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Isolation and characterisation of P-EPTX-Ap1a and P-EPTX-Ar1a: Pre-synaptic neurotoxins from the venom of the northern (*Acanthophis praelongus*) and Irian Jayan (*Acanthophis rugosus*) death adders

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ABSTRACT

The neurotoxicity observed following death adder envenoming has been thought to be solely due to the presence of potent post-synaptic neurotoxins. Clinically, these effects are often poorly reversed by death adder antivenom or anticholinesterase, particularly when patients present with established paralysis. This suggests that either the post-synaptic neurotoxins are irreversible/'pseudo' irreversible, or the venom contains pre-synaptic neurotoxins that do not respond to antivenom. To support the later hypothesis, a pre-synaptic neurotoxin (P-EPTX-Aa1a) has recently been isolated from the venom of *Acanthophis antarcticus*. We examined *Acanthophis praelongus* and *Acanthophis rugosus* venoms for the presence of pre-synaptic neurotoxins. P-EPTX-Ap1a (40,719 Da) and P-EPTX-Ar1a (40,879 Da) were isolated from *A. praelongus* and *A. rugosus* venoms, respectively. P-EPTX-Ap1a and P-EPTX-Ar1a are comprised of three different subunits, α, β1 and β2. The two toxins displayed similar levels of PLA_2 activity which was almost solely attributed to the α subunit in both toxins. P-EPTX-Ap1a (20–100 nM) and P-EPTX-Ar1a (20–100 nM) caused inhibition of indirect twitches of the skeletal muscle preparation without affecting contractile responses to nicotinic receptor agonists. Interestingly, only the α subunit of both toxins (300 nM) displayed neurotoxic activity. Inhibition of PLA_2 activity markedly reduced the effect of the toxins on muscle twitch height. These results confirm that P-EPTX-Ap1a and P-EPTX-Ar1a are pre-synaptic neurotoxins and represent the second and third such toxins to be isolated from death adder venom. The presence of pre-synaptic neurotoxins in *Acanthophis* sp. venoms indicates that treatment strategies for envenoming by these snakes needs to be reassessed given the likelihood of irreversible neurotoxicity.

1. Introduction

Death adders (*Acanthophis* genus) are found throughout Australia, the Torres Straight Islands, Papua New Guinea, Irian Jaya, and the Indonesian islands; Seram, Halmahera, Obi and Tanimbar. Although belonging to the Elapidae family, they resemble snakes of the Viperidae family in appearance and habit [1]. They are characterized by a somewhat flattened, almost rectangular head and thick muscular bodies ending in a thin rat-like tail. Death adder envenomings are a rare occurrence in Australia however these are still a significant health problem in Papua New Guinea [2–4]. Clinical symptoms of envenoming by *Acanthophis* sp. include paralysis of extra ocular muscles, abdominal pain, headache, drowsiness, enlargement of regional lymph nodes and death occurs through respiratory failure from paralysis of the voluntary muscles [1,5]. Myotoxicity is another important symptom of envenoming by some death adders. Two clinical studies reported myotoxic activity following the envenomings by *Acanthophis* sp. in Papua New Guinea and the northern death adder (*Acanthophis praelongus*) [5,6]. Patients developed renal failure and displayed elevated creatine kinase levels, indicating rhabdomyolysis and the presence of myotoxic activity in the venom [7].

The neurotoxicity observed following envenoming by *Acanthophis* sp. has been widely accepted to be due to the presence of post-synaptic neurotoxins, this has been supported by the fact that the neurotoxicity can be reversed by administration of antivenom or anticholinesterase [8]. However, there have been reports some envenomed patients have responded poorly to antivenom and

Abbreviations: ACh, acetylcholine; BSA, bovine serum albumin; CCh, carbachol; MALDI-TOF, matrix-assisted laser desorption ionization time-of-fight; PLA_2, phospholipase A_2; P-EPTX-Aa1a, P-elapitoxin-Aa1a; RP-HPLC, reverse-phase high-pressure liquid chromatography.

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neostigmine [9]. This suggests that either the α-neurotoxins may not be completely reversible, or that the venom contains pre-synaptic neurotoxins (i.e. β-neurotoxin) which, due to their mode of action, are unresponsive to anticholinesterase and antivenom.

Pre-synaptic 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 resulting in dysfunction of transmission at the neuromuscular junction [10]. Numerous pre-synaptic neurotoxins have been isolated from the venom of the major families of venomous snakes (i.e. Crotalidae, Elapidae and Viperidae). Australian snake (i.e. Elapids) venoms contain PLA_2 enzymes, which differ in enzymatic activity and pharmacological effects. These include textilotoxin from the Australian brown snake (*Pseudonaja textilis*) which contains five subunits [11], taipoxin, from the Australian coastal taipan (*Oxyuranus scutellatus*) which contains three subunits [12] and notexin, from the Australian tiger snake (*Notechis scutatus scutatus*) which is a single chain PLA_2 [13].

Previously we showed that *Acanthophis rugosus* (Irian Jayan death adder) and *A. praelongus* venoms caused time-dependent inhibition of indirect twitches and blocked contractile response to exogenous acetylcholine and carbachol [14]. Thus, suggesting the presence of post-synaptic neurotoxins.

