Isolation and Pharmacological Characterization of Cannitoxin, a Presynaptic Neurotoxin from the Venom of the Papuan Taipan (Oxyuranus scutellatus canni)

Sanjaya Kuruppu, Shane Reeve, Yajnavalka Banerjee, R. Manjunatha Kini, A. Ian Smith, and Wayne C. Hodgson

Monash Venom Group, Department of Pharmacology, Monash University, Victoria, Australia (S.K., W.C.H.); Department of Biochemistry and Molecular Biology, Monash University, Victoria, Australia (S.R., A.I.S.); and Department of Biological Sciences, Faculty of Science, National University of Singapore, Singapore, Singapore (Y.B., R.M.K.)

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ABSTRACT

The Papuan taipan (Oxyuranus scutellatus canni) is widely distributed throughout much of Papua New Guinea. Although neurotoxicity is a major symptom of envenomation, no neurotoxins have been isolated from this venom. Using a series of size exclusion chromatography steps, we report the isolation of cannitoxin, a presynaptic neurotoxin (44,848 Da) that represents approximately 16% of the whole venom. The toxin displayed high phospholipase A₂ (PLA₂) activity (330 ± 5 μmol/min/mg) and caused concentration-dependent (11–66 nM) inhibition of indirect (0.2 ms; 0.1 Hz; supramaximal V) twitches of the chick biventer cervicis nerve-muscle preparation without effecting nicotinic receptor agonists. Prior addition of CSL Taipan antivenom (5 U/ml) or inhibition of phospholipase A₂ activity by incubation with 4-bromophenacyl bromide prevented the inhibition of twitches. Cannitoxin is composed of three different subunits, α, β, and γ, with the possibility of two β isomers. However, only the α subunit displayed in vitro neurotoxic activity of its own. Thus, cannitoxin is similar in structure and pharmacology to taipoxin, which has been isolated from the closely related Australian species O. scutellatus scutellatus (coastal taipan).

Three species of taipans (genus Oxyuranus) have been identified and are considered to be the world’s most venomous snakes (Sutherland and Tibballs, 2001). These are the Australian coastal taipan (Oxyuranus scutellatus), inland taipan (Oxyuranus microlepidotus), and Papuan taipan (Oxyuranus scutellatus canni) that are found throughout the northern coastal region of Australia (Worrell, 1970), central Australia (Sutherland and Tibballs, 2001), and in the low-lying and mountainous terrain of Papua New Guinea (O’Shea, 1996), respectively. The rank order of potency of the venoms based on murine LD₅₀ (s.c.) data is as follows: O. microlepidotus (0.025 mg/kg) (Broad et al., 1979) > O. scutellatus canni (0.0505 mg/kg) (Sutherland and Tibballs, 2001) > O. scutellatus scutellatus (0.999 mg/kg) (Broad et al., 1979). However, t₉₀ (i.e., time to produce 90% inhibition of indirect twitches) data obtained in the chick biventer cervicis nerve-muscle (CBCNM) preparation indicated that O. scutellatus scutellatus venom is more neurotoxic than that of O. scutellatus canni venom (Crachi et al., 1999b). They also showed that CSL Taipan antivenom (CTPV) was markedly more effective in neutralizing the neurotoxic effects of the Papuan or coastal taipan venoms than that of the inland taipan (Crachi et al., 1999b).

Presynaptic neurotoxins act at the motor nerve terminal to either facilitate (e.g., dendrotoxin) or inhibit (e.g., β-bungarotoxin, taipoxin, and paradoxin) the release of neurotransmitter. All of the presynaptic inhibitors have phospholipase A₂ (PLA₂) activity (Harris, 1991). Taipoxin and paradoxin were isolated from the venoms of O. scutellatus scutellatus (Fohlman et al., 1976) and O. microlepidotus, respectively. Taipoxin is a ternary complex of three subunits, α, β, and γ-taipoxin, that exists in a stoichiometry of 1:1:1 held together by noncovalent interactions. The α subunit is the only subunit with toxic activity on its own. The β subunit is neutral and exists in two isoforms (i.e., β₁ and β₂), which are interchangeable in the complex. The γ subunit is the largest of the three subunits, and it seems to be glycosylated (Fohl-
man et al., 1976). Paradoxin has a similar structure to taipoxin (Fohlman, 1979); however, this neurotoxin has been less well studied, and no reports of the presence of two β isoforms have been located in the literature.

