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Characterisation of the heterotrimeric presynaptic phospholipase A_2 neurotoxin complex from the venom of the common death adder (*Acanthophis antarcticus*)

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ABSTRACT

While Australo-Papuan death adder neurotoxicity is generally considered to be due to the actions of reversible competitive postsynaptic α-neurotoxins, the neurotoxic effects are often poorly reversed by antivenom or anticholinesterases. This suggests that the venom may contain a snake presynaptic phospholipase A_2 (PLA_2) neurotoxin (SPAN) that binds irreversibly to motor nerve terminals to inhibit neurotransmitter release. Using size-exclusion liquid chromatography under non-reducing conditions, we report the isolation and characterisation of a high molecular mass SPAN complex, P-elapitoxin-Aa1a (P-EPTX-Aa1a), from the venom of the common death adder *Acanthophis antarcticus*. Using the chick biventer-cervicis nerve-muscle preparation, P-EPTX-Aa1a (44,698 Da) caused inhibition of nerve-evoked twitch contractions while responses to cholinergic agonists and KCl remained unaffected. P-EPTX-Aa1a also produced significant fade in tetanic contractions and a triphasic timecourse of neuromuscular blockade. These actions are consistent with other SPANs that inhibit acetylcholine release. P-EPTX-Aa1a was found to be a heterotrimeric complex composed of α, β and γ-subunits in a 1:1:1 stoichiometry with each subunit showing significant *N*-terminal sequence homology to the subunits of taipoxin, a SPAN from *Oxyuranus s. scutellatus*. Like taipoxin, only the α-chain produced any signs of neurotoxicity or displayed significant PLA_2 enzymatic activity. Preincubation with monovalent death adder antivenom or suramin, or inhibition of PLA_2 activity by incubation with 4-bromophenacyl bromide, either prevented or significantly delayed the onset of toxicity by P-EPTX-Aa1a. However, antivenom failed to reverse neurotoxicity. Early intervention with antivenom may therefore be important in severe cases of envenomation by *A. antarcticus*, given the presence of potent irreversible presynaptic neurotoxins.

1. Introduction

The Elapidae family consists of a variety of highly venomous snakes, many of which are found in Australia. The common death adder *Acanthophis antarcticus* (Serpentes: Elapidae: Acanthophiinae) is usually found in the coastal areas of the states of Queensland, New South Wales (NSW), South Australia and Western Australia [1,2] and is considered an elapid, despite its viper-like appearance and behaviour. Other *Acanthophis* spp. have also been identified in Irian Jaya, Papua New Guinea and some eastern Indonesian islands [2]. Symptoms of envenomation from *Acanthophis* mainly involve neurotoxic symptoms, including ptosis and general flaccid muscle paralysis, with death resulting from respiratory failure [2,3]. Although some cases of weak haemolytic and anticoagulant activity have been reported [4,5], there have been no serious clinical cases of coagulopathies resulting from envenomation, as is common with bites from many other venomous Australian elapid snakes. Envenomation does not produce any clinical signs of myotoxicity, with studies confirming that there is no evidence of skeletal muscle damage [6,7]. More recent studies have found myotoxicity present in the directly stimulated chick biventer-cervicis nerve-muscle (CBCNM) preparation after incubation with some species of death adder, however *A. antarcticus* whole venom was devoid of myotoxic activity [8]. Significantly, delayed-onset neurotoxicity is a problem in death adder envenomations, occasionally with late presentation of neurotoxicity as the first feature of envenoming [2,9,10]. This has resulted in a recommended hospital admission period of 24 h for patients in Papua New Guinea and both central and northern Australia, and for children in any region [11].

Abbreviations: ACh, acetylcholine; ANOVA, analysis of variance; 4BPB, 4-bromophenacyl bromide; CBCNM, chick biventer-cervicis nerve-muscle; CCh, carbachol; ESI-Q-TOF, electrospray ionisation quadrupole time-of-flight; MALDI-TOF, matrix-assisted laser desorption ionisation time-of-flight; NSW, New South Wales; PLA_2, phospholipase A_2; P-EPTX-Aa1a, P-elapitoxin-Aa1a; RP-HPLC, reverse-phase high-pressure liquid chromatography; SPAN, snake presynaptic phospholipase A_2 neurotoxin; TFA, trifluoroacetic acid; t_{90}, time to 90% neuromuscular blockade; V_e, elution volume; V_o, void volume.

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The recommended primary treatment for death adder envenomation is CSL monovalent death adder antivenom, which has been raised against *A. antarcticus* venom [12,13], although cross-neutralisation occurs with 'monovalent' taipan and brown snake antivenoms [14]. Death adder antivenom appears to be quite effective in preventing the progression of neurotoxicity from all species of death adder in Australia and Papua New Guinea [2,3], showing a rapid reversal of paralysis. However, some reports have suggested that the neurotoxic effects can be poorly reversed by antivenom or anticholinesterase (neostigmine) if the patient presents late after envenomation [15]. In support, an *in vitro* study involving *A. antarcticus*, *A. praelongus* and *A. pyrrhus* venoms found that all three venoms produced rapid postsynaptic neurotoxicity but antivenom displayed varying efficacy to reverse toxicity following neuromuscular blockade over a 4-h period. Indeed, there was only a 22% recovery of contractile responses following complete neuromuscular blockade with *A. antarcticus*, the venom to which the antivenom is raised [16].

Acanthophis venoms have long been considered to be composed of predominantly postsynaptic α-neurotoxins (for a review see [17]). However, rapidly developing neurotoxicity from postsynaptic α-neurotoxins conceals the action of any underlying snake presynaptic phospholipase A_2 (PLA_2; EC 3.1.1.4) neurotoxins (so-called 'β-neurotoxins' or SPANs), that bind to motor nerve terminals to inhibit neurotransmitter release [18], or myotoxins that may be present in the venom. These toxins typically have slower onsets of activity but bind irreversibly [19,20]. In the case of patients with delayed-onset or slowly developing neurotoxicity, SPANS or myotoxins may play a significant role in the speed of recovery following antivenom therapy due to the irreversible nature of their actions, and may go some way to explain the above resistance to antivenom therapy. While a number of monomeric PLA_2 (12–15 kDa) proteins have been isolated from death adder venom (for a review see [17]), none of these have been characterised pharmacologically. Recently, however, we identified the presence of high molecular mass presynaptic neurotoxic fractions within the venom of *A. antarcticus* geographic variants and other Australo-Papuan *Acanthophis* spp. [21]. This study therefore aimed to biochemically and pharmacologically characterise the high molecular mass SPAN complex present within *A. antarcticus* (NSW variant) venom.