In terms of PLA_2 components, acanthin I and II, both potent inhibitors of platelet aggregation have been isolated from *A. antarcticus* venom [15]. In addition, acanthoxin A_1 and A_2, two PLA_2 isoforms with weak neurotoxic activity, have been isolated from *A. antarcticus* venom [16]. Three PLA_2 isoenzymes, praelongins 2bIII, 2cII and 2cIV, with antiplatelet activity have also been isolated from *A. praelongus* venom [17]. Acanmyotoxin-1, the first death adder PLA_2 myotoxin was isolated from *A. rugosus* [18]. Subsequently, acanmyotoxin-2 and acanmyotoxin-3 were isolated from the venom of *A.* sp. Seram [19].

Recently, P-EPTX-Aa1a [20], a pre-synaptic PLA_2 neurotoxin was isolated from the venom of the common death adder (*A. antarcticus*). P-EPTX-Aa1a, which displayed modest PLA_2 activity, inhibited nerve-evoked contractions in a skeletal muscle preparation without affecting responses to cholinergic agonists. This is the first pre-synaptic neurotoxin isolated from a death adder venom.

A subsequent study examining the HPLC profiles of 9 death adder venoms including 4 geographical variants of *A. antarcticus* venoms (i.e. New South Wales, Queensland, South Australia and Western Australia) identified a high molecular weight component, which corresponded to the elution time for P-EPTX-Aa1a, in seven of the nine venoms, including *A. praelongus* and *A. rugosus*. Interestingly, the venoms of *A. wellsi* and *A. pyrrhus* appeared to be devoid of this component [21]. The presence of a pre-synaptic toxin would explain the clinical outcomes of patients envenomed by some species of death adders who do not respond adequately to anticholinesterases or antivenom.

The aim of this study was to isolate and characterise the PLA_2 pre-synaptic neurotoxins from *A. praelongus* and *A. rugosus* venoms. It is important to understand the composition and mechanism of action of these venom components so that appropriate treatments can be administered to envenomed patients.

2. Materials and methods

2.1. Venom preparation

Freeze-dried venoms were gifted from Venom Supplies (Venom Supplies Pty Ltd., Tanunda, SA, Australia). Venom was dissolved in Milli-Q water and insoluble material was removed by centrifugation at 16,000 × *g* at 4 °C for 8 min.

2.2. Fractionation of venom

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

2.2.1. Size exclusion chromatography

Venom (3 mg) was applied to a Superdex G-75 column (13 μm; 10 mm × 300 mm; GE Healthcare, Buckinghamshire, UK) equilibrated with ammonium acetate buffer (0.1 M; pH 6.8). The sample was eluted at a flow rate of 0.6 ml/min. The eluant was monitored at 280 nm. The fractions were collected and freeze-died prior to being screened for neurotoxicity as per Section 2.3.

2.2.2. Reverse phase-high performance liquid chromatography

The active freeze-dried, purified component (40 μg) obtained as described in Section 2.2.1 was reconstituted in Milli-Q water and applied to a Phenomenex (Torrance, CA, USA) Jupiter analytical C18 column (150 mm × 2 mm; 5 μm; 300 Å) after equilibrating with solvent A (0.1% trifluoroacetic acid (TFA)). The sample was eluted with the following gradient conditions of solvent B (90% acetonitrile in 0.09% TFA): 0–20% for 0–5 min (4% gradient), 20–60% for 5–45 min (1% gradient), and then 60–80% for 45–50 min (4% gradient) at a flow rate of 0.2 ml/min. The eluant was monitored at 280 and 214 nm and freeze-dried.

2.3. Chick biventer cervicis nerve–muscle preparation

Chickens (4–10 day old males) were sacrificed by CO_2 asphyxiation and biventer cervicis nerve–muscle preparations were dissected. These were mounted in 5 ml organ baths containing physiological salt solution (118.4 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, 25 mM $NaHCO_3$ and 11.1 mM glucose). The solution was maintained at 34 °C and bubbled with carbogen (95% O_2 and 5% CO_2) under 1 g resting tension.

Motor nerves within the biventer were stimulated every 10 s (0.2 ms duration) at supramaximal voltage using a Grass S88 stimulator. *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 potassium chloride (KCl; 40 mM for 30 s) were obtained in the absence of stimulation. 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 (20–100 nM), subunits (300 nM) or venom (10 μg/ml) was left in contact with the preparation until responses to nerve stimulation were abolished or for a maximum of 4 h if total twitch blockade did not occur. In separate experiments, $MgCl_2$ (5 mM) was added to suppress twitch contraction to approximately 30% for at least 30 min prior to the addition of toxin (100 nM). At the conclusion of the experiment, responses were measured via a Grass force displacement transducer (FT03) and recorded on a MacLab System.

Given the 'all or nothing' nature of the failure of neurotransmission which occurs in the skeletal muscle preparation in the presence of snake venoms/toxins, the time taken to reduce the amplitude of the indirect twitches by 90% (t_{90}) was calculated to provide a quantitative measure (i.e. potency) of the neurotoxins [22]. This parameter enables comparison of the potency of previously published post- and pre-synaptic neurotoxins [22,23]. Where indicated, the PLA_2 activity of the toxin was inhibited by alkylation with 4-bromophenacylbromide (4-BPB). Toxin (20 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. The mixture was incubated for 16 h at 30 °C and examined for neurotoxic activity as above.

2.4. Molecular mass determination of P-EPTX-Ap1a and P-EPTX-Ar1a

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 6.8). The column was calibrated with the series of known standard (6500–66,000 Da) using the molecular weight marker kit for gel filtration chromatography (MW-GF-70; Sigma–Aldrich, St. Louis, MO, USA). The eluant was monitored at 280 nM, and a flow rate of 0.6 ml/min was used. Void volume (V_o) 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_o ratio.

