

Isolation and pharmacological characterization of a phospholipase A₂ myotoxin from the venom of the Irian Jaya death adder (*Acanthophis rugosus*)

¹Janith C. Wickramaratna, ^{2,3}Bryan G. Fry, ⁴Marie-Isabel Aguilar, ²R. Manjunatha Kini & ^{*1}Wayne C. Hodgson

¹Monash Venom Group, Department of Pharmacology, P.O. Box 13E, Monash University, Victoria 3800, Australia;

²Department of Biological Sciences, Faculty of Science, National University of Singapore, Singapore 119260; ³Australian

Venom Research Unit, Department of Pharmacology, University of Melbourne, Victoria 3010, Australia and ⁴Department of Biochemistry and Molecular Biology, P.O. Box 13D, Monash University, Victoria 3800, Australia

1 It has long been thought that death adder venoms are devoid of myotoxic activity based on studies done on *Acanthophis antarcticus* (Common death adder) venom. However, a recent clinical study reported rhabdomyolysis in patients following death adder envenomations, in Papua New Guinea, by a species thought to be different to *A. antarcticus*. Consequently, the present study examined *A. rugosus* (Irian Jaya death adder) venom for myotoxicity, and isolated the first myotoxin (acanmyotoxin-1) from a death adder venom.

2 *A. rugosus* (10–50 µg ml⁻¹) and acanmyotoxin-1 (MW 13811; 0.1–1 µM) were screened for myotoxicity using the chick directly (0.1 Hz, 2 ms, supramaximal V) stimulated biventer cervicis nerve-muscle (CBCNM) preparation. A significant contracture of skeletal muscle and/or inhibition of direct twitches were considered signs of myotoxicity. This was confirmed by histological examination.

3 High phospholipase A₂ (PLA₂) activity was detected in both *A. rugosus* venom (140.2 ± 10.4 µmol min⁻¹ mg⁻¹; n=6) and acanmyotoxin-1 (153.4 ± 11 µmol min⁻¹ mg⁻¹; n=6). Both *A. rugosus* venom (10–50 µg ml⁻¹) and acanmyotoxin-1 (0.1–1 µM) caused dose-dependent inhibition of direct twitches and increase in baseline tension (n=4–6). In addition, dose-dependent morphological changes in skeletal muscle were observed.

4 Prior incubation (10 min) of CSL death adder antivenom (5 units ml⁻¹; n=4) or inactivation of PLA₂ activity with 4-bromophenacyl bromide (1.8 mM; n=4) prevented the myotoxicity caused by acanmyotoxin-1 (1 µM).

5 Acanmyotoxin-1 (0.1 µM; n=4) displayed no significant neurotoxicity when it was examined using the indirectly (0.1 Hz, 0.2 ms, supramaximal V) stimulated CBCNM preparation.

6 In conclusion, clinicians may need to be mindful of possible myotoxicity following death adder envenomation in Irian Jaya.

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Abbreviations: 4-BPB, 4-bromophenacyl bromide; CBCNM, chick biventer cervicis nerve-muscle; CCh, carbachol; PLA₂, Phospholipase A₂; TFA, trifluoroacetic acid

Introduction

Death adders (genus *Acanthophis*) are unique among Australian snakes in both morphology and behaviour. Although classified into the Elapidae family of snakes they are viper-like in appearance and habit (Campbell, 1966). They are characterized by a somewhat flattened, almost triangular head and a short, stout body terminating to a thin rat-like tail (Cogger, 2000). This makes them among the most specialized of all elapids and closely convergent in many respects with members of the family Viperidae.

Death adders are the widest ranging of the Australian elapids, being found not only in continental Australia, but

North throughout the Torres Straight Islands, Papua New Guinea, Irian Jaya and the Indonesian islands of Seram, Halmahera, Obi and Tanimbar. Although there have been up to 12 species and three subspecies of death adders described thus far (Hoser, 1998), considerable debate remains about species identification (Wuster *et al.*, 1999). Of these, only the venoms of the common (*A. antarcticus*) and northern (*A. praelongus*) death adders have been studied in some detail. However, recently the venoms of the major species and regional variants have been investigated by liquid chromatography/mass spectrometry (Fry *et al.*, 2002). This study revealed a great diversity in the venoms.

Previously, using the chick isolated stimulated biventer cervicis nerve-muscle (CBCNM) preparation, we have shown that Irian Jaya death adder (*A. rugosus*) venom (1–

*Author for correspondence;

E-mail: wayne.hodgson@med.monash.edu.au

10 µg ml⁻¹) caused time-dependent inhibition of indirect twitches and blocked contractile responses to exogenous acetylcholine and carbachol (Fry *et al.*, 2001). Thus, suggesting the presence of postsynaptic neurotoxins. In addition, the efficacy of CSL death adder antivenom against the *in vitro* neurotoxicity of *A. rugosus* venom was studied. It was found that CSL death adder antivenom (1 unit ml⁻¹), raised against *A. antarcticus* venom, was markedly less effective against *A. rugosus* venom compared to *A. hawkiei*, *A. praelongus* and *A. pyrrhus* venoms (Fry *et al.*, 2001). However, a higher concentration of antivenom (5 units ml⁻¹) completely neutralized the *in vitro* neurotoxicity of *A. rugosus* venom (Fry *et al.*, 2001). To date, no components have been studied from *A. rugosus* venom.

In contrast to *A. rugosus* venom, *A. antarcticus* venom has previously been examined for lethality, neurotoxicity, myotoxicity and its effects on blood coagulation, both experimentally and clinically (Kellaway, 1929a, b; Campbell, 1966; Broad *et al.*, 1979; Mebs & Samejima, 1980; Sutherland *et al.*, 1981). In addition, five postsynaptic neurotoxins have been isolated and sequenced from *A. antarcticus* venom (Sheumack *et al.*, 1979; Kim & Tamiya, 1981a, b; Sheumack *et al.*, 1990; Tyler *et al.*, 1997). In terms of phospholipase A₂ (PLA₂) components, acanthin I and II, both potent inhibitors of platelet aggregation have been isolated from *A. antarcticus* venom (Chow *et al.*, 1998). In addition, acanthoxin A1 and A2, two PLA₂ isoforms with weak neurotoxic activity, have been isolated from *A. antarcticus* venom (van der Weyden *et al.*, 1997; 2001). Three PLA₂ isoenzymes, praelongins 2bIII, 2cII and 2cIV, with antiplatelet activity have also been isolated from *A. praelongus* venom (Sim, 1998). However, no myotoxic components have been isolated from any death adder venom to date.