2. Materials and methods

2.1. Venom/toxin preparation and storage

Lyophilised pooled *A. antarcticus* venom (NSW variant) was provided by the Australian Reptile Park (Gosford, NSW). Death adders were collected from their natural habitats in the Sydney metropolitan region. To minimise the effects of individual variations in venom [22], venom was collected, pooled and lyophilised (100 mg dry weight) by the supplier. Lyophilised venom and isolated components were stored at −20 °C until required.

2.2. Size-exclusion liquid chromatography under non-reducing conditions

Bioassay-guided isolation of the high molecular mass SPAN complex using fast-perfusion liquid chromatography of whole venom was performed using a Superdex G-75 column (10 mm × 300 mm, 13 μm; GE Healthcare, Sydney, NSW, Australia) employing methods described previously [21]. The purified SPAN complex was rerun under the same conditions to ensure purity. The approximate molecular mass of the SPAN complex was then determined following calibration of the Superdex G-75 column with protein standards ranging from 6.5 to 66 kDa, as described previously [21]. The void volume (V_o) of the column was determined by running blue dextran (2000 kDa), and the elution volume (V_e) was calculated for each molecular mass marker. The molecular mass range of SPAN complexes were then determined from interpolation of a plot of log molecular mass versus retention ratio (V_e/V_o) of the standards.

2.2.1. Reverse-phase high-pressure liquid chromatography (RP-HPLC)

Subunits of the high molecular mass SPAN complex were isolated from the purified size-exclusion fraction using a Chromolith RP-18e column (4.6 mm × 100 mm; Merck KGaA, Darmstadt, Germany). Fractions were eluted using an acetonitrile/trifluoroacetic acid (TFA) gradient (Buffer A: 0.1% [v/v] TFA, Buffer B: acetonitrile/0.085% [v/v] TFA). The gradient employed was: 0–10 min, 2% B; 10–50 min, 2–98% B; at a flow rate of 4 ml/min. The eluant was monitored at 280 and 214 nm.

2.2.2. Bicinchoninic acid protein (BCA) assay

Following lyophilisation, protein concentrations were determined using the Quantipro™ BCA assay kit (Sigma–Aldrich). Protein contents between 5 and 200 μg/ml were detected at 560 nm by a Titertek Multiscan Plus MKII plate reader (Flow Laboratories Australasia, North Ryde, NSW). A standard curve was created using bovine serum albumin from which protein concentrations were interpolated.

2.3. Mass determination of toxin subunits

2.3.1. Matrix-assisted laser desorption ionisation time-of-flight (MALDI-TOF) mass spectrometry

Lyophilised RP-HPLC samples were analysed to determine mass and confirm purity using a Shimadzu AXIMA TOF^2 (Shimadzu Oceania, Rydalmere, NSW, Australia). The system was operated in positive linear mode. Sinapinic acid (20 mg/ml in 40% acetonitrile, 0.1% TFA) was used as the matrix. Samples were resuspended in 50% [v/v] acetonitrile, 1% [v/v] TFA and spotted with an equal volume of matrix, then allowed to dry. Data was collected with a 337 nm nitrogen laser. The signal was averaged and processed using Shimadzu Launchpad 2.7 software (Shimadzu Oceania, Rydalmere, NSW, Australia). All spectra were calibrated externally with appropriate protein standard mixtures (insulin, 5729.61 Da; cytochrome c, 12,361.96 Da and apomyoglobin, 16,952.27 Da).

2.3.2. Electrospray ionisation quadrupole time-of-flight (ESI-Q-TOF) mass spectrometry

Once purity was confirmed, the definitive mass of RP-HPLC samples was determined using a QSTAR Elite hybrid Q-TOF mass spectrometer system (Applied Biosystems/MDS Sciex, Foster City, CA, USA) equipped with a nanospray source. Lyophilised samples were dissolved into 10 μL of solvent A (2% [v/v] acetonitrile, 0.2% [v/v] formic acid) and were loaded into an Eksigent AS-1 autosampler connected to a Tempo nanoLC system (Eksigent Technologies, Dublin, CA, USA). Sample was passed into a C18 reverse-phase trap column (Michrom Bioresources, Auburn, CA, USA) connected to a 10-way switching valve at a flow rate of 20 μl/min, and the trap was washed for 3 min. Proteins were eluted from the trap at a flow rate of 500 nl/min, and then flowed into a New Objective IntegraFrit column (100 mm, 75 μm ID) packed with ProteoPep 2 C18 resin. At the moment of switching, an acetonitrile gradient was initiated (Solvent A: 2% [v/v] acetonitrile, 0.2% [v/v] formic acid. Solvent B: 98% [v/v] acetonitrile, 0.2% [v/v] formic acid. Gradient: 0–30 min, 5–95% B; 30–32 min, 95% B; 32–35 min, 95–5% B; 35–45 min, 0% B) to elute bound protein from the column. Eluted proteins flowed into a MicroIonSpray II-mounted 75 μm ID emitter tip that tapered to 15 μm. Charged proteins were then

ionised by nanoelectrospray with a potential of 2300 V into the source of the QSTAR with a temperature of 150 °C. A mass range of 500–2000 Da was scanned every second. The masses of proteins were determined as an average of the masses of multiply charged ions. Raw data were processed using the Analyst® QS version 2.0 (Applied Biosystems/MDS Sciex, Foster City, CA, USA).

2.4. N-terminal sequence determination

N-terminal sequencing of purified toxin subunits was performed by the Biomolecular Research Facility at the University of Newcastle, NSW, Australia using an Applied Biosystems Procise HT Protein Sequencer, and the Australian Proteome Analysis Facility (APAF) using an Applied Biosystems/PerkinElmer Procise 494 Procise protein sequencer. Samples was loaded onto a biobrene-treated, precycled discs and subjected to N-terminal (Edman) sequencing. Sequence homology of purified toxin subunits with existing proteins was determined using a Blastp search of the UniProt Knowledgebase (http://ca.expasy.org/tools/blast) followed by a ClustalW multiple alignment.