2.5. Mass spectrometry

MALDI-TOF MS analysis was performed using an Applied Biosystem (Foster City, CA, USA) 4700 ToF ToF Proteomics Analyser. The instrument was operated in positive polarity in linear mode using sinapinic acid matrix (Agilent Technologies; Palo Alto, CA, USA) 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 4000 series Data Explorer software.

2.6. Determination of PLA_2 activity

PLA_2 activity of the toxin and toxin subunits was determined using a secretory PLA_2 colourmetric assay kit (Cayman Chemical; MI, USA) according to manufacturer's instructions. 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 thio ester bond at the *sn*-2 position by PLA_2 are detected using DTNB (5,5′-dithio-bis-(2-nitrobenzoic acid)). Colour changes were monitored at 405 nm in a fusion α microplate reader (PerkinElmer; MA, USA), sampling every minute for a 5 min period. PLA_2 activity was expressed as micromoles of phosphatidylcholine hydrolyzed per minute per milligram of enzyme. For some samples, the PLA_2 activity of venom was inhibited by alkylation with 4-BPB (as above).

2.7. Chemical and drugs

The following drugs were used: ACh, BSA, CCh, *d*-tubocurarine, 4-BPB, ammonium acetate, acetone and cacodylic acid (Sigma Chemical Co., MO, USA); KCl, NaCl, $NaHCO_3$ and glucose (Ajax Finechem, New South Wales, Australia); TFA (Auspep, Victoria, Australia); acetonitrile, KH_2PO_4 and $CaCl_2$ (Merck, Darmstadt, Germany).

2.8. Analysis of results and statistics

Statistical analysis was performed using the Prism 5.0 software package (GraphPad Software; San Diego, CA, USA). Twitch height and contractile responses to agonists were expressed as a percentage of the corresponding value prior to the administration of toxins. For twitch height, statistical difference was determined by a one-way analysis of variance (ANOVA) of the change in twitch height at the 240 min time point followed by Tukey's multiple comparison test. Statistical significance was indicated were $P < 0.05$.

3. Result

3.1. Isolation and purification of pre-synaptic neurotoxins from *A. praelongus* and *A. rugosus* venoms

A. praelongus and *A. rugosus* venoms were fractionated by size exclusion chromatography on a Superdex G-75 column. The venom profile, of both species, indicated six to seven major peaks which were individually collected (Fig. 1a and b). Screening in the chick biventer cervicis nerve–muscle preparation confirmed pre-synaptic neurotoxic activity in the first peak of each venom which was subsequently named: P-EPTX-Ap1a (*A. praelongus*) and P-EPTX-Ar1a (*A. rugosus*) based on the suggested nomenclature of King et al. [24]. The samples were freeze-dried and then reconstituted in Milli-Q water. To verify the purity and determine the homogeneity and location of the isolated toxins, P-EPTX-Ap1a and P-EPTX-Ar1a were re-administered under the same conditions on the Superdex G-75 column (Fig. 1c and d). P-EPTX-Ap1a (8.97% of whole venom) and P-EPTX-Ar1a (7.25% of whole venom) eluted as clean peaks with retention time of 17.98 and 17.97 min (Table 1), respectively.

3.2. Separation of toxin subunits using reverse phase-high performance liquid chromatography

P-EPTX-Ap1a and P-EPTX-Ar1a were broken up into individual subunit using RP-HPLC on a Phenomenex Jupiter analytical column. Each toxin produced three peaks that eluded at different retention times. These components were collected for molecular weight determination by MALDI-TOF (see below) and analysis of PLA_2 activity (Fig. 1e and f). The subunits were designated as α, β1 and β2 based on their PLA_2 activity and neurotoxic effects (Table 1).

3.3. Molecular mass determination

3.3.1. Size exclusion chromatography in nondenaturing media

The molecular mass of P-EPTX-Ap1a and P-EPTX-Ar1a was determined by size exclusion chromatography on a Superdex G-75 column calibrated with the following protein standards: BSA (66,000 Da), carbonic anhydrase (29,000 Da), cytochrome c (12,400 Da), and aprotinin (6500 Da). The V_o of the column was determined as 8.17 ml using blue dextran (2,000,000 Da) and the V_e of P-EPTX-Ap1a and P-EPTX-Ar1a were each calculated as

Table 1
PLA_2 activity, retention times of isolated fractions/toxin subunits and molecular weights from MALDI-TOF.

Toxin/subunit	PLA_2 activity (μmol/min/mg)	Retention time (min)	Molecular weight (Da)
P-EPTX-Ap1a	101.7 ± 9.9	17.98[a]	40,719
β_1-P-EPTX-Ap1a	9.5 ± 0.6	20.93[b]	13,449
α-P-EPTX-Ap1a	220.4 ± 19.2	30.76[b]	13,804
β_2-P-EPTX-Ap1a	6.7 ± 1.1	33.9[b]	13,466
P-EPTX-Ap1a + 4-BPB	2.5 ± 0.3**	N/A	N/A
P-EPTX-Ar1a	115.2 ± 4.1	17.97[a]	40,879
β_1-P-EPTX-Ar1a	2.6 ± 0.5	21.24[b]	13,475
β_2-P-EPTX-Ar1a	32.1 ± 1.2	24.23[b]	13,592
α-P-EPTX-Ar1a	174.3 ± 14.3	31.08[b]	13,812
P-EPTX-Ar1a + 4-BPB	13.1 ± 1.5**	N/A	N/A

[a] Data from size exclusion HPLC.
[b] Data from reverse phase HPLC.
** $P < 0.05$, significantly different compared with P-EPTX-Ap1a and P-EPTX-Ar1a in the absence of 4-BPB, one-way ANOVA.