It has long been thought that death adder venoms are devoid of myotoxic activity based on studies done on *A. antarcticus* venom. This venom had no myotoxic activity in Rhesus monkeys (*Macaca fascicularis*) (Sutherland *et al.*, 1981). Mebs & Samejima (1980) fractionated *A. antarcticus* venom by size exclusion chromatography. None of the isolated fractions were capable of causing myoglobinuria in mice after subcutaneous injection. Furthermore, *A. antarcticus* venom (30 µg ml⁻¹) had no myotoxic activity *in vitro* in the directly stimulated CBCNM preparation (Wickramaratna & Hodgson, 2001). However, a recent clinical study reported myotoxic activity *in vivo* following death adder envenomations, in Papua New Guinea, by a species thought to be different to *A. antarcticus* (Lalloo *et al.*, 1996). In this study there was one patient who developed renal failure following delayed presentation after a suspected death adder bite. There were significantly elevated creatine kinase levels (median of 411 IU l⁻¹, range of 164–4220 IU l⁻¹) in two thirds of envenomed patients (Lalloo *et al.*, 1996). However, these levels may not be clinically important in terms of causing renal failure (GK Isbister, personal communication 2002). Renal failure and elevated creatine kinase levels suggest rhabdomyolysis and the presence of myotoxic activity in the venom (Sutherland *et al.*, 1981).

The first aim of this study was to examine the venom from death adders (*A. rugosus*) found in Irian Jaya (West Papua) to determine any possible myotoxic activity. Secondly, to isolate and pharmacologically characterize myotoxins from this venom. Thirdly, to determine the effectiveness of CSL

death adder antivenom, which has been raised against *A. antarcticus* venom, in neutralizing myotoxic activity.

Methods

Venom preparation and storage

A. rugosus venom was purchased from Venom Supplies Pty. Ltd., South Australia. Freeze dried venom and stock solutions of venom prepared in 0.1% bovine serum albumin in 0.9% saline (BSA) were stored at -20°C until required.

Fractionation of venom

Freeze dried venom was dissolved in distilled water and filtered through a 0.45 µm Millipore (Bedford, MA, U.S.A.) filter. Reverse phase high performance liquid chromatography (RP-HPLC) separations were performed on the BIOCAD Perfusion Chromatography Workstation (Applied Biosystems, CA, U.S.A.) using Phenomenex Jupiter preparative (250 × 21.2 mm, 10 µ, 300 Å) and semi-preparative (250 × 10 mm, 5 µ, 300 Å) C18 columns. The column was equilibrated with solvent A (0.1% trifluoroacetic acid - TFA) and the sample then eluted with the following gradient conditions of solvent B (90% acetonitrile in 0.09% TFA) and solvent A at a flow rate of 10 ml min⁻¹: 0 to 60% over 60 min (1% gradient) and then 60 to 80% in 5 min (4% gradient). The eluant was monitored at 214 and 280 nm.

The purified component was re-run on a Hewlett Packard series 1100 ChemStation (Agilent Technologies, CA, U.S.A.) using a Phenomenex Jupiter analytical (150 × 2 mm, 5 µ, 300 Å) C18 column. The column was equilibrated with solvent A (0.1% TFA) and loaded with 100 µl of 100 µg ml⁻¹ isolated component. The sample was then eluted with the following gradient conditions of solvent B (90% acetonitrile in 0.09% TFA) and solvent A at a flow rate of 0.2 ml min⁻¹: 0 to 20% over 5 min (4% gradient), 20 to 60% in 40 min (1% gradient) and then 60 to 80% over 5 min (4% gradient). The eluant was monitored at 214 nm.

Molecular mass determination by electrospray mass spectrometry

The sample was dissolved in 50% acetonitrile and analysed using a Perkin-Elmer Sciex API 300 (PE-Sciex, Thronton, Canada) triple quadrupole instrument equipped with an ionspray interface. The ionspray voltage was set at 4600 V and the orifice potential at 30 V. Nitrogen gas was used as a curtain gas with a flow rate of 0.6 l min⁻¹ while compressed air was the nebulizer gas. The sample (10 µl) was injected manually into the LC-MS system and analysed in positive ion mode. Data processing was performed with the aid of the software package Biomultiview (PE-Sciex, Thronton, Canada).

Amino acid sequence determination

Pure peptide (400 µg) was dissolved in 400 µl of 6 M guanidinium hydrochloride and then 8 µl of 2-mercaptoethanol was added. The sample was then vortexed and briefly centrifuged. Subsequently, 80 µl of 4-vinylpyridine was then added, nitrogen gas passed over the sample for 2 min, the

sample sealed airtight and then incubated at 37°C for 2 h. The reduced/alkylated peptide was N-terminally sequenced using Edman degradation chemistry on an Applied Biosystems 494 pulsed-liquid-phased sequencer (Applied Biosystems, CA, U.S.A.).

Determination of phospholipase A₂ activity

The PLA₂ activity of whole venom and isolated component was determined using a secretory PLA₂ colourimetric assay kit (Cayman Chemical, U.S.A.). The assay uses the 1,2-dithio analogue of diheptanoyl phosphatidylcholine as a substrate. Free thiols generated upon hydrolysis of the thio ester bond at the *sn*-2 position by PLA₂ are detected using DTNB (5,5'-dithiobis(2-nitrobenzoic acid)). Colour changes were monitored by the CERES900C microplate reader (Bio-Tek Instruments, U.S.A.) at 405 nm, sampling every min for a 5 min period. PLA₂ activity was expressed as micromoles of phosphatidylcholine hydrolysed per min per milligram of enzyme.

Inactivation of PLA₂ activity with 4-bromophenacyl bromide

PLA₂ activity of acanmyotoxin-1 was inhibited by alkylation with 4-bromophenacyl bromide (4-BPB). Acanmyotoxin-1 (0.1 mM) was made up in sodium cacodylate-HCl buffer (50 µl, 0.1 M, pH 6.0), and 4-BPB made up in acetone was added to give a final concentration of 1.8 mM (Abe *et al.*, 1977; Bell *et al.*, 1998; Crachi *et al.*, 1999a). Each vial containing the above solution was then incubated at 30°C for 16 h. As a positive control, acanmyotoxin-1 (0.1 mM) made up in sodium cacodylate-HCl buffer was incubated with acetone. As a negative control, sodium cacodylate-HCl buffer was incubated with 1.8 mM 4-BPB in acetone.