2.5. Isolated chick biventer-cervicis nerve-muscle preparation

Isolated fractions were tested for neurotoxic and myotoxic activity using the isolated chick biventer-cervicis nerve-muscle (CBCNM) preparation [23]. Male Australorps chicks aged 1–7 days were euthanised with CO_2 and exsanguinated. The biventer-cervicis muscle with attached nerve was dissected and placed in an organ bath (8 ml) under 1 gram of resting tension. The organ bath contained Krebs-Henseleit solution of the following composition (in mM): NaCl, 118.4; KCl, 4.7; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $NaHCO_3$, 25.0; D-glucose, 11.1; $CaCl_2$, 2.5, which was bubbled with carbogen (95% O_2 and 5% CO_2) and maintained at 34 °C. Indirect stimulation was applied to the motor nerve using supramaximal *ca.* 30 V square-wave pulses of 0.2 ms duration at 0.1 Hz with a Grass S88 stimulator. Contractions were measured using an isometric force transducer (ADInstruments, Belle Vista, NSW, Australia). The nerve-evoked muscle tissue was allowed to equilibrate for 30 min. Exclusive electrical stimulation of the nerve was ensured by complete blockade of twitches using 10 μM *d*-tubocurarine. Washout was then repeated until twitch tension returned to its original amplitude. Contractures to various exogenous agonists were subsequently recorded in the absence of electrical stimulation before, and after, incubation with toxin. The final bath concentrations and periods of incubation were as follows: acetylcholine (ACh, 1 mM) for 30 s; carbachol (CCh, 20 μM) for 60 s; potassium chloride (KCl, 40 mM) for 30 s [24]. After each agonist incubation period, washout was repeated until a stable baseline was observed. Following addition of toxin, twitch tension amplitude was monitored for 4 h, or until twitches were abolished. Time-matched or vehicle controls confirmed that muscle fatigue was not significant up to 4 h after introduction of muscles into the organ bath. Muscle tension from isometric force transducers was amplified using a ML221 bridge amplifier (ADInstruments) and recorded using a Powerlab 2/25 system (ADInstruments) connected to a Macintosh computer. Data were digitised at 140 Hz, modified with a 50/60 Hz mains filter and a 20–200 Hz low-pass filter. Muscle tension was recorded using Chart v5.5.4 software (ADInstruments).

2.5.1. Antivenom and suramin reversibility studies

The efficacy of monovalent death adder antivenom (CSL Biotherapies, Melbourne, Australia) or suramin to neutralise the activity of the toxin was assessed by preincubating the organ bath with 5 U/ml of antivenom or 0.3 mM suramin for 10 min prior to addition of venom or toxin. Reversibility studies were also performed by the addition of 5 U/ml monovalent death adder antivenom at 90% inhibition of twitch contractions (t_{90}) or 0.3 mM suramin at 50% inhibition of twitch contractions (t_{50}). An additional study observed the reversibility of toxicity using suramin (0.3 mM) after the toxin was incubated for 30 min and then washed from the bath.

2.5.2. Myotoxicity studies

The myotoxic effects of venom or toxin were examined using direct stimulation of the biventer-cervicis muscle. Muscles were directly stimulated every 10 s with square-wave pulses of 2 ms duration at supramaximal voltage (*ca.* 30 V) using electrodes placed around the belly of the muscle. Muscle contractions due to any nerve-evoked release of ACh were blocked by the addition of 10 μM *d*-tubocurarine, which remained in the organ bath for the duration of the experiment. Toxin was left in contact with the preparation until blockade of twitch contractions occurred, or for a 270 min period. Venom or toxins were considered to be myotoxic if they inhibited twitches elicited by direct stimulation or caused an Increase in baseline muscle tension [24].

2.5.3. Low quantal content studies

The presence of Mg^{2+} in the bathing solution facilitates a more pronounced triphasic action of SPANs on twitch contractions [25,26]. The safety margin for neurotransmitter release was therefore reduced in separate experiments by the addition of 9–17 mM Mg^{2+} to the bath [27]. Increasing concentrations of $MgCl_2$ were titrated to suppress twitch contractions to between 10 and 30% of initial amplitude. After twitch contractions had stablised for at least 30 min toxin was then added.

2.5.4. Tetanic fade studies

In separate experiments, the ability of the CBCNM preparation to maintain tetanic contractions in response to a short tetanic train of stimuli in the presence of the toxin was investigated. Prior to, and following, addition of toxin to the bath, three 50 Hz, 3 s trains every 10 s were interspersed at 0% (control), 25% (t_{25}), 50% (t_{50}), 75% (t_{75}) and 90% (t_{90}) neuromuscular blockade. These tetanic trains were interspersed between normal 0.05 ms 0.1 Hz stimulation. The amount of fade in the tetanic response was calculated by comparing the amplitude of the initial (t_{initial}) and final tension (t_{final}) during the tetanic train. Tetanic fade was determined from the following equation:

$$\%\ \text{Tetanic Fade} = 100 \times \left(1 - \frac{t_{\text{final}}}{t_{\text{initial}}}\right) \quad (1)$$

2.6. Secretory phospholipase A_2 ($sPLA_2$) activity assay

The $sPLA_2$ activities of whole venom and isolated toxins were determined using a colorimetric $sPLA_2$ assay kit (Cayman Chemical Ltd, Ann Arbor, MI) as previously described [21]. $sPLA_2$ activity was calculated as micromoles of substrate (1,2-dithio analog of diheptanoyl phosphohatidylcholine) hydrolysed per minute per milligram of enzyme (μmol/min/mg), with bee (*Apis mellifera*) venom as a positive control.

2.7. Chemical modification of His^{48} using 4-bromophenacyl bromide

The PLA_2 enzymatic activity of the toxin was inhibited by alkylation of the His^{48} residue using 4-bromophenacyl bromide (4BPB) as previously described [28]. 330 μg of toxin was prepared in 615 μl of 0.1 M Tris–HCl/0.7 M EDTA buffer (pH 8.0), to which 10 μl of 4BPB (0.75 mg/ml in ethanol) was subsequently added,