Envenoming by the Papuan taipan is a significant health problem in Papua New Guinea, with 70 to 80 patients being admitted to the Port Moresby General Hospital alone each year (Connolly et al., 1995). Symptoms of envenoming include local tender lymphadenopathy, abdominal pain, coagulopathy, and neurotoxicity (Connolly et al., 1995). However, research on the venom of the Papuan taipan has been limited to a basic pharmacological examination (Crachi et al., 1999a,b), and no neurotoxins have been isolated so far. In particular, it is not known whether the venom contains a presynaptic neurotoxin similar to paradoxin and taipoxin, which have been isolated from the two closely related species. This study reports the isolation and characterization of canitoxin, a presynaptic neurotoxin from the venom of the Papuan taipan.

Materials and Methods

All chromatography separations were performed using a Shimadzu (Kyoto, Japan) high-performance liquid chromatography system (LC-10ATvp pump and SPD-10AVP detector).

Size Exclusion Chromatography

Freeze-dried venom was dissolved in ammonium acetate buffer (0.1 M; pH 5.0), and insoluble material was removed by centrifugation at 5000g for 5 min. The supernatant was applied to a Superdex G-75 column (13 μm; 10 x 300 mm; GE Healthcare, Little Chalfont, Buckinghamshire, UK) equilibrated with ammonium acetate buffer. The sample was eluted at a flow rate of 0.5 ml/min. The purified component was rerun under the same conditions to ensure purity. The eluant was monitored at 280 nm.

Separation of Canitoxin Subunits Using Reverse Phase-High Performance Liquid Chromatography

The freeze-dried, purified component was reconstituted in Milli-Q water (Millipore Corporation, Billerica, MA) and applied to a Phenomenex (Torrance, CA) Jupiter analytical C18 column (150 x 2 mm; 5 μm; 300 A) after equilibrating with solvent A (0.1% trifluoroacetic acid). The sample was eluted with the following gradient conditions of solvent B at a flow rate of 0.2 ml/min: 0 to 20% over 5 min, 20 to 60% in 40 min, and then 60 to 80% over 5 min (Wickramaratna et al., 2004). The eluant was monitored at 280 and 214 nm.

Molecular Mass Determination

Molecular mass was determined by both size exclusion chromatography and mass spectrometry.

Size Exclusion Chromatography on Nondenaturing Media

The molecular mass of the complex was determined by gel filtration on a Superdex G-75 column equilibrated with ammonium acetate buffer (0.1 M; pH 5.0), and insoluble material was removed by centrifugation. The supernatant was applied to a Superdex 75/100 column (Amersham, Uppsala, Sweden) equilibrated with phosphate buffer (0.1 M; pH 5.0), and the column was calibrated with the series of known standards (6500–66,000 Da) using the molecular weight marker kit for gel filtration chromatography (lot no. 093K9307; MW-GF-70; Sigma-Aldrich, St. Louis, MO). The eluant was monitored at 280 nm, and a flow rate of 0.6 ml/min was used. Void volume ($V_v$) of the column was determined by running blue dextran, and the elution volume ($V_e$) was calculated for each molecular weight marker before injecting the purified component (0.5 mg). The molecular weight of the toxin was determined from a plot of log (mol. wt.) versus $V_e/V_v$ ratio.

Mass Spectrometry. MALDI-TOF MS analysis was performed with an Applied Biosystems (Foster City, CA) Voyager-DE STR BioSpectrometry Workstation. The instrument was operated in positive polarity in linear mode using sinapinic acid matrix (Agilent Technologies, Palo Alto, CA) for low-resolution protein analysis. Matrix (1 μl) was spotted on the sample plate and allowed to air-dry; sample (1 μl) diluted in acetonitrile/water (1:1) containing 0.1% (v/v) formic acid was subsequently spotted on dried matrix and allowed to air-dry. Data from 500 laser shots (337-nm nitrogen laser) were collected, and the signal was averaged and processed with the instrument manufacturer’s Data Explorer software.