Chick isolated biventer cervicis nerve-muscle preparation

Male White leg horn chicks aged between 5 and 9 days were killed with CO₂ and both biventer cervicis nerve-muscle preparations were removed. These were mounted under 1 g resting tension in organ baths (5 ml) containing Krebs solution of the following composition (mM): NaCl, 118.4; KCl, 4.7; MgSO₄, 1.2; KH₂PO₄, 1.2; CaCl₂, 2.5; NaHCO₃, 25 and glucose, 11.1. The Krebs solution was bubbled with carbogen (95% O₂ and 5% CO₂) and maintained at 34°C. Indirect twitches were evoked by stimulating the motor nerve every 10 s with pulses of 0.2 ms duration at a supramaximal voltage (Harvey *et al.*, 1994) using a Grass S88 stimulator. After a 30 min equilibration period, d-tubocurarine (10 µM) was added. Subsequent abolition of twitches confirmed selective stimulation of nerves. Twitches were then re-established by thorough washing. Contractile responses to acetylcholine (ACh; 1 mM for 30 s), carbachol (CCh; 20 µM for 60 s) and potassium chloride (KCl; 40 mM for 30 s) were obtained in the absence of stimulation (Harvey *et al.*, 1994). Electrical stimulation was then recommenced and the preparations were allowed to equilibrate for a further 30 min period before commencement of the experiment. Venom or toxin was left in contact with the preparations until complete twitch blockade occurred, or for a 3 h period. Contractile responses to ACh, CCh and KCl were then obtained as previously described.

In experiments determining myotoxicity, direct twitches were evoked by stimulating the muscle directly every 10 s with pulses of 2 ms duration at a supramaximal voltage (Harvey *et al.*, 1994). To achieve selective stimulation of muscle, d-tubocurarine (10 µM) was added and left in the organ bath for the duration of the experiment. *A. rugosus* venom (10–50 µg ml⁻¹), acanmyotoxin-1 (0.1–1 µM), 4-BPB modified acanmyotoxin-1 (1 µM) or relevant controls were left in contact with the preparations for a 3 h period. Where indicated, CSL death adder antivenom (5 units ml⁻¹) was added 10 min prior (Barfaraz & Harvey, 1994; Crachi *et al.*, 1999b; Fry *et al.*, 2001; Wickramaratna & Hodgson, 2001) to the addition of acanmyotoxin-1 (1 µM). A significant contracture of skeletal muscle (i.e. a rise in baseline) and/or inhibition of direct twitches were considered signs of myotoxicity (Harvey *et al.*, 1994).

Morphological studies

After the conclusion of the functional myotoxic experiments the tissues were quickly placed in Tissue Tek and frozen with liquid nitrogen. The tissues were stored at –80°C until required. Using a Leica CM1800 cryostat, tissues were cut into transverse sections (14 µm) and placed onto gelatin-coated slides. Tissue sections were post fixed for 15 min in a solution containing 4% paraformaldehyde in phosphate buffered saline (PBS; (mol l⁻¹) NaCl, 0.137; KH₂PO₄, 0.002; and Na₂HPO₄, 0.008). Tissue sections were routinely stained with haematoxylin and eosin and examined under a light microscope (Olympus BH-2, Olympus Optical Co., Japan). Areas exhibiting typical pathological changes were photographed using an Olympus C-35AD (Olympus Optical Co., Japan) camera and Kodak film (Ektachrome P1600).

Chemicals and drugs

The following drugs and chemicals were used: acetonitrile (Fisher Scientific, U.K.); acetylcholine chloride, 4-bromophenacyl bromide (4-BPB), bovine serum albumin (BSA), cacodylic acid (sodium cacodylate), carbamylcholine chloride (carbachol), d-tubocurarine chloride; eosin, Mayer's Haemalum (Sigma Chemical Co., St. Louis, MO, U.S.A.); trifluoroacetic acid, 4-vinylpyridine (Fluka Chemika-Biochemika, Buchs, Switzerland). Sequencing grade chemicals were obtained from Applied Biosystems (Singapore). Except where indicated, stock solutions were made up in distilled water. 4-BPB was made up in acetone. Death adder antivenom, which is raised against *A. antarcticus* venom in horses, was obtained from CSL Ltd (Melbourne, Australia). All reagents were of analytical grade.

Analysis of results and statistics

For isolated tissue experiments, responses were measured *via* a Grass force displacement transducer (FT03) and recorded on a MacLab System. For both neurotoxicity and myotoxicity studies, twitch height was expressed as a percentage of the pre-treated twitch height. Statistical difference was determined by a one-way analysis of variance (ANOVA) on the twitch height at the 180 min time point. Likewise, a one-way ANOVA was performed on the contractile response induced by the venom and acanmyotoxin-1 at the 180 min

time point. Contractile responses to ACh, CCh and KCl were expressed as a percentage of the respective initial response. These were analysed using either Student's paired *t*-tests or, where stated, compared against the control response *via* a one-way ANOVA. All ANOVAs were followed by a Bonferroni *post hoc* test. Statistical significance was indicated when $P < 0.05$.

Results

Isolation and purification of acanmyotoxin-1

Acanmyotoxin-1 was isolated from *A. rugosus* venom by successive RP-HPLC separations. The initial fractionation of *A. rugosus* venom using a Phenomenex Jupiter preparative column produced eleven major peaks. The eleventh peak was subjected to further purification by RP-HPLC. In order to determine homogeneity and location of acanmyotoxin-1 in relation to other peaks of the whole venom both *A. rugosus* venom and acanmyotoxin-1 were run on the same conditions using a Phenomenex Jupiter analytical column (Figure 1a,b). Acanmyotoxin-1 eluted as a clean peak separating away from minor contaminants at about 47.5% acetonitrile or at 32.5 min.

Purity and molecular mass determination

Homogeneity and molecular mass of acanmyotoxin-1 were determined by electrospray mass spectrometry (Figure 2). The mass spectra of purified acanmyotoxin-1 displayed several charged states and these could be reconstructed into a single molecular mass of 13811.38 ± 0.81 daltons.