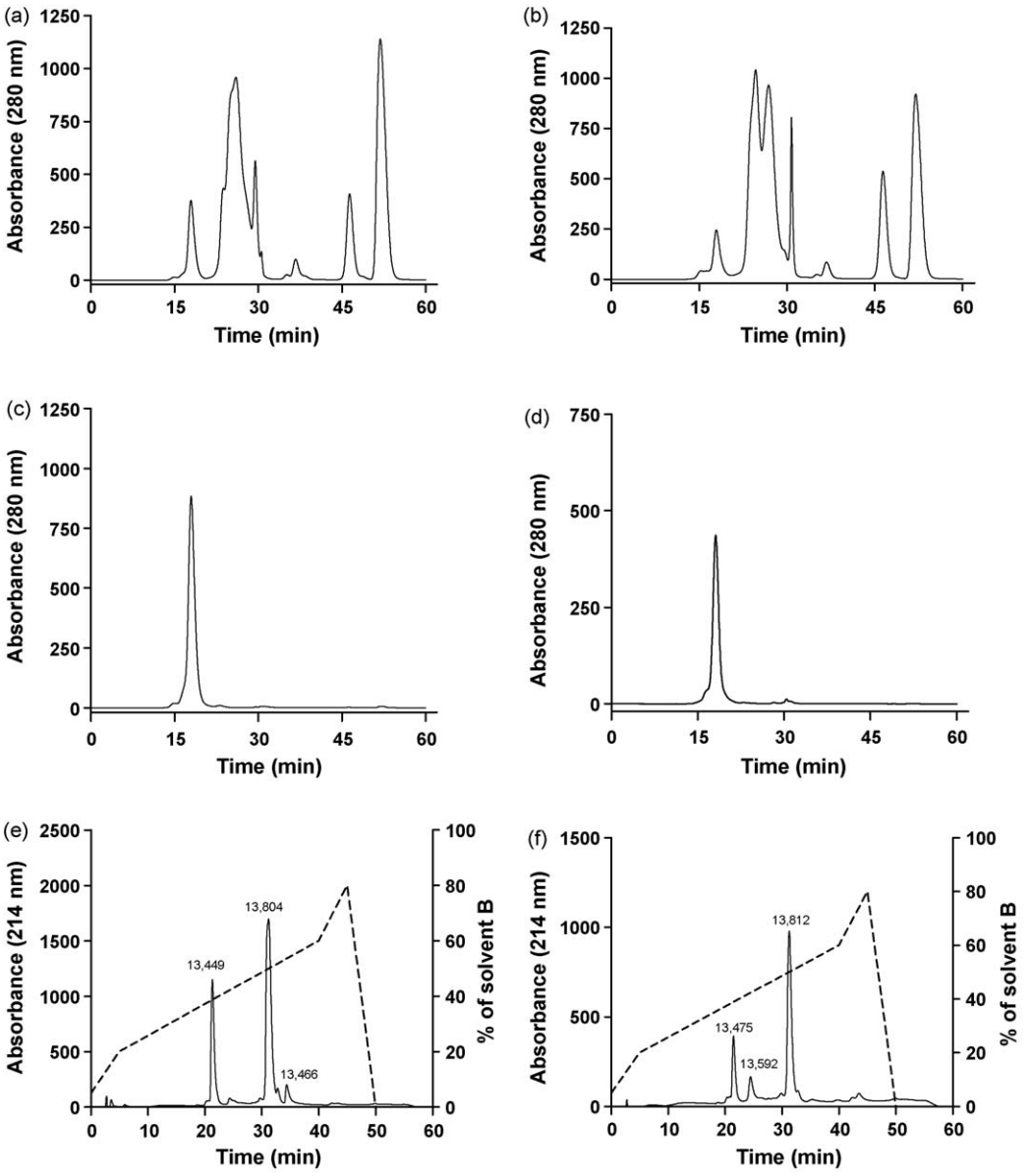

Fig. 1. Size exclusion chromatograph of (a) *A. praelongus* venom and (b) *A. rugosus*, (c) P-EPTX-Ap1a and (d) P-EPTX-Ar1a run on a Superdex G-75 column equilibrated with ammonium acetate (0.1 M; pH 6.8) at a flow rate of 0.6 ml/min. RP-HPLC chromatograph of (e) P-EPTX-Ap1a and (f) P-EPTX-Ar1a run on a Jupiter analytical C18 column equilibrated with solvent A (0.1% TFA) and eluted with the following gradient of solvent B (90% acetonitrile in 0.09% TFA) and solvent A: 0–20% for 0–5 min, 20–60% for 5–45 min and 60–80% for 45–50 min. Flow rate of 0.2 ml/min.

10.9 ml. The molecular mass of both toxins was determined from a plot of log mol. wt. versus corresponding V_e/V_o ratio (of the protein standards described above) to be approximately 41,000 Da (Fig. 2).

