molecular mass of 8517.38 ± 0.44 Da as assessed by ESI/MS (Fig. 3), which precisely matched the calculated mass for the reduced form of denmotoxin (8517.8 Da). This reduced synthetic denmotoxin was subjected to glutathione-mediated oxidation, followed by acidification and RP-HPLC purification. The oxidized peptide eluted as a major component ~ 10 min earlier than the reduced form (data not shown); therefore, for greater purification, the gradient was modified (Fig. 3). The oxidized synthetic denmotoxin was homogeneous, with a molecular mass of 8507.70 ± 0.22 Da (Fig. 3), which exactly matched the mass of the native protein. Furthermore, the native and synthetic proteins were shown to be identical based on co-elution profiles by RP-HPLC (Fig. 3) and CD spectroscopy analysis (data not shown). To compare the pharmacological action of the synthetic and native denmotoxin proteins, we tested both preparations in CBCM experiments. Our results indicate that they displayed identical physicochemical and pharmacological properties.

—Denmotoxin caused time- and concentration-dependent blockage of the

In contrast, denmotoxin showed a very weak effect on neuromuscular transmission in MHD preparations. The blockage produced by denmotoxin in MHD was up to 100-fold smaller than that produced in CBCM, and denmotoxin was not able to produce complete blockage of indirectly stimulated twitches up to 11.76 μM (Fig. 4). Moreover, unlike irreversible denmotoxin-induced blockage in CBCM, that in MHD was rapidly reversed by washing (Fig. 4). In contrast, the positive control (α-bungarotoxin) used in MHD experiments virtually irreversibly blocked with a similar dose-response curve as in CBCM experiments (Fig. 4). Thus, denmotoxin induces partial and reversible blockage of neurotransmission in MHD. In agreement with this finding, we observed no paralysis or other biological effects in mice after intraperitoneal or intracerebroventricular injection of the toxin at up to 20 and 10 mg/kg, respectively. Taken together, these results indicate a clear species-dependent toxicity and susceptibility of chick muscle nAChR to denmotoxin.

—We determined the three-dimensional structure of denmotoxin, a unique 3FTX with an unusually long N-terminal segment, by crystallization and

In an attempt to obtain denmotoxin in larger quantities, we synthesized it by solid-phase peptide synthesis using Fmoc chemistry. The synthesis of denmotoxin without using chemical ligation was challenging, as the protein is a 77-amino acid residue long β-sheeted polypeptide that is rich in disulfide bridges and therefore prone to aggregation and incorrect disulfide formation. To enhance the assembly, UV monitoring of deprotection and automatic extension of the deprotection time to 20 min and of the coupling time to 30 min were used when the deprotection was not sufficient after two successive 3-min deprotections. Using this method, the overall yield of synthesis was. 30%, which is much higher than often obtained with peptides of this length. Peptide synthesis allowed us to obtain considerable quantities of the toxin without resorting to traditional purification methods. It should be emphasized that the oxidized synthetic peptide has all the biochemical characteristics of the natural toxin and was virtually indistinguishable from the native toxin in organ bath experiments. These results ruled out the possibility that the 3(a)ivty tf tative denmotoxin dma tb nuesihow

such as acetylcholine. We compared the amino acid sequences of chick (α