N-terminal amino acid sequence

The N-terminal amino acid sequence of acanmyotoxin-1 was determined (Table 1). The location of half-cystines was typical of elapid PLA₂ enzymes. The N-terminal sequence of acanmyotoxin-1 was compared with other protein sequences at the National Center for Biotechnology Information (NCBI) database using the BLAST service. Acanmyotoxin-1 shared highest identity with taipoxin α chain (75%) from the coastal taipan (*Oxyuranus s. scutellatus*) and Pa-1G (65%) from the Australian king brown snake (*Pseudechis australis*). Acanmyotoxin-1 shared lower identity with other previously isolated death adder PLA₂ components such as acanthin II (55%) and acanthin I (51%).

Phospholipase A₂ activity

High PLA₂ activity was detected in both *A. rugosus* venom and the isolated component acanmyotoxin-1. *A. rugosus* venom had a specific activity of $140.2 \pm 10.4 \mu\text{mol min}^{-1} \text{mg}^{-1}$ ($n = 6$) while acanmyotoxin-1 had a specific activity of $153.4 \pm 11 \mu\text{mol min}^{-1} \text{mg}^{-1}$ ($n = 6$). The positive control, bee venom PLA₂ had a specific activity of $287.5 \pm 17.5 \mu\text{mol min}^{-1} \text{mg}^{-1}$ ($n = 4$). 4-BPB modified acanmyotoxin-1 had no PLA₂ activity ($n = 6$).

Chick isolated biventer cervicis nerve-muscle preparation

Neurotoxic studies The positive control and presynaptic neurotoxin, paradoxin (0.07 μM) caused time-dependent

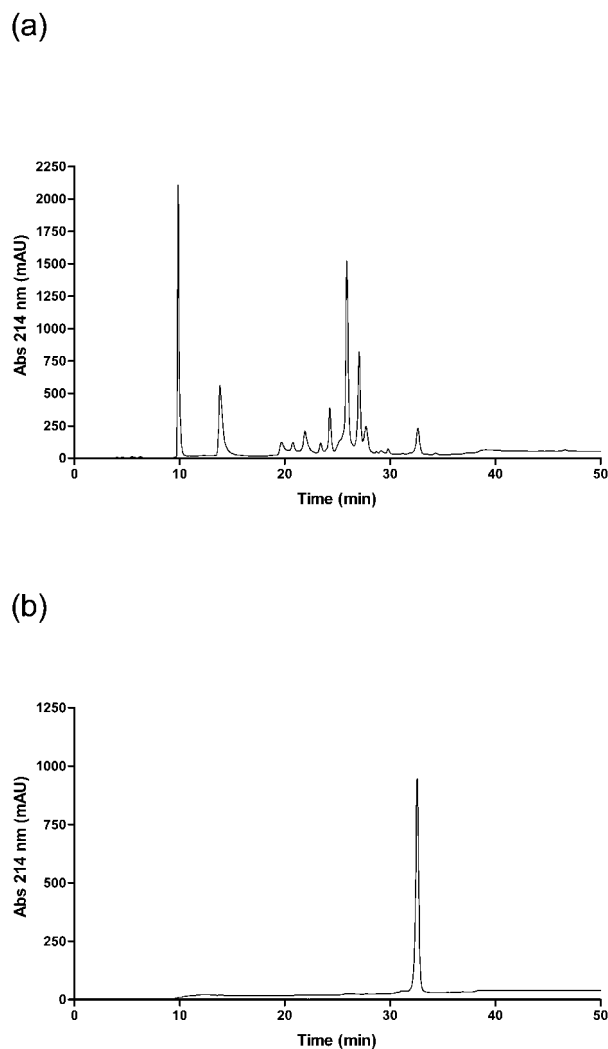


Figure 1 RP-HPLC chromatograph of (a) *A. rugosus* venom or (b) acanmyotoxin-1 run on a Jupiter analytical C18 column, equilibrated with solvent A (0.1% TFA) and eluted with the following gradient conditions of solvent B (90% acetonitrile in 0.09% TFA) and solvent A: 0 to 20% over 5 min, 20 to 60% in 40 min and then 60 to 80% over 5 min.

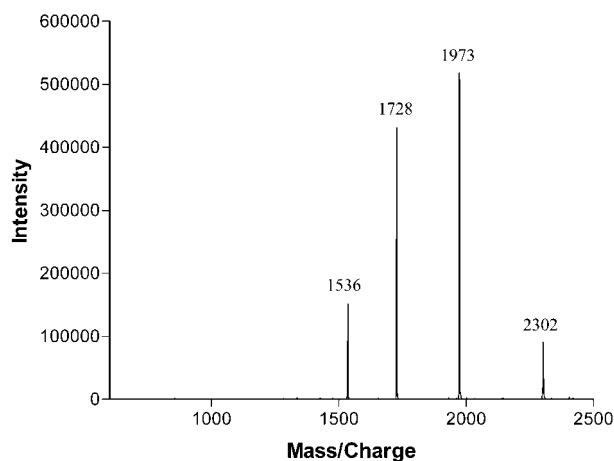


Figure 2 Electrospray mass spectrometry of acanmyotoxin-1. The spectrum shows a series of multiple-charged ions, related to molecules bearing 6–9 protons.

Table 1 N-terminal sequence of PLA₂ components isolated from death adder and some other elapid snake venoms

Common name	Species	PLA ₂ component	N-terminal sequence
Irian Jayan death adder	<i>A. rugosus</i>	acanmyotoxin-1	NLLQIGIMKR CANKRRRPVF HYRDYGCYC
Common death adder	<i>A. antarcticus</i>	acanthin I*	DLFQFGGMIG CANKGARSWL SYVNYGCYC
Common death adder	<i>A. antarcticus</i>	acanthin II*	NLYQFGGMIO CANKGARSWL SYVNYGCYC
Common death adder	<i>A. antarcticus</i>	acanthoxin A1**	NLYQFGGMIO CANKGARSWL SYVNYGCYC
Common death adder	<i>A. antarcticus</i>	acanthoxin A2**	DLFQFGGMIG CANKGARSWL SYVNYGCYC
Northern death adder	<i>A. praelongus</i>	acanthoxin B†	DLFQFGFMIO CANKGSRPVF
Desert death adder	<i>A. pyrrhus</i>	acanthoxin C†	NLFQFGGMIG CANKGTRSWL SYVNYGCYC
Coastal taipan	<i>Oxyuranus s. scutellatus</i>	taipoxin α chain††	NLLQFGFMIR CANRRSRPVW HYMDYGCYC
Australian king brown snake	<i>Pseudechis australis</i>	Pa-1G‡	NLIQFGNMIQ CANKGSRPTR HYMDYGCYC

*Chow *et al.* (1998); **van der Weyden *et al.* (1997); †van der Weyden *et al.* (2000); ††Lind & Eaker (1982); ‡Takasaki *et al.* (1990).