CBCNM Preparation

Chickens (4–10-day-old males) were sacrificed with CO2, and both biventer cervicis nerve-muscle preparations were dissected. These were mounted under 1-g resting tension in 5-ml organ baths containing physiological salt solution of the following composition: 118.4 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO4, 1.2 mM KH2PO4, 2.5 mM CaCl2, 25 mM NaHCO3, and 11.1 mM glucose. The solution was maintained at 34°C and bubbled with carbogen (95% O2 and 5% CO2).

Motor nerves were stimulated every 1 s (0.2-ms duration) at supramaximal voltage using a Grass S88 stimulator (Harvey et al., 1994). d-Tubocurarine (10 μM) was added, and the subsequent abolition of twitches confirmed the selective stimulation of nerves. Responses to nerve stimulation were re-established by thorough washing. Contractile responses to acetylcholine (ACh; 1 mM for 30 s), carbachol (CCh; 20 μM for 60 s), and KCl (40 mM for 30 s) were obtained in the absence of stimulation (Harvey et al., 1994). The preparations were then equilibrated for at least 30 min with continuous nerve stimulation (as described above) before the addition of toxin. In all experiments, toxin (11–66 nM), subunits (100 nM), or venom (10 μg/ml) was left in contact with the preparations until responses to nerve stimulation were abolished or for a maximum of 5 h if total twitch blockade did not occur. At the conclusion of the experiment, responses to ACh, CCh, and KCl were obtained as described previously. Time taken to reduce the amplitude of the indirect twitches by 90% ($t_{90}$) was calculated to provide a quantitative measure of neurotoxicity (Crachi et al., 1999a).

Where indicated, CTPV (5 U/ml) was added 10 min before the addition of toxin. Reversibility of the venom was tested by adding TPAV (5 U/ml) at $t_{90}$ after the addition of venom (10 μg/ml). Anti-venom was left in contact with the tissue for 2 h.

Determination of PLA2 Activity

$PLA_2$ activity of the venom, canitoxin, and canitoxin subunits was determined using a colorimetric assay kit (Cayman Chemical, Ann Arbor, MI) designed to test the activity of secretory $PLA_2$s. This assay uses the 1,2-dithio analog of diheptanoyl phosphatidylcholine, which serves as a substrate for $PLA_2$ enzymes. Free thiols generated following the hydrolysis of the thioester bond at the sn-2 position by $PLA_2$ are detected using 5,5'-dithio-bis-(2-nitrobenzoic acid). Color changes were monitored using a CERES900C microplate reader (Bio-Tek Instruments, Winooski, VT) at 405 nm, sampling every minute for a 5-min period. $PLA_2$ activity was expressed as micromoles of phosphatidylcholine hydrolyzed per minute per milligram of enzyme.

$PLA_2$ Inhibition with 4-Bromophenacyl Bromide

$PLA_2$ activity of venom was inhibited by alkylation with 4-bromophenacyl bromide (4-BPB). Toxin (11 nM) made up in sodium cacodylate-HCl buffer (0.1 M; pH 6.0) and 4-BPB made up in acetone were added to produce a final concentration of 1.8 mM (Abe et al., 1977; Bell et al., 1998; Crachi et al., 1999a). Vials containing the above-mentioned mixture were incubated for 16 h at 30°C. Where indicated, toxin made up in sodium cacodylate-HCl buffer incubated with acetone was used as a vehicle control for 4-BPB (Wickramaratna et al., 2003).
N-Terminal Amino Acid Sequence Determination

Purified peptides were loaded into the sequencing chamber of a Procise N-terminal amino acid sequencer (Applied Biosystems), and the amino acid sequence was determined (Edman degradation, phenylthiohydantoin derivatization chemistry, and separation of derivatized amino acids by RP-HPLC) using the manufacturer’s recommended methods and reagents.