3.3.2. Mass spectrometry

The individual subunits of P-EPTX-Ap1a obtained in Section 3.2 were found to be of the following molecular mass by MALDI-TOF analysis: 13,449 Da (β_1), 13,804 Da (α) and 13,466 Da (β_2), while the subunits of P-EPTX-Ar1a were 13,475 Da (β_1), 13,592 Da (β_2) and 13,812 Da (α) (Table 1). The sum of the molecular masses of subunits resulted in a mass of 40,719 and 40,879 Da for P-EPTX-Ap1a and P-EPTX-Ar1a, respectively.

3.4. Chick biventer cervicis nerve–muscle preparation

3.4.1. Whole venom studies

Both *A. rugosus* and *A. praelongus* venoms (10 μg/ml; $n = 5$) abolished indirect twitches of the chick biventer cervicis nerve–muscle preparation (Fig. 3). The time taken to cause 90% inhibition (i.e. t_{90} values) was significantly less for the venom of *A. rugosus* (Table 2). Both venoms (10 μg/ml) abolished contractile responses to exogenous ACh (1 mM) and CCh (20 μM), but had no significant effect on responses to KCl (40 mM) (Fig. 3). Vehicle had no significant inhibitory effect on twitches or on the contractile responses to exogenous agonists ($n = 5$; one-way ANOVA, $P < 0.05$).

3.4.2. Isolated toxin studies

P-EPTX-Ar1a (20 and 100 nM) caused significant concentration-dependent decreases in twitch height compared to vehicle ($n = 5$–6; one-way ANOVA, $P < 0.05$; Fig. 4b). In contrast, P-EPTX-Ap1a (20 and 100 nM, $n = 5$) did not display a significant difference in the level of inhibition produced by the concentrations tested ($n = 5$; one-way ANOVA, $P < 0.05$; Fig. 4a). The time taken by P-EPTX-Ap1a (20 and 100 nM) to produce 90% inhibition of twitch height (i.e. t_{90}) was significantly shorter compared to P-EPTX-Ar1a at 20 nM (Table 2). Neither toxin had a significant inhibitory effect

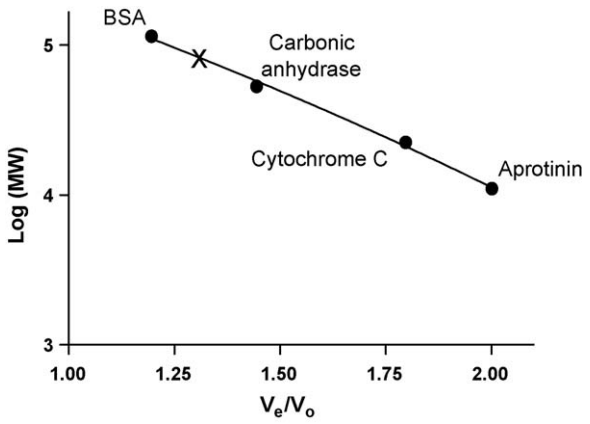

Fig. 2. Plot of log molecular weight versus V_e/V_o of a series of molecular weight standards run on a Superdex G 75 column equilibrated with ammonium acetate (0.1 M; pH 6.8) at a flow rate of 0.6 ml/min. V_o of the column was 8.17 ml. Cross indicates P-EPTX-Ap1a and P-EPTX-Ar1a.

on the tissue response to ACh, CCh, or KCl, indicating an action at the pre-synaptic nerve terminal (Fig. 4c).

The addition of Mg^{2+} (5 mM) to the physiological solution unmasked a triphasic effect of the toxins (100 nM) on twitch height. This was characterized by a transient inhibitory phase, followed by a transient facilitatory phase, prior to complete inhibition (Fig. 5).

The prior incubation of 4-BPB with either P-EPTX-Ar1a (20 nM) or P-EPTX-Ap1a (20 nM) prevented the complete inhibition of indirect twitches over the period of 4 h with 53 ± 12 and 30 ± 9% reduction in twitch response, respectively (Fig. 6). Both modified

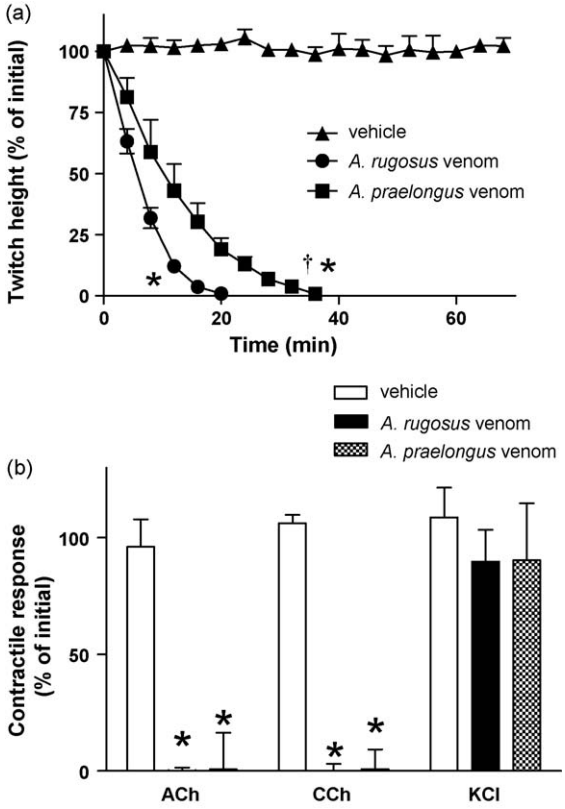

Fig. 3. Effect of *A. praelongus* (10 μg/ml; $n = 5$), *A. rugosus* venom (10 μg/ml; $n = 5$) or vehicle ($n = 5$) on (a) nerve-mediated twitches or (b) contractile responses to exogenous ACh, CCh and KCl in the chick isolated biventer cervicis nerve–muscle preparation. $^{\dagger}P < 0.05$, significantly different from *A. rugosus* venom 10 μg/ml, one-way ANOVA. $^{*}P < 0.05$, significantly different from vehicle, one-way ANOVA.