†No functional studies were done on these toxins.

inhibition of indirect twitches, whereas acanmyotoxin-1 (0.1 μM) had no significant inhibitory effect on twitch height compared to the vehicle, 0.1% bovine serum albumin in 0.9% saline ($n=4-8$; one-way ANOVA, $P<0.05$; Figure 3a). Paradoxin (0.07 μM), acanmyotoxin-1 (0.1 μM) and vehicle had no significant effect on the contractile responses to exogenous ACh (1 mM), CCh (20 μM) and KCl (40 mM) ($n=4-8$; Student's paired *t*-test, $P<0.05$; Figure 3b). However, acanmyotoxin-1 (0.1 μM) caused a significant increase in baseline tension compared to the vehicle ($n=4-8$; one-way ANOVA, $P<0.05$; data not shown).

Myotoxic studies *A. rugosus* venom (30–50 μg ml⁻¹) caused a significant inhibition of direct twitches compared to the vehicle ($n=4-8$; one-way ANOVA, $P<0.05$; Figure 4a). This effect was dose-dependent with *A. rugosus* venom (50 μg ml⁻¹) causing a significant inhibition of direct twitches compared to *A. rugosus* venom at 10 μg ml⁻¹ ($n=4-5$; one-way ANOVA, $P<0.05$; Figure 4a). In addition, *A. rugosus* venom (10–50 μg ml⁻¹) induced a significant increase in baseline tension compared to the vehicle control ($n=4-8$; one-way ANOVA, $P<0.05$; Figure 4b). Again this effect was dose-dependent with *A. rugosus* venom (50 μg ml⁻¹) causing a significantly greater contraction compared to *A. rugosus* venom at 10 μg ml⁻¹ ($n=4-5$; one-way ANOVA, $P<0.05$; Figure 4b).

Acanmyotoxin-1 (1 μM) caused a significant inhibition of direct twitches compared to the vehicle, whereas acanmyotoxin-1 (0.1 μM) had no significant effect on the twitch height ($n=4-6$; one-way ANOVA, $P<0.05$; Figure 5a). In addition, acanmyotoxin-1 (0.1–1 μM) induced a significant increase in baseline tension compared to the vehicle ($n=4-6$; one-way ANOVA, $P<0.05$; Figure 5b). This response was dose-dependent with acanmyotoxin-1 (1 μM) causing a significantly greater contraction than acanmyotoxin-1 at 0.1 μM ($n=4$; one-way ANOVA, $P<0.05$; Figure 5b).

Antivenom studies Prior incubation (10 min) of CSL death adder antivenom (5 units ml⁻¹) prevented the inhibition of direct twitches and the increase in baseline tension caused by acanmyotoxin-1 (1 μM; $n=4$; Figures 6a,b).

4-BPB modified acanmyotoxin-1 studies Acanmyotoxin-1 (1 μM) plus vehicle (acetone) significantly inhibited direct twitches compared to 4-BPB plus vehicle (sodium cacodylate; $n=4-6$; one-way ANOVA, $P<0.05$; Figure 6a). However,

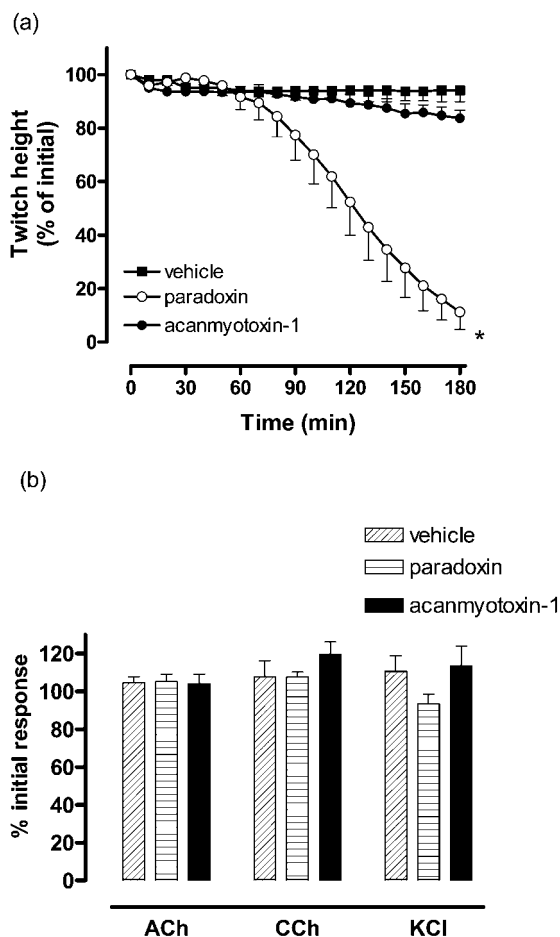


Figure 3 The effect of acanmyotoxin-1 (0.1 μM; $n=4$), paradoxin (positive control; 0.07 μM; $n=4$) or vehicle ($n=8$) on (a) indirect twitches or (b) contractile responses to exogenous ACh, CCh and KCl in the CBCNM preparation. * $P<0.05$, significantly different from vehicle, one-way ANOVA.

acanmyotoxin-1 (1 μM) plus 4-BPB had no significant inhibitory effect on direct twitches compared to 4-BPB plus vehicle ($n=4-6$; one-way ANOVA, $P<0.05$; Figure 6a). In addition, acanmyotoxin-1 (1 μM) plus vehicle induced a significant increase in baseline tension compared to 4-BPB plus vehicle, while acanmyotoxin-1 (1 μM) plus 4-BPB had no significant effect on the baseline tension ($n=4-6$; one-way ANOVA, $P<0.05$; Figure 6b).