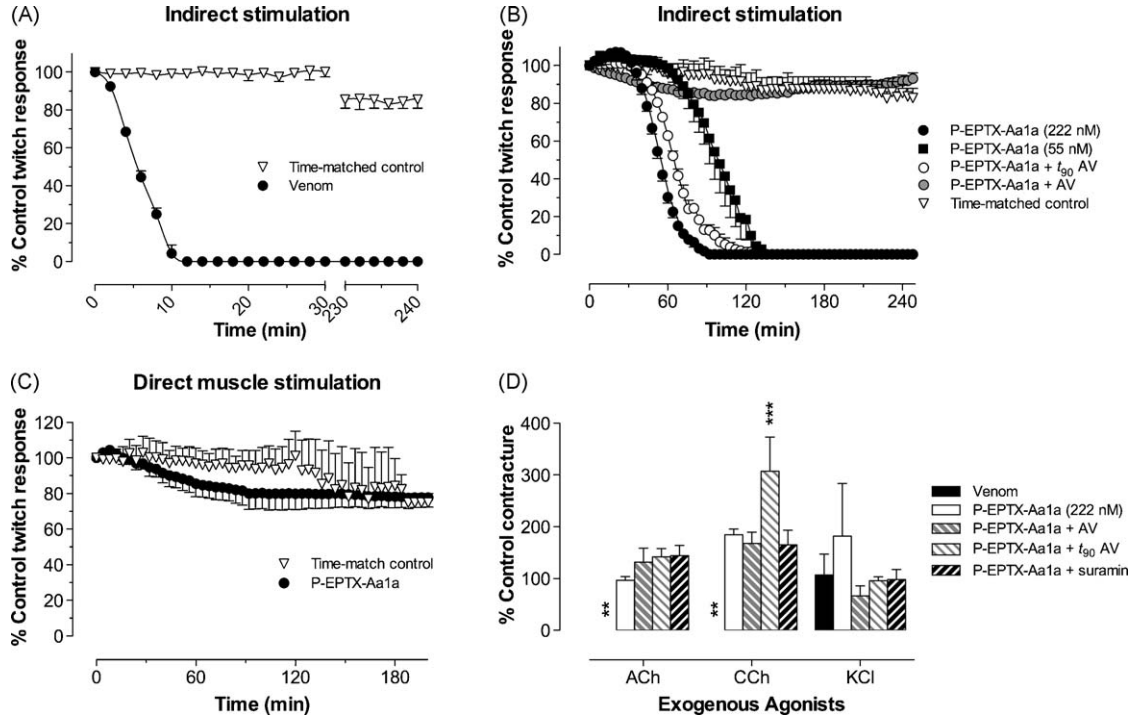

Fig. 1. Effects of P-EPTX-Aa1a on neuromuscular transmission in the chick biventer-cervicis nerve-muscle preparation. (A) Rapid inhibition of indirectly stimulated (nerve-evoked) fast twitch contractions by 10 μg/ml whole *A. antarcticus* (NSW variant) venom (closed circles, $n = 2$), with no significant changes in twitch tension in time-matched controls (open triangles, $n = 3$). Note the split *x*-axis. (B) Concentration-dependent inhibition of indirectly stimulated fast twitch contractions by P-EPTX-Aa1a (55 nM, closed squares, $n = 4$; 222 nM, closed circles, $n = 4$). Addition of 5 U/ml monovalent death adder antivenom, 10 min prior to application of 222 nM P-EPTX-Aa1a, successfully prevented onset of toxicity (gray circles, $n = 4$), whereas antivenom applied at t_{90} failed to reverse the inhibition of twitch contractions (open circles, $n = 4$). (C) Lack of effect of 222 nM P-EPTX-Aa1a on twitch contractions evoked by direct muscle stimulation (closed circles, $n = 4$), in comparison to time-matched controls (open triangles, $n = 4$). All muscle contractions were recorded in the presence of 10 μM *d*-tubocurarine to prevent any effects of nerve-evoked release of ACh. (D) Effect of venom and P-EPTX-Aa1a on contractile responses to exogenous ACh, CCh and KCl. Data shows the percentage of control slow fibre contracture responses to exogenous agonists after complete inhibition of twitch contractions by 10 μg/ml venom (closed bars, $n = 2$) or 222 nM toxin alone (open bars, $n = 4$), or following application of 5 U/ml antivenom at t_{90} (thick gray striped bars, $n = 4$) or 0.3 mM suramin (black striped bars, $n = 4$). In the case of 5 U/ml antivenom preincubated 10 min prior to addition of 222 nM P-EPTX-Aa1a (thin gray striped bars, $n = 4$), data was recorded at 300 min. Note that only whole venom completely inhibited responses to ACh and CCh. Data represent the mean ± SEM. $^{**}p < 0.01$, $^{***}p < 0.001$, significantly different from control response, one-way ANOVA. For clarity, only data points recorded every 4 min are displayed in panels B and C.

and incubated at 25 °C for 13 h. The modified toxin was collected and lyophilised prior to assaying for $sPLA_2$ activity and neurotoxicity.

2.8. Chemicals and drugs

Unless otherwise stated, all chemicals and drugs were purchased from Sigma–Aldrich, Castle Hill, NSW, Australia.

2.9. Data analysis

Twitch contraction amplitude was expressed as a percentage of initial amplitude, prior to addition of the toxin. Contractile responses to exogenous agonists were expressed as a percentage of their amplitude prior to addition of the toxin. To compare the neurotoxicity of venoms, the time taken to cause 90% inhibition of nerve-mediated twitch contractions (t_{90}) was determined as a quantitative measure of potency. Values for t_{90} were calculated for each experiment by determining the elapsed time after toxin addition at 10% of the initial twitch contraction amplitude, and then the means and standard error of the means were calculated. Where indicated, statistical significance was determined by one-way analysis of variance (ANOVA) or paired Student's *t*-test. All ANOVAs were followed by a Bonferroni-corrected multiple *t*-test. Statistical analyses were performed using the PRISM 5.0 software package (GraphPad Software, San Diego, CA, USA). A *p*-value of 0.05 was employed for all statistical analyses.

3. Results

3.1. Toxicity of whole venom

Whole *A. antarcticus* venom (10 μg/ml) produced rapid and complete inhibition of nerve-evoked twitch contractions within 9 ± 1 min ($n = 2$; Fig. 1A), whereas with time-matched controls there was no significant fatigue of twitch tension over a 240 min period (Fig. 1A). Whole *A. antarcticus* venom displayed classical postsynaptic neurotoxic activity, with complete inhibition of responses to exogenous nicotinic agonists ($p < 0.01$, $n = 2$; Fig. 1D).