Table 2
Time to 90% inhibition (t_{90}) of twitch height as an indication of venom/toxin potency.

	t_{90} (min)
A. praelongus venom (10 μg/ml)	27.5 ± 1.9*
P-EPTX-Ap1a (20 nM)	96 ± 9.8**
P-EPTX-Ap1a (100 nM)	79.5 ± 8.0**
P-EPTX-Ap1a (20 nM) with 4-BPB	>240
P-EPTX-Ap1a (20 nM) with vehicle	100 ± 13.4
P-EPTX-Ap1a (100 nM) with Mg^{2+}	70.5 ± 1.9
A. rugosus venom (10 μg/ml)	12.4 ± 0.9
P-EPTX-Ar1a (20 nM)	132 ± 23.6
P-EPTX-Ar1a (100 nM)	79.6 ± 6.3**
P-EPTX-Ar1a (20 nM) with 4-BPB	>240
P-EPTX-Ar1a (20 nM) with vehicle	118.5 ± 14.3
P-EPTX-Ar1a (100 nM) with Mg^{2+}	69.5 ± 10.2

Values are mean ± s.e.m., $n = 4$–6.
* $P < 0.05$ compared to *A. rugosus venom* (10 μg/ml).
** $P < 0.05$ compared to P-EPTX-Ar1a (20 nM).

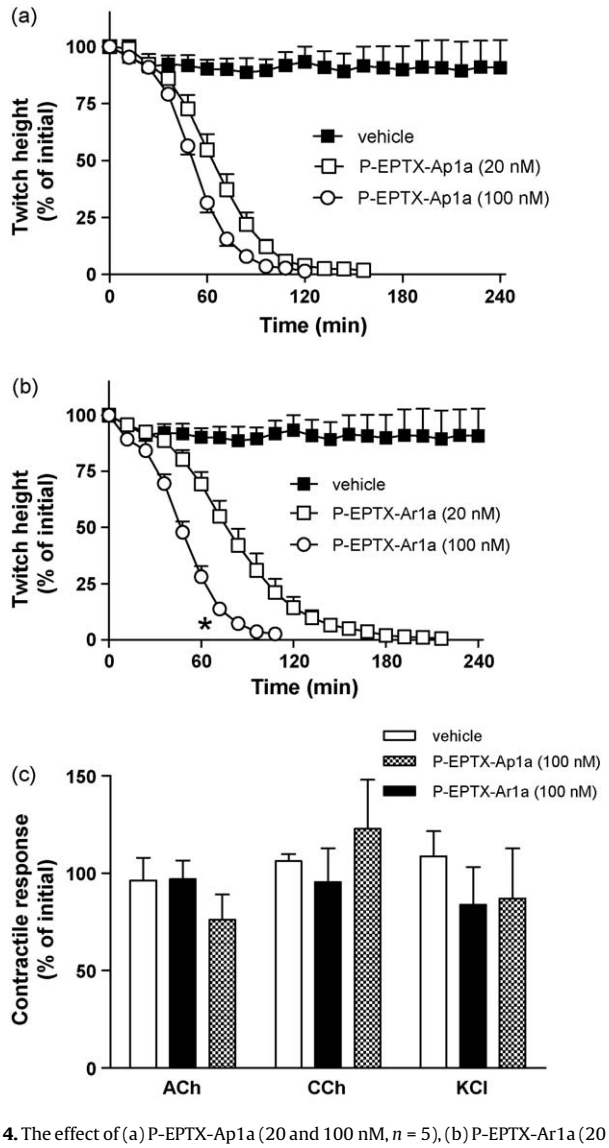

Fig. 4. The effect of (a) P-EPTX-Ap1a (20 and 100 nM, $n = 5$), (b) P-EPTX-Ar1a (20 and 100 nM, $n = 6$), or vehicle ($n = 5$) on nerve-mediated twitches in the chick isolated biventer cervicis nerve–muscle preparation. (c) Effect of P-EPTX-Ap1a (100 nM, $n = 5$), P-EPTX-Ar1a (100 nM, $n = 6$) or vehicle ($n = 5$) on contractile responses to exogenous ACh, CCh and KCl in the chick isolated biventer cervicis nerve–muscle preparation. $^{*}P < 0.05$, significantly different compared with toxin in the concentration of 20 nM, one-way ANOVA.

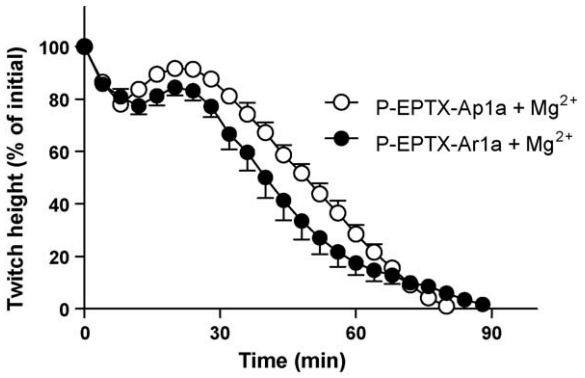

Fig. 5. The effect of P-EPTX-Ap1a or P-EPTX-Ar1a (100 nM), in the presence of Mg^{2+} (5 mM, $n = 4$), on nerve-mediated twitches in the chick isolated biventer cervicis nerve–muscle preparation.