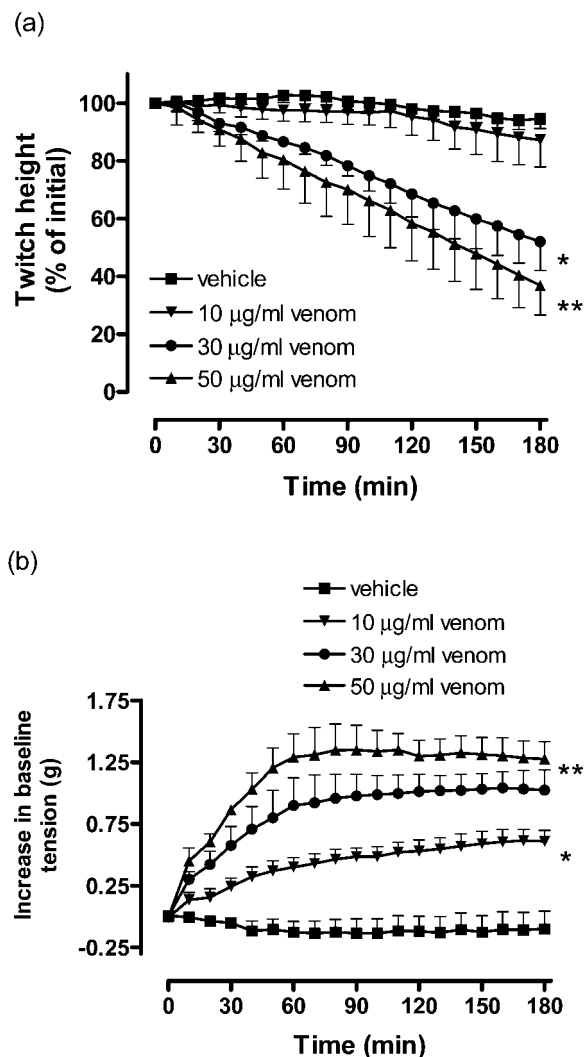


Figure 4 The effect of *A. rugosus* venom (10–50 µg ml⁻¹; *n*=4) or vehicle (*n*=8) on (a) direct twitches or (b) baseline tension of the CBCNM preparation. **P*<0.05, significantly different from vehicle, one-way ANOVA. ***P*<0.05, significantly different from *A. rugosus* venom (10 µg ml⁻¹), one-way ANOVA.

Morphological studies

Light microscopy studies of tissues exposed to *A. rugosus* venom (10–50 µg ml⁻¹) and acanmyotoxin-1 (0.1–1 µM) showed dose-dependent morphological changes in skeletal muscle compared to the vehicle control tissues (Figures 7a,b,c,d; data not shown for 10 and 50 µg ml⁻¹ *A. rugosus* venom). These changes included muscle fibre damage and vacuolation of the muscle cells. Prior incubation of CSL death adder antivenom (5 units ml⁻¹) prevented morphological changes from occurring due to acanmyotoxin-1 (1 µM; Figure 7e). No detectable morphological changes were seen in tissues equilibrated with antivenom alone (data not shown). While acanmyotoxin-1 (1 µM) plus vehicle (i.e. acetone) induced morphological changes similar to acanmyotoxin-1 (1 µM) alone, no detectable morphological changes were seen in acanmyotoxin-1 (1 µM) plus 4-BPB or vehicle plus 4-BPB treated tissues (data not shown).

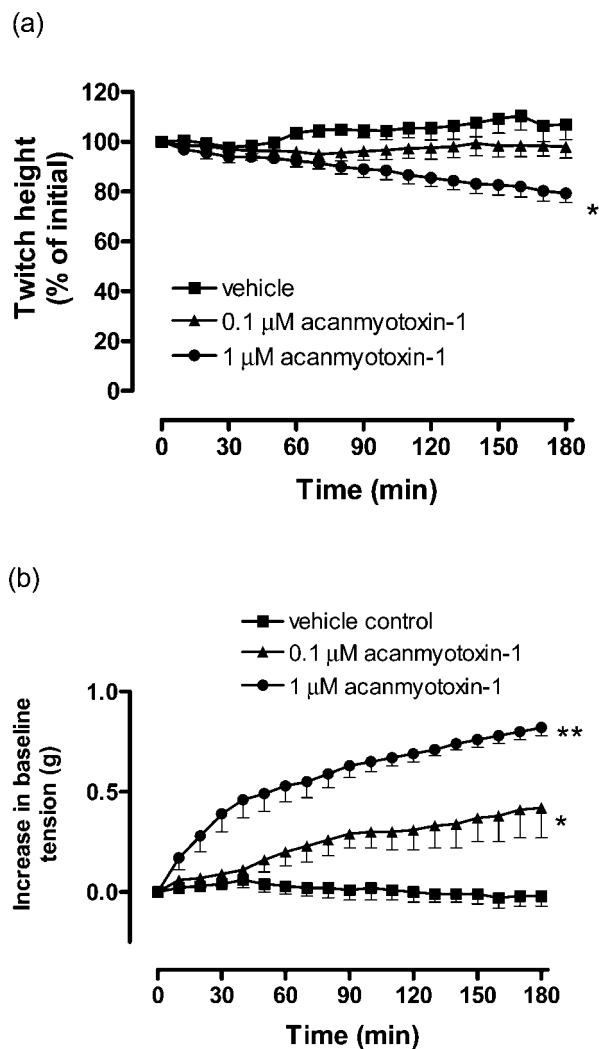


Figure 5 The effect of acanmyotoxin-1 (0.1–1 µM; *n*=4) or vehicle (*n*=6) on (a) direct twitches or (b) baseline tension of the CBCNM preparation. **P*<0.05, significantly different from vehicle, one-way ANOVA.

Discussion

Until recently, research on death adders has been largely focused on the venom from the Australian *A. antarcticus* (common death adder). Both *in vivo* and *in vitro* studies have shown that this venom has no significant myotoxic activity (Mebs & Samejima, 1980; Sutherland *et al.*, 1981; Wickramaratna & Hodgson, 2001). Therefore, it has been thought that death adder venoms are devoid of myotoxic activity. However, a recent clinical study reported evidence of rhabdomyolysis in patients following death adder envenomations, in Papua New Guinea, by a species not closely aligned with *A. antarcticus* (Lalloo *et al.*, 1996). Consequently, the present study examined *A. rugosus* venom for myotoxic activity, and isolated the first myotoxin from a death adder venom.