3.2. Venom fractionation under non-denaturing conditions

Prior to separation of whole *A. antarcticus* venom by size-exclusion FPLC the Sephadex G-75 column was calibrated using a set of molecular weight markers. Using blue dextran (2000 kDa), the void volume (V_o) of the Sephadex G-75 column was determined to be 7.6 ml while the elution volume (V_e) of a range of known standards was determined and the molecular mass of unknown complexed was then determined by interpolation of a plot of log molecular mass *vs.* V_e/V_o ratio (Fig. 2B). Under non-reducing conditions, fractionation of whole *A. antarcticus* venom produced a characteristic pattern of four major peaks (Fig. 2A). This was similar to that seen with other geographic variants of *A. antarcticus* and certain other Australo-Papuan species of *Acanthophis* [21]. The retention time of the earliest eluting fraction was 12.8–16.0 min,

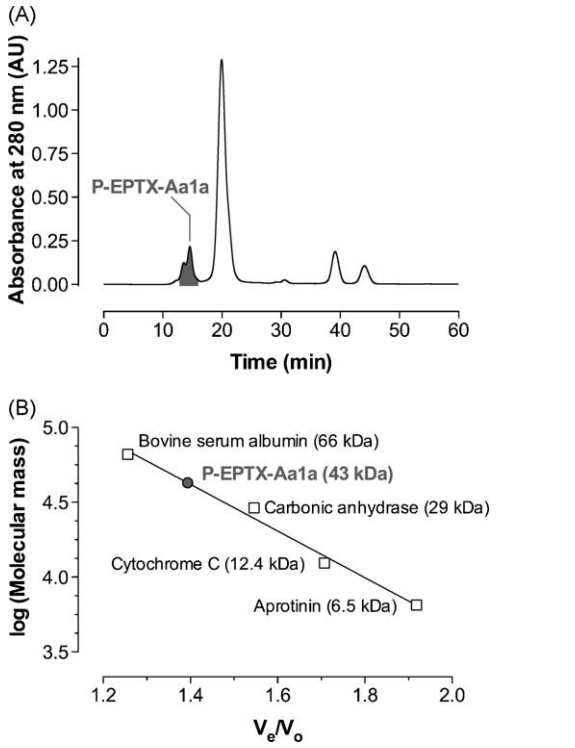

Fig. 2. Separation of the P-EPTX-Aa1a complex under non-reducing conditions. (A) Representative size-exclusion FPLC chromatogram of *Acanthophis antarcticus* (NSW variant) venom employing a Superdex G-75 column with an isocratic flow of 0.1 M ammonium acetate (pH 6.8) at 0.75 ml/min. The shaded fraction, corresponding to P-EPTX-Aa1a, was collected for further characterisation. (B) Determination of the molecular mass of P-EPTX-Aa1a. Log molecular weight versus retention ration (V_e/V_o) standard curve for the calibration of the Superdex G-75 column using a series of molecular weight standards. The molecular mass of P-EPTX-Aa1a was determined to be *ca.* 43 kDa.

with the peak absorbance at 14.6 min (Fig. 2A), corresponding to a molecular mass of *ca.* 43 kDa (Fig. 2B).

SPAN complexes typically have molecular weights ranging between 21 kDa (the covalently linked heterodimer β-bungarotoxin from *Bungarus fasciatus*; [29]) and *ca.* 87 kDa (the heterohexameric textilotoxin; [30]). Therefore the 43 kDa fraction was collected for further biochemical characterisation and neurotoxicity testing. The yield of the early eluting fraction, as determined by BCA assay, was 6.5% (w/w) *A. antarcticus* venom (195 μg from *ca.* 3 mg of whole venom). Toxicity screening in the CBCNM preparation indicated that this fraction contained prejunctional neurotoxic activity (see Section 3.3), while the second peak (eluting at 17.4–21.5 min, *ca.* 3–13 kDa) caused a postsynaptic neurotoxic action due to inhibition of responses to the exogenous nicotinic agonists, ACh and CCh (data not shown). Accordingly, the 43 kDa fraction was named P-elapitoxin-Aa1a (P-EPTX-Aa1a) using the rational nomenclature system for naming toxins from spiders and other venomous animals [31]. The activity descriptor prefix 'P' indicates SPANs with a presynaptic action to inhibit neurotransmitter release, 'elapitoxin' is the generic name for toxins from the family Elapidae, 'Aa' are the genus and species descriptor for *A. antarcticus*, '1' represents the first family of toxins with this activity ('1' was chosen to represent multimeric SPANs vs. '2' for monomeric SPANs), and 'a' denotes the first paralog (isoform) found.

3.3. Neurotoxicity studies with P-EPTX-Aa1a

P-elapitoxin-Aa1a (55–222 nM) produced a concentration-dependent inhibition of nerve-evoked twitch contractions of the