Chemicals and Drugs

The following drugs were used: acetylcholine chloride, bovine serum albumin, carbamylcholine chloride (carbachol), d-tubocurarine, 4-bromophenacyl bromide, ammonium acetate, molecular weight marker kit (6500–66,000), and cacodylic acid (Sigma-Aldrich); KCl (Ajax Chemicals, Sydney, Australia); trifluoroacetic acid (Auspep, Melbourne, Australia); acetonitrile (Ajax Finechem, Seven Hills, New South Wales, Australia); CSL Taipan antivenom (CSL Ltd., Melbourne, Australia); and acetone (BDH Chemicals, Victoria, Australia).

Analysis of Results and Statistics

In isolated tissue experiments, responses were measured via a Grass PTO3 force displacement transducer and recorded on a PowerLab system (ADInstruments Pty Ltd., Castle Hill, Australia). Twitch height and contractile responses to agonists were expressed as a percentage of the corresponding value before the addition of toxin. Statistical difference was determined by a one-way analysis of variance (ANOVA) on the twitch height at the 300-min time point and on the contractile responses to exogenous agonists. All ANOVAs were followed by a Bonferroni post hoc test, and statistical significance was indicated where \( P < 0.05 \).

Results

Chick Biventer Studies: Whole Venom

Venom from the Papuan taipan displays postsynaptic neurotoxic activity (Crachi et al., 1999a) that is prevented by CSL Taipan antivenom (Crachi et al., 1999b). In the current study, taipan antivenom (5 U/ml) added at \( t_{90} \) (44 ± 8.2 min) failed to reverse or prevent further inhibition of indirect twitches over a period of 2 h (Fig. 1a). However, antivenom restored the response of the tissue to exogenous ACh and CCh (Fig. 1b). This confirmed the presence of a presynaptic neurotoxin and indicated that only the postsynaptic neurotoxic effects of the venom can be reversed by taipan antivenom. Therefore, we decided to purify and characterize the presynaptic neurotoxin.

Size Exclusion Chromatography

Cannitoxin was isolated from the venom of the Papuan taipan following successive separations by size exclusion chromatography. Initial fractionation on a Superdex G-75 column produced six main peaks (Fig. 2a). Screening in the chick biventer cervicis nerve-muscle preparation indicated the presence of four presynaptic toxins using size exclusion chromatography and RP-HPLC. Cannitoxin elutes as clean peak with an approximate retention time of 20 min (Fig. 2b), and it accounts for approximately 16% of the whole venom.
peaks in MALDI spectra (data not shown). MALDI analysis also indicated a possible glycosylation in one of the subunits.

In addition, individual subunits purified by RP-HPLC were analyzed by MALDI-TOF. The subunits were found to be of the following molecular masses: 13,242 Da (α9252), 13,276 Da (α9253), and 17,762 Da (α9253). MALDI-TOF analysis showed the subunit (17,762 Da) to be heterogeneously glycosylated (data not shown), confirming the data mentioned above. These masses are in agreement with those observed upon initial MALDI-TOF analysis of the protein complex and were also confirmed by electrospray ionization-MS. The sum of the molecular masses of α, γ, and β1 or β2 subunits results in an average mass of 44,848 Da, which is in agreement with that determined by size exclusion chromatography.

N-Terminal Amino Acid Sequence

Partial N-terminal amino acid sequence of the isolated subunits of canntoxin was determined using Edman degradation (Table 1). These were compared with the protein sequences of the corresponding subunits of taipoxin at the National Center for Biotechnology Information database using the BLAST service. The subunits showed sequence identity in the order β1 (100%) > γ (86%) > α (85%), based on the partial N-terminal sequence. Since the other subunit is structurally similar to β1, we identified this subunit as β2. The N-terminal sequence of β2 taipoxin was not listed in the database or in the literature; hence, comparisons cannot be made.