Acanyotoxin-1 was isolated as a single peak from *A. rugosus* venom by successive RP-HPLC separations. As seen from the RP-HPLC chromatogram of the *A. rugosus* venom,

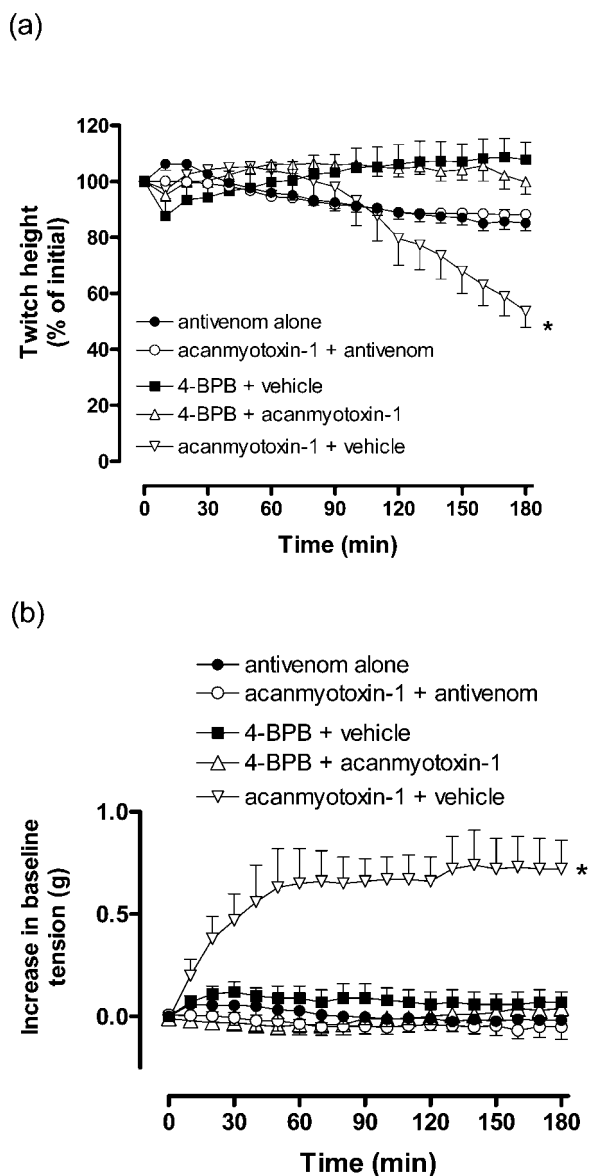


Figure 6 The effect of acanmyotoxin-1 (1 μM; *n*=4) or vehicle (BSA; *n*=4) in the presence of antivenom (5 units ml⁻¹), and effect of acanmyotoxin-1 (1 μM; *n*=4) or vehicle (sodium cacodylate; *n*=6) incubated in 4-BPB (1.8 mM) on (a) direct twitches or (b) baseline tension of the CBCNM preparation. Positive control was acanmyotoxin-1 (1 μM; *n*=4) incubated in vehicle (acetone). **P*<0.05, significantly different from 4-BPB plus vehicle, one-way ANOVA.

acanmyotoxin-1 is also the last major peak to elute at the given conditions. Using electrospray mass spectrometry the molecular mass of acanmyotoxin-1 was determined to be 13811 daltons. It is well documented that elapid snake venom PLA₂ components usually have molecular mass in the range of 12–14 kDa (Dawson & Hemington, 1967; Sim, 1998). While the molecular mass of acanmyotoxin-1 is consistent with other snake venom PLA₂ components it is about 1 kDa bigger than PLA₂ components previously isolated from *A. antarcticus* and *A. praelongus* venoms. Comparison of the N-terminal sequences showed that acanmyotoxin-1 shared highest identity with taipoxin α chain (75%) and Pa-1G (65%). The taipoxin α chain is the subunit of a potent

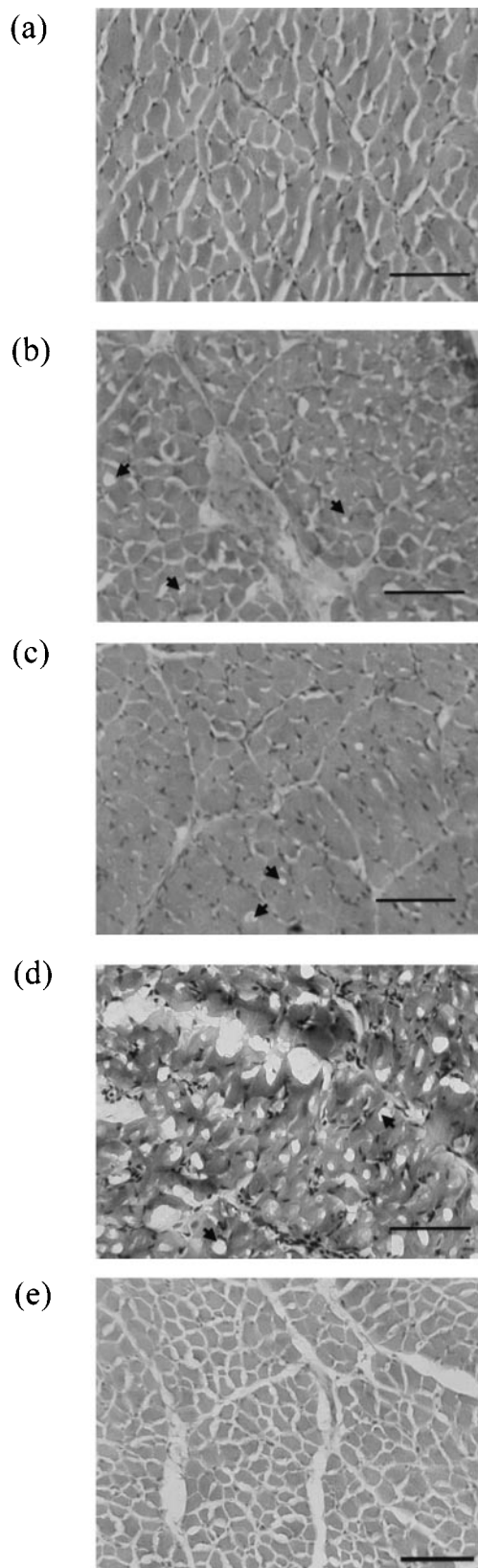


Figure 7 Transverse sections of CBCNM preparations exposed to (a) vehicle (BSA); (b) *A. rugosus* venom (30 μg ml⁻¹); (c) acanmyotoxin-1 (0.1 μM); (d) acanmyotoxin-1 (1 μM); (e) acanmyotoxin-1 (1 μM) in the presence of antivenom (5 units ml⁻¹). Scale bars, 100 μm in all micrographs. Arrowheads indicate prominent vacuoles.

presynaptic neurotoxin possessing myotoxic and PLA₂ activity (Harris & Maltin, 1982; Lind & Eaker, 1982), and Pa-1G is a myotoxic PLA₂ component (Geh *et al.*, 1992). Interestingly, lower sequence identity was seen with the antiplatelet active death adder PLA₂ components.