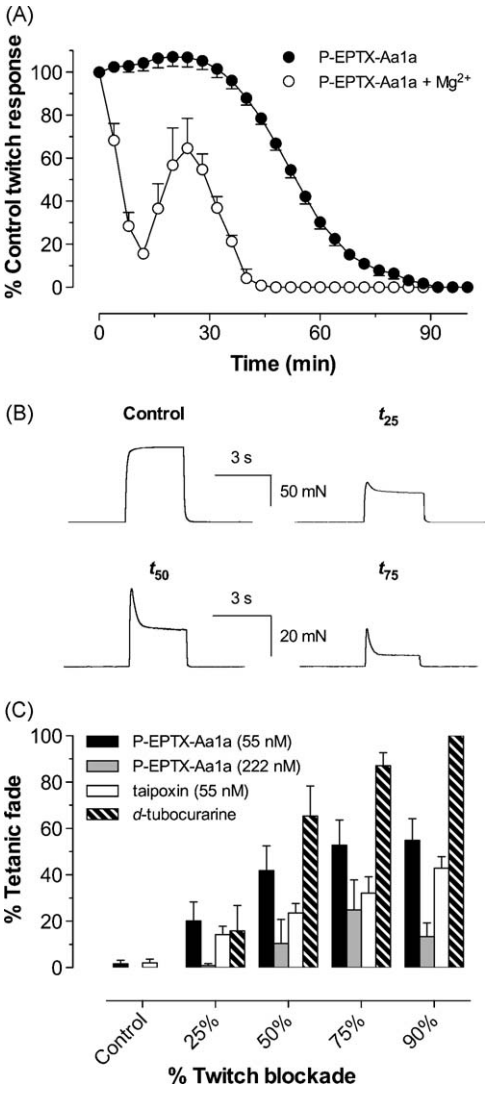

Fig. 3. P-EPTX-Aa1a produces both triphasic changes in twitch tension and tetanic fade during neuromuscular blockade in the CBCNM preparation. (A) Inhibition of indirectly stimulated fast twitch contractions by 222 nM P-EPTX-Aa1a with (open circles, $n = 3$), and without (closed circles, $n = 4$), a reduction in quantal content using 9–17 mM $[Mg^{2+}]_o$. The $[Mg^{2+}]_o$ was titrated to achieve 10–30% of control twitch contractions. Note the triphasic action evident following incubation with raised $[Mg^{2+}]$, typical of SPANs. (B) Representative tension traces showing increasing degrees of tetanic fade during 3 s 50 Hz tetanic trains in the course of the development of neuromuscular blockade by 55 nM P-EPTX-Aa1a. Note the change in calibration in the lower two panels at 50 and 75% neuromuscular blockade. (C) Comparison of the action of P-EPTX-Aa1a (55 nM, closed bars, $n = 4$; 222 nM, gray bars, $n = 3$) with taipoxin (55 nM, open bars, $n = 4$) and *d*-tubocurarine (1 μM, striped bars, $n = 4$). Percent tetanic fade was calculated according to Eq. (1) in Section 2. Data represent the mean ± SEM.

CBCNM preparation (Fig. 1B). The t_{90} value for 222 nM P-EPTX-Aa1a for inhibition of twitch contractions was 74 ± 3.5 min ($n = 4$) that increased to 121 ± 7 min ($n = 4$) using 55 nM P-EPTX-Aa1a. A slight triphasic timecourse of action characterised by depression-enhancement-blockade of neurotransmission was evident as has been previously reported for a variety of other SPANs in mammalian nerve-muscle preparations [19,32]. This triphasic action was appreciably enhanced under conditions of low quantal content (high external $[Mg^{2+}]$), and the t_{90} value for inhibition of twitch contractions was also significantly reduced to 41 ± 1 min ($p < 0.001$, unpaired Student's *t*-test, $n = 3$; Fig. 3A).

Importantly, following complete neuromuscular blockade, P-EPTX-Aa1a failed to inhibit responses to the exogenous

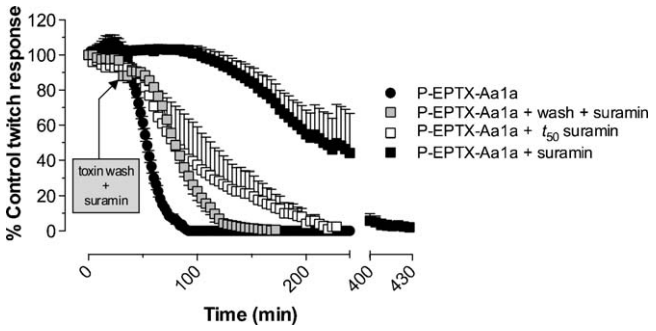

Fig. 4. Neutralisation and reversibility of toxin-induced neuromuscular blockade by suramin in the CBCNM preparation. Suramin preincubated for 10 min prior to the addition of 222 nM P-EPTX-Aa1a (closed squares, $n = 6$) significantly delayed the onset of neurotoxicity. However suramin applied at t_{50} (open squares, $n = 4$) or at 30 min following washout of the toxin (see arrow, gray squares, $n = 4$) prolonged, but did not prevent, neuromuscular blockade. 222 nM P-EPTX-Aa1a (closed circles, $n = 4$) is included for comparison. Note the split *x*-axis. Data represent the mean ± SEM. For clarity, only data points recorded every 4 min are displayed.

cholinergic agonists ACh and CCh or the depolarising agonist KCl ($n = 4$; Fig. 1D). Interestingly, the responses to carbachol were enhanced in many fractions, but this has been previously reported in this preparation [33–35] and may reflect time-dependent sensitization of the tissue to carbachol. Furthermore, P-EPTX-Aa1a failed to induce any significant change in baseline tension (data not shown) or directly stimulated muscle twitches of the biventer-cervicis muscle ($n = 4$; Fig. 1C). These actions are consistent with the presence of a SPAN complex that inhibits neurotransmitter release without any additional signs of myotoxicity.

Further support that P-EPTX-Aa1a is a SPAN complex was provided by the ability of the toxin to induce fade in tetanic contractions that contributes to muscle fatigue at more physiological frequencies of nerve stimulation (Fig. 3B and C). Tetanic fade in controls in response to a 3 s 50 Hz train was minimal in all experiments with a range of 0–6% (1 ± 1%, $n = 7$). In the presence of the positive control, 1 μM *d*-tubocurarine, isometric tension rapidly faded to a plateau level. With P-EPTX-Aa1a and taipoxin tetanic fade developed gradually in the initial 500–1000 ms of the tetanic train to a plateau that was maintained to the end of the train (Fig. 3B). P-EPTX-Aa1a (55 nM) produced concentration-dependent tetanic fade of 53 ± 11% at t_{75} ($n = 4$; Fig. 3C). This was less than the positive control, 1 μM *d*-tubocurarine (87 ± 11% at t_{75}, $n = 4$), but greater than the classical heterotrimeric SPAN, taipoxin (32 ± 14% at t_{75}, $n = 4$, 55 nM) previously shown to cause tetanic fade [36]. Interestingly, lower concentrations produced a greater degree of tetanic fade ($n = 4$; Fig. 3C).

3.4. Neutralisation and reversibility of P-EPTX-Aa1a neurotoxicity

CSL Monovalent death adder antivenom (5 U/ml) was effective in preventing the development of toxicity with P-EPTX-Aa1a (Fig. 1B). However, reversal of P-EPTX-Aa1a toxicity by antivenom applied at t_{90} was unsuccessful (Fig. 1B), highlighting the irreversible binding of SPANs. Preincubation with the polysulphonated naphthylurea anti-trypanosomal drug, suramin (0.3 mM), previously shown to inhibit SPAN but not postsynaptic α-neurotoxin toxicity [37,38], produced a 4.3-fold increase in the time to t_{90} of P-EPTX-Aa1a to 315 ± 51 min ($p < 0.01$, $n = 4$), but unfortunately failed to completely inhibit neuromuscular blockade (Fig. 4). The irreversible nature of toxin binding following only short incubation periods [19,39] was further highlighted by experiments where unbound toxin was washed from the bath after 30 min but neuromuscular blockade still occurred despite subsequent addition of 0.3 mM suramin (t_{90} = 119 ± 7.2 min, $n = 4$; Fig. 4). Suramin administered at t_{50} was even less effective at reversing neurotoxicity with 90% neuromuscular blockade reached at 146 ± 37 min ($n = 4$; Fig. 4). Consistent with its prejunctional action, agonist responses were not