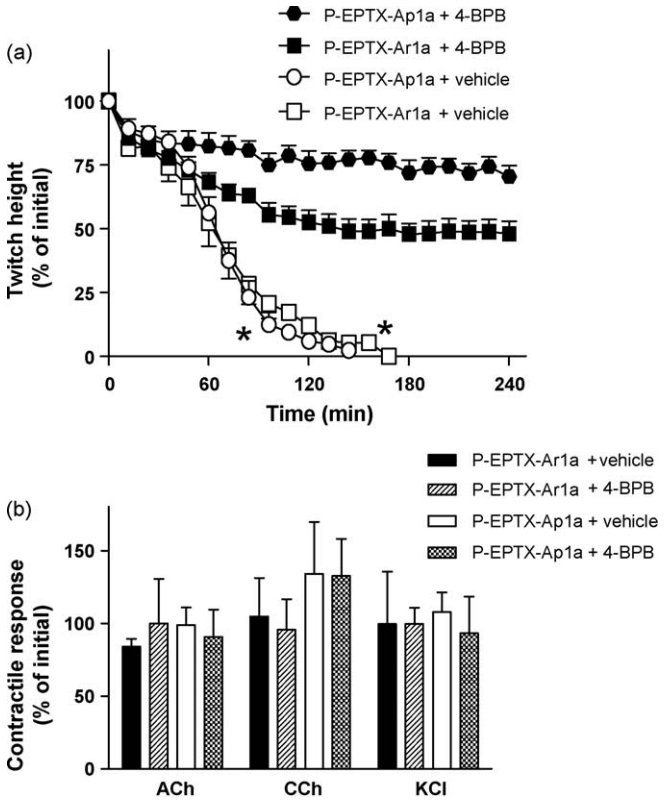

Fig. 6. The effect of P-EPTX-Ap1a or P-EPTX-Ar1a in the presence of 4-BPB (1.8 nM, $n = 6$) or vehicle ($n = 5$) on (a) nerve-mediated twitches or (b) contractile responses to exogenous ACh, CCh and KCl in the chick isolated biventer cervicis nerve–muscle preparation. $^{*}P < 0.05$, significantly different from corresponding toxin in the presence of 4-BPB, one-way ANOVA.

toxins did not affect the contractile responses to exogenous nicotinic receptor agonists.

3.4.3. Subunits of P-EPTX-Ar1a or P-EPTX-Ap1a

The β_1 and β_2 subunits (300 nM) of P-EPTX-Ar1a or P-EPTX-Ap1a had no significant inhibition on indirect twitches of chick biventer cervicis nerve–muscle preparation. However, the α subunits (300 nM) from both toxins significant inhibited twitch height compared with vehicle (Fig. 7).

3.5. Phospholipase A_2 activity

PLA_2 activity was detected in both P-EPTX-Ar1a and P-EPTX-Ap1a ($n = 4$). The positive control, bee venom showed a specific activity of 309.8 ± 12.1 μmol/min/mg ($n = 4$). 4-BPB significantly inhibited the PLA_2 activity of P-EPTX-Ar1a and P-EPTX-Ap1a ($n = 4$). The α-subunit seems to be the only subunit with a significant level of activity in both toxins (Table 1).

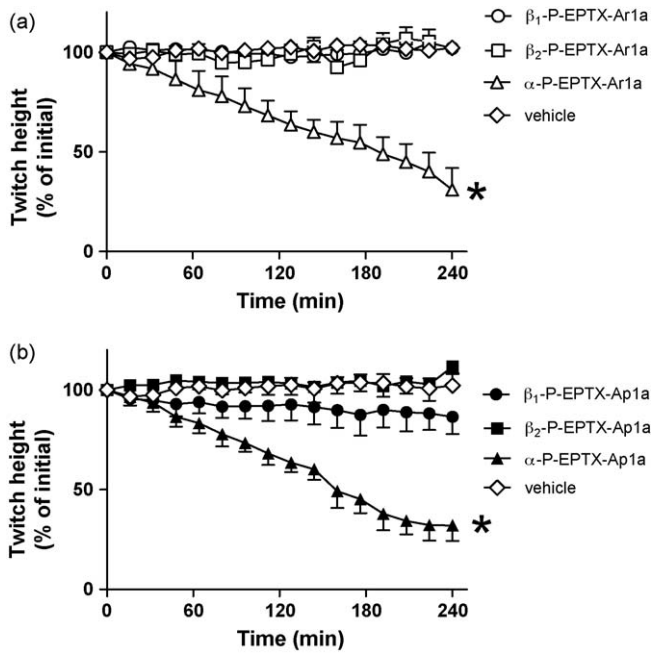

Fig. 7. Effect of α, β1 and β2 subunits of (a) P-EPTX-Ar1a or (b) P-EPTX-Ap1a (300 nM, $n = 3$–4) on nerve-mediated twitches. $^{*}P < 0.05$, significantly different from vehicle, one-way ANOVA.