Due to the sequence homology and molecular mass resemblance of acanmyotoxin-1 to other elapid venom PLA₂ components, the specific activity of acanmyotoxin-1 was determined. High PLA₂ activity was detected in both *A. rugosus* venom and the isolated component. Given the high PLA₂ activity of *A. rugosus* whole venom it is possible that the whole venom may have other components with high PLA₂ activity.

A. rugosus venom and acanmyotoxin-1 were examined for *in vitro* myotoxicity using the directly stimulated CBCNM preparation. Both *A. rugosus* venom and acanmyotoxin-1 caused dose-dependent inhibition of direct twitches. Furthermore, both *A. rugosus* venom and acanmyotoxin-1 induced a dose-dependent increase in baseline tension. An inhibition of direct twitches and a rise in baseline tension is indicative of myotoxic activity (Harvey *et al.*, 1994). In addition to these results, light microscopy studies of tissues exposed to *A. rugosus* venom and acanmyotoxin-1 showed obvious morphological changes in skeletal muscle compared to tissues exposed to the vehicle. Together, these results suggest that both *A. rugosus* venom and acanmyotoxin-1 cause myotoxicity.

Several studies have shown that some elapid venom PLA₂ components, such as notexin and notechis II-5, are myotoxic as well as presynaptically neurotoxic (Harris & Johnson, 1978; Harris, 1991; Dixon & Harris, 1996). Therefore, acanmyotoxin-1 was examined for *in vitro* neurotoxicity using the indirectly stimulated CBCNM preparation. Acanmyotoxin-1 (0.1 µM) had no significant inhibitory effect on the indirect twitch height compared to the vehicle control, thus, suggesting it to be lacking in any detectable neurotoxic activity at this concentration. However, acanmyotoxin-1 (0.1 µM) caused a significant increase in baseline tension compared to vehicle. Given the myotoxic activity, it was not possible to further examine the neurotoxic activity of acanmyotoxin-1 at a higher concentration. In contrast to acanmyotoxin-1 (0.1 µM), paradoxin (0.07 µM) caused almost full inhibition of the indirect twitches over 3 h. Electrophysiological studies are required to further examine the neurotoxic activity of acanmyotoxin-1.

Due to habitat destruction and consequential decrease in species population levels, death adder envenomations are a rare occurrence in Australia, although these are still a significant health problem in Papua New Guinea (Currie *et al.*, 1991; Sutherland, 1992; Laloo *et al.*, 1995; 1996). CSL death adder antivenom is indicated for use in envenomation by any death adder species (AMH, 1998; White, 1998). Since *A. antarcticus* venom lacks myotoxic activity, and given that death adder antivenom has been raised against *A. antarcticus* venom, it was of clinical relevance to examine the efficacy of death adder antivenom against the *in vitro* myotoxicity of

acanmyotoxin-1. Previously, we have studied the efficacy of CSL death adder antivenom against the *in vitro* neurotoxicity of *A. rugosus* venom (Fry *et al.*, 2001). Prior incubation of antivenom (5 units ml⁻¹) prevented the inhibition of direct twitches and the increase in baseline tension caused by acanmyotoxin-1 (1 µM). Furthermore, antivenom prevented morphological changes from occurring due to acanmyotoxin-1 (1 µM). Thus, CSL death adder antivenom is effective in neutralizing the *in vitro* myotoxic activity of acanmyotoxin-1.

In order to determine whether the PLA₂ activity of acanmyotoxin-1 is necessary for the myotoxic action, acanmyotoxin-1 was subjected to 4-BPB modification. Many studies have shown that PLA₂ activity can be inhibited by selective acylation of His-48 using 4-BPB (Volwerk *et al.*, 1974; Abe *et al.*, 1977). When acanmyotoxin-1 was incubated with 4-BPB, the enzymatic activity as well as myotoxic activity was abolished. Thus, suggesting that PLA₂ activity is essential for the myotoxic activity of acanmyotoxin-1. In contrast to acanmyotoxin-1, some studies have shown that Lys-49 PLA₂ components lack catalytic activity on artificial substrates (Soares *et al.*, 2000; 2001). However, 4-BPB modification of these Lys-49 PLA₂ components prevented some of their pharmacological effects (Soares *et al.*, 2000; 2001). Thus, it was suggested that inhibition of the pharmacological effects by 4-BPB modification were not due to the inhibition of enzymatic activity (Soares *et al.*, 2000). Recently, it has been shown that some Lys-49 PLA₂ components are catalytically active on biological substrates (Soares *et al.*, 2002). Therefore, the observed reduction in pharmacological effects after 4-BPB modification of Lys-49 PLA₂ components may still be the result of inhibition of the catalytic activity. However, it is possible that His-48 may be important in the pharmacological site of PLA₂ components.

In conclusion, *A. rugosus* venom caused dose-dependent *in vitro* myotoxicity in the CBCNM preparation. Acanmyotoxin-1 is the first myotoxic component to be isolated from any death adder venom. Although CSL death adder antivenom has been raised against *A. antarcticus* venom it is effective in neutralizing the myotoxic activity of acanmyotoxin-1. Furthermore, studies with 4-BPB suggest that PLA₂ activity is essential for the myotoxic activity of acanmyotoxin-1. Given the results of this study clinicians may need to be mindful of possible myotoxicity following death adder envenomation in Irian Jaya. In light of this finding, other death adder venoms should be examined for myotoxic activity.

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