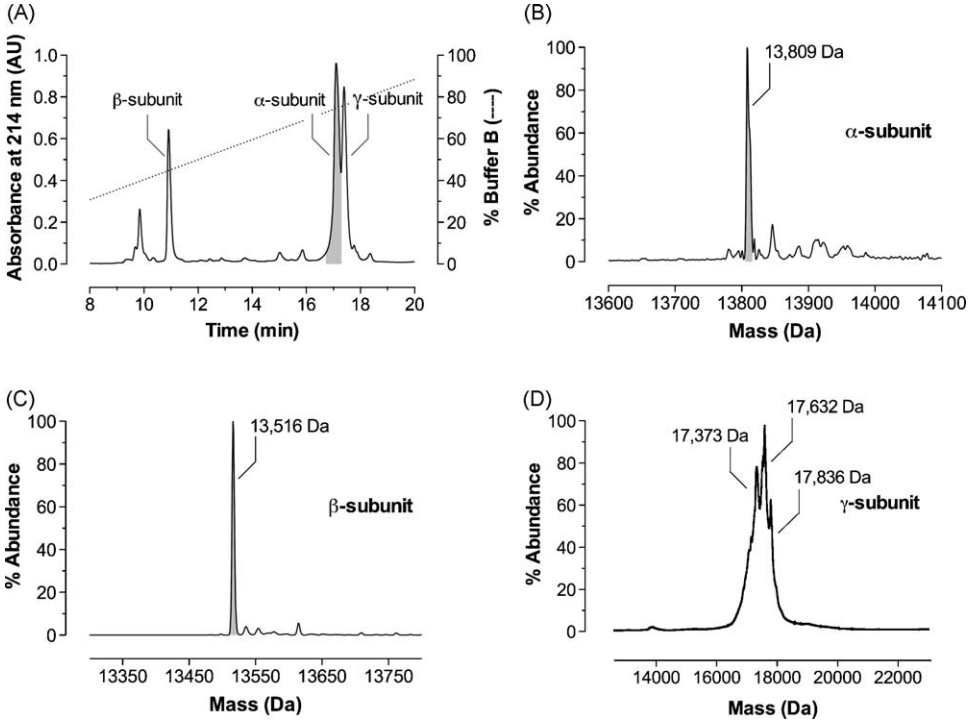

Fig. 5. Isolation and mass determination of P-EPTX-Aa1a subunits. (A) Purification of P-EPTX-Aa1a into component subunits using C18 reverse-phase HPLC employing an acetonitrile/TFA gradient. A representative chromatogram at 214 nm is shown. Subunits are labelled α, β and γ. (B and C) ESI-Q-TOF mass spectrometry, showing deconvoluted spectra of the α-subunit (panel B, 13,809 Da) and β-subunit (panel C, 13,516 Da). (D) MALDI-TOF mass spectrometry of the γ-subunit showing a spectrum consistent with heterogeneous glycosylation, with a nominal mass of 17,373 Da. The sum of masses is 44,698 Da, which is consistent with the estimated mass from size-exclusion chromatography.

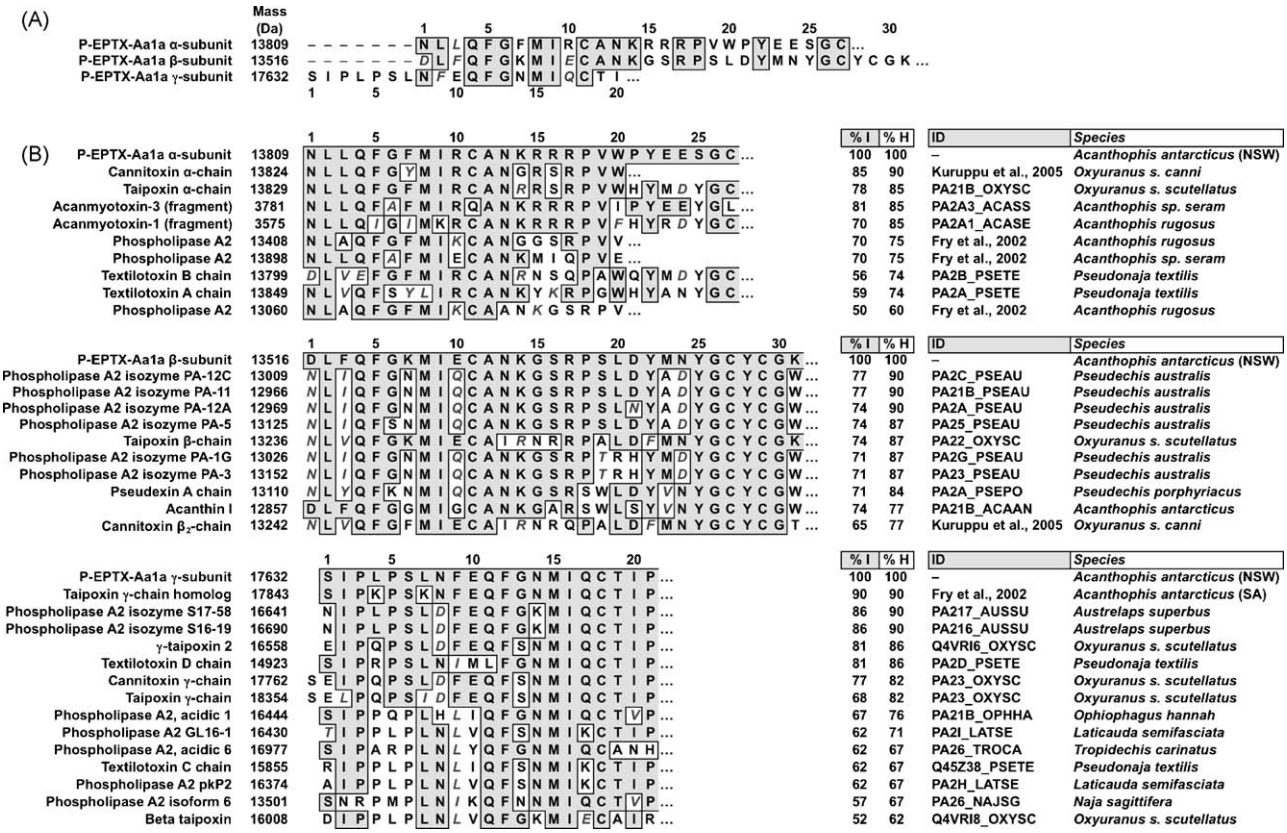

Fig. 6. Sequence alignment of P-EPTX-Aa1a subunits. (A) Alignment of the three subunits of P-EPTX-Aa1a. (B) Alignment of individual P-EPTX-Aa1a subunits with known sequences of other snake sPLA2 enzymes and neurotoxins. Conserved residues are boxed in gray while conservative substitutions are shown in gray italic text. Percentage identity (%I) is relative to each respective P-EPTX-Aa1a subunit while percentage homology (%H) includes conservatively substituted residues. 'ID' indicates the UniprotKB/SwissProt ID code or source reference. 'Species' refers to the venom source.

significantly inhibited following application of antivenom or suramin in the above experiments (Fig. 1D).