Chick Biventer Studies

Canntoxin. Canntoxin (11–66 nM) caused concentration-dependent inhibition of indirect twitches in the CBCNM preparation (Fig. 4a), but it had no effect on the response of the tissue to ACh, CCh, or KCl (Fig. 4b), indicating its action at the presynaptic terminal. Canntoxin incubated with 4-BPB, or TPAV added before the addition of canntoxin, resulted in prevention of the toxin-induced inhibition of indirect twitches (Fig. 4a), but it had no significant effect on the response of the tissue to exogenous agonists (Fig. 4b). Vehicle (i.e., BSA) and canntoxin in the presence of vehicle (i.e., acetone) had no effect on the agonist responses (data not shown).

Subunits of Canntoxin. The γ and β2 subunits (100 nM) of canntoxin had no significant effect on indirect twitches of the CBCNM preparation. However, the α subunit (100 nM) caused a slight but significant inhibition of indirect twitches compared with the vehicle (Fig. 5a). None of the subunits had a significant effect on the response of the tissue to agonists (Fig. 5b).

Phospholipase A2 Activity

Venom had a specific activity of 330 ± 5 μmol/min/mg, whereas that of the positive control (i.e., bee venom) was...
344 ± 26 μmol/min/mg. 4-BPB significantly inhibited the PLA₂ activity of taipoxin, determined previously (Fohlman, 1979), is included for comparison. The α-subunit seems to be the only subunit with a significant level of activity in cannitoxin and taipoxin (Table 2).

Discussion

This study describes the isolation and the pharmacological characterization of the first presynaptic neurotoxin, cannitoxin, from the venom of O. scutellatus canni. The venom from the Papuan taipan has postsynaptic neurotoxic activity (Crachi et al., 1999a) that is neutralized by the prior addition of taipan antivenom (Crachi et al., 1999b). However, in the current study, antivenom added at the t₉₀ time point failed to reverse the indirect twitches, indicating the presence of a presynaptic neurotoxin(s). Such neurotoxins are unable to be reversed by the addition of antivenom once they have been bound and internalized (Fohlman et al., 1976) due to the physical damage to the presynaptic membrane by phospholipid hydrolysis.

The molecular mass of cannitoxin was determined as 45,000 Da using a series of known molecular weight standards on nondenaturing media. RP-HPLC of cannitoxin produced four main peaks, the molecular masses of...
which do not add up to 45,000 Da. The similar molecular mass of the \( \beta_1 \) and \( \beta_2 \) components suggests the possibility of isomerism, which is confirmed by the high sequence similarity (90%) between these two peptides. Furthermore, a combined molecular mass of 44,848 Da (consisting of the sum of \( \alpha, \gamma \), plus \( \beta_1 \) or \( \beta_2 \) subunits) is in agreement with the molecular mass estimated from size exclusion chromatography. The average molecular mass of cannitoxin can thus be calculated as 44,912 Da. MALDI-TOF analysis revealed the \( \gamma \) subunit of cannitoxin to be a glycoprotein as evidenced by the areas of heterogenous glycosylation observed in the MALDI spectra. High similarity of the partial N-terminal sequences was observed between the corresponding subunits of taipoxin and cannitoxin. Therefore, it seems that cannitoxin is similar in structure to that of taipoxin, isolated from the coastal taipan, which consists of an \( \alpha \) subunit, two isomers (see comments above) of the \( \beta \) subunit, and a glycosylated \( \gamma \) subunit (Fohlman et al., 1977).

Cannitoxin was examined for in vitro neurotoxic effects using the CBCNM preparation. Cannitoxin caused concentration-dependent inhibition of the indirect twitches, with a lack of effect on contractile responses to exogenous nicotinic agonists, confirming its presynaptic activity. Reduction of twitches by cannitoxin is triphasic with an initial decrease and a transient increase followed by the complete inhibition of indirect twitches. This triphasic effect is commonly associated with other presynaptic neurotoxins such as taipoxin, notexin, and \( \beta \)-bungarotoxin (Harris, 1991). The initial two phases seem to be independent of PLA\(_2\) activity (Harvey, 1990), and they are particularly evident when the safety factor of transmission is lowered by reducing the \( \mathrm{Ca}^{2+} \) or increasing the \( \mathrm{Mg}^{2+} \) content of the bathing medium (Chang et al., 1977). Therefore, cannitoxin is similar in pharmacology and toxicology to taipoxin, which causes the inhibition of nerve-mediated twitches in the CBCNM with a lack of effect on contractile responses to exogenous nicotinic agonists (Crachi et al., 1999).