4. Discussion

Whilst death adder neurotoxicity has been thought to be primarily due to the presence of reversible, competitive post-synaptic neurotoxins, clinical reports have indicated cases that responded poorly to treatment with antivenom and/or neostigmine [9]. This is surprising as it would be expected that, given their mode of action, these toxins would respond to this treatment protocol. However, a recent study has reported the characterisation and isolation of a pre-synaptic neurotoxin (i.e. P-EPTX-Aa1a) from the venom of the common death adder (*A. antarticus*) [20], a class of toxins that was previously thought to be absent from this genus. In addition, a recent study has indicated the likely presence of pre-synaptic neurotoxins in a number of species of death adder [21]. In the present study, we report the isolation and characterisation of pre-synaptic PLA_2 neurotoxins from the venoms of *A. praelongus* and *A. rugosus*.

Size exclusion chromatography, determined the molecular mass of P-EPTX-Ap1a and P-EPTX-Ar1a to be 41 kDa. This value corresponded well with the combined molecular masses of three subunits isolated from RP-HPLC determined by MALDI-TOF (i.e. 40,719 Da for P-EPTX-Ap1a and 40,879 Da for P-EPTX-Ar1a, respectively). In addition, the toxins display a similar molecular weight to that of P-EPTX-Aa1a, a heterotrimeric complex of 44,698 Da composed of three subunits, recently isolated from the venom of the common death adder [20]. It is well documented that elapid snake venom PLA_2 components usually have a molecular mass in the range of 12–14 kDa [17]. This was supported by MALDI-TOF analysis of the individual subunits for P-EPTX-Ap1a and P-EPTX-Ar1a which showed a range of mass from 13,449–13,812 Da. MALDI-TOF spectra did not indicate any areas of heterogenous glycosylation to aid identification of the γ subunit of both toxins. Thus, we labeled the two subunits with low PLA_2

activity as β1 and β2 subunit, respectively. The similar molecular mass of the α subunit of P-EPTX-Ap1a and P-EPTX-Ar1a is consistent with snake venom pre-synaptic PLA_2 neurotoxin components isolated from *A. antarcticus* [20] and *O. scutellatus canni* venoms [25].

Many elapid venom PLA_2 components are pre-synaptically neurotoxic [26]. Hence, P-EPTX-Ap1a and P-EPTX-Ar1a were examined for in vitro neurotoxicity using a skeletal muscle preparation. Both toxins inhibited indirect twitches but did not inhibit the contractile responses to exogenous ACh, CCh or KCl. This pharmacological profile indicates a pre-synaptic mode of action. Under conditions of reduced safety margin for neurotransmitter release, the reduction of indirect twitches by P-EPTX-Ap1a and P-EPTX-Ar1a was triphasic, characterized by an initial decrease and a transient increase followed by the complete inhibition of twitches. This triphasic effect is commonly observed with many other pre-synaptic neurotoxins, taipoxin, notexin, and β-bungarotoxin [27]. The initial two phases seem to be independent of PLA_2 activity [28], and are particularly evident when the safety factor of transmission is lowered by reducing the Ca^{2+} or increasing the Mg^{2+} content of the bathing medium [29].

P-EPTX-Ap1a, P-EPTX-Ar1a and their subunits were examined for PLA_2 activity. Studies from our laboratory have shown that the venom of *A. praelongus* possesses higher PLA_2 activity than that of *A. rugosus* [30]. In the current study, there was no significant difference between the PLA_2 activity of P-EPTX-Ar1a and P-EPTX-Ap1a indicating the difference observed between the whole venoms is likely to be due to the presence of other PLA_2 components. To test whether the PLA_2 activity of P-EPTX-Ap1a and P-EPTX-Ar1a was essential for their neurotoxic effects, we subjected the toxins to 4-BPB modification. Previous studies have shown that PLA_2 activity can be inhibited by selective alkylation of the His-48 residue using 4-BPB [31,32]. The complete block of indirect twitches of the chick preparation was significantly attenuated when P-EPTX-Ap1a and P-EPTX-Ar1a were incubated with 4-BPB prior to addition. However, a partial decrease in twitch height was observed for both alkylated toxins. This finding is in agreement with previous studies which have shown that chemical inactivation of the PLA_2 active site by 4-BPB inhibits the effects of pre-synaptic neurotoxins such as P-EPTX-Aa1a [20], taipoxin [33], β-bungarotoxin [29,32] and cannitoxin [25].

Pre-synaptic neurotoxins with PLA_2 activity can be classified based on their structures as being single, diametric, or multichain complexes [34]. 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 [34]. Previous studies have shown that the activity of the toxic subunit is far less than the activity of native toxin [35]. Therefore, the subunits of P-EPTX-Ap1a and P-EPTX-Ar1a were examined for PLA_2 activity and neurotoxicity. The current study clearly showed that the α-subunit of both toxins were the only components to display marked activity as well as the lack of PLA_2 activity and neurotoxicity in the β1 and β2 subunits supporting previous studies of P-EPTX-Aa1a [20] and cannitoxin [25].

In conclusion, P-EPTX-Ap1a and P-EPTX-Ar1a are the first pre-synaptic PLA_2 neurotoxin complexes to be characterized from *A. praelongus* and *A. rugosus* venom, respectively, and the second and third pre-synaptic neurotoxins isolated from *Acanthophis* spp. venoms. Both toxins caused in vitro neurotoxicity in a skeletal muscle preparation which was markedly reduced by 4-BPB indicating that PLA_2 activity is essential for their neurotoxicity. Given the results of this study clinicians may need to be mindful of persistent neurotoxicity following death adder envenoming. Early intervention with antivenom may be important in severe death adder neurotoxicity.

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