PLA\(_2\) enzymes are found in the venoms of snakes of all families (Harris, 1991); hence, the whole venom, cannitoxin, and its subunits were examined for PLA\(_2\) activity. The level of activity displayed by cannitoxin was approximately 3-fold less than that of the whole venom, suggesting the presence of other venom components with PLA\(_2\) activity. Although there is no quantitative relationship between the potency and PLA\(_2\) activity of presynaptic neurotoxins, inhibition of enzymatic activity is known to prevent their toxic effects (Yang, 1997). Thus, to test whether the PLA\(_2\) activity of cannitoxin is essential for its toxic effects, it was subjected to 4-BPB modification. Previous studies have shown that PLA\(_2\) activity can be inhibited by selective acylation of His48 residue using 4-BPB (Volwerk et al., 1974; Abe et al., 1977). When cannitoxin was incubated with 4-BPB, PLA\(_2\) activity as well as the neurotoxic effects was abolished. This suggests that PLA\(_2\) activity is essential for the neurotoxic effects of cannitoxin. Similarly, modification by 4-BPB inhibits the effects of other presynaptic neurotoxins such as taipoxin (Fohlman et al., 1979) and \( \beta \)-bungarotoxin (Abe et al., 1977). Further similarity between cannitoxin and taipoxin is highlighted by the PLA\(_2\) activity being largely confined to the \( \alpha \)-subunit of canni- toxin.

Given the medical importance of this species, it was important to assess the efficacy of the commercially available taipan antivenom in neutralizing the effects of cannitoxin. Prior addition of antivenom prevented the toxin-induced inhibition of indirect twitches in the CBCNM preparation. Addition of antivenom after the addition of toxin was not undertaken in this study since a similar experiment involving whole venom confirmed the irreversible nature of presynaptic neurotoxins.

Presynaptic neurotoxins with PLA\(_2\) activity can be classified based on structure as being single, dimeric, or multichain complexes (Yang, 1997). Multichain neurotoxins consist of several different polypeptide chains held together by noncovalent interactions, with at least one of the subunits having toxic activity on its own (Yang, 1997). However, previous studies have shown that the activity of the toxic subunit is far less in comparison with the native toxin (Fohlman et al., 1976; Francis et al., 1993). Therefore, the subunits of cannitoxin were tested for their neurotoxic effects. Despite having a high level of PLA\(_2\) activity, only the \( \alpha \) subunit at a much higher concentration (100 nM) caused a slight but statistically significant decrease in the indirect twitches. This further proves the lack of any quantitative relationship between enzymatic and toxic activity as well as the possibility of the other subunits acting as chaperones (Fohlman et al., 1976). The toxic effects and PLA\(_2\) activity of the \( \beta_1 \) isosubunit could not be tested because of its low abundance in the venom and hence the small amount isolated. However, because the \( \beta_1 \) and \( \beta_2 \) subunits of cannitoxin are isomers and given the high sequence identity between \( \beta_1 \) taipoxin and \( \beta_1 \) cannitoxin, it is unlikely that the latter subunit has any neurotoxic activity. Neither \( \beta \) subunit of taipoxin is known to have toxic activity (Fohlman et al., 1976).

In conclusion, cannitoxin is the first neurotoxin to be isolated from the venom of the Papuan taipan. Cannitoxin is a presynaptic neurotoxin consisting of three different subunits held together by noncovalent interactions. The toxic effects of cannitoxin can be neutralized by commercially available CTPV and are dependent on its PLA\(_2\) activity. Cannitoxin is a major neurotoxic component of the venom; thus, it is likely to be responsible for much of the neurotoxic effect observed in envenomed patients.

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References


Address correspondence to: Dr. Wayne C. Hodgson, Monash Venom Group, Department of Pharmacology, Monash University, Victoria 3800, Australia. E-mail: wayne.hodgson@med.monash.edu.au