3.5. Fractionation of the P-EPTX-Aa1a complex

Given that SPAN complexes are commonly derived of between 2 and 6 subunits [30,40], P-EPTX-Aa1a subunits were separated under reducing conditions using C18 RP-HPLC to determine the subunit composition. This resulted in three major peaks (Fig. 5A). The BCA protein assay failed to detect any significant quantities of protein in the early eluting peak at 9.6–10.1 min, and this peak was subsequently excluded from further investigation. The three most abundant peaks, eluting at 16.7, 10.8, and 17.3 min, were named the α-, β-, and γ-subunits of P-EPTX-Aa1a, respectively, based on their homology to the molecular masses (see Section 3.5) and N-terminal amino acid sequences (see Section 3.6) of known multimeric SPAN subunits.

3.6. Mass spectrometry of P-EPTX-Aa1a subunits

ESI-Q-TOF mass spectrometry determined the definitive mass of the α- and β-subunits to be 13,809 Da and 13,516 Da, respectively (Figs. 5B and C). MALDI-TOF mass spectrometry determined that the γ-subunit was most likely heterogeneously glycosylated with a nominal mass of 17,373 Da (Fig. 5D). The sum of the α-, β- and γ-subunit masses resulted in an average mass of 44,698 Da, which is comparable with the mass estimated from calibration of the size-exclusion column (*ca.* 43 kDa), suggesting a 1:1:1 binding stoichiometry.

3.7. N-terminal sequencing of P-EPTX-Aa1a subunits

Partial N-terminal sequences of the α-, β-, and γ-subunits were determined by Edman degradation. The α- and β-subunits showed a high degree of homology to each other but the γ-subunit possessed a seven residue extension at the N-terminus (Fig. 6A). All three subunits were then subjected to a Blastp analysis of the UniProt Knowledgebase and found to show significant homology to a range of SPAN neurotoxin subunits (Fig. 6B). Comparing the three subunit sequences with corresponding N-terminal fragments of other snake proteins, the subunits of P-EPTX-Aa1a displayed the highest overall homology to the heterotrimeric SPAN taipoxin from the coastal taipan *Oxyuranus s. scutellatus* [41], with the α-, β- and γ-chains of taipoxin showing 85%, 87% and 90% homology to the corresponding subunits of P-EPTX-Aa1a. Individual subunits of P-EPTX-Aa1a also showed lesser homology to subunits of the heteromultimeric SPANs cannitoxin from *Oxyuranus s. canni* [35] and textilotoxin from *Pseudonaja textilis* [42].

3.8. Neurotoxicity studies with P-EPTX-Aa1a subunits

Of the three subunits isolated from P-EPTX-Aa1a, only the α-subunit (740 nM) produced inhibition of nerve-evoked twitch contractions compared with the time-matched control, resulting in 79 ± 10% neuromuscular blockade after 270 min (n = 4; Fig. 7A). This neuromuscular blockade occurred in the absence of any significant inhibition of responses to exogenous agonists (Fig. 7C). There was a complete lack of toxicity apparent with both β- and γ-subunits at this high concentration. Equimolar recombination of these subunits determined that the α-subunit was important in presenting any

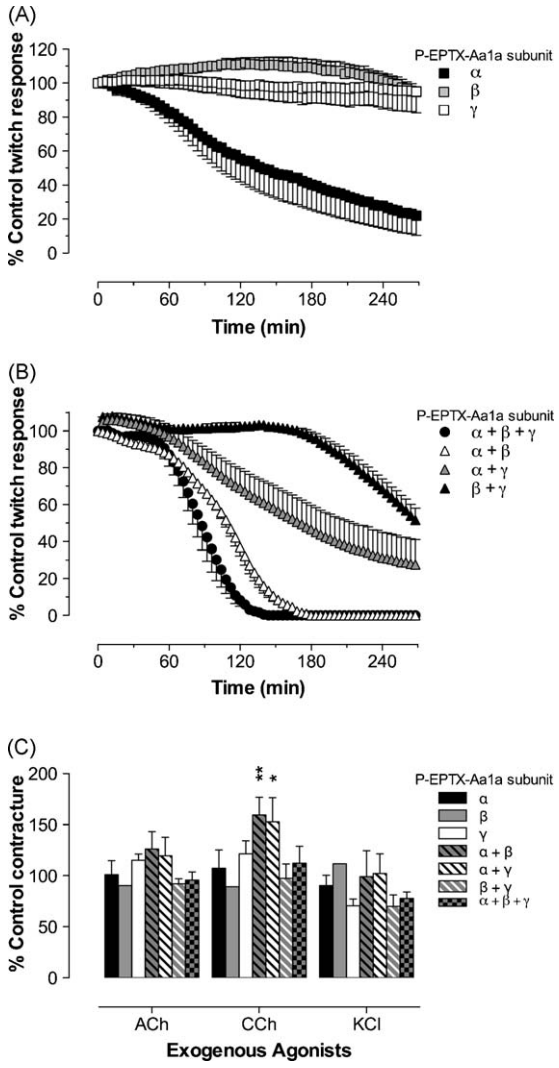

Fig. 7. Neurotoxicity of individual P-EPTX-Aa1a subunits in the CBCNM preparation. Inhibition of indirectly stimulated fast twitch contractions by the (A) individual α-, β-, and γ-subunits of P-EPTX-Aa1a subunits (740 nM, squares) and (B) equimolar combinations of two (triangles) or three (closed circles) subunits at 150 nM. Data represents the mean ± SEM of 4 experiments. (C) Effect of P-EPTX-Aa1a subunits (individual and recombined) on contractile responses to exogenous ACh, CCh and KCl. Data shows the percentage of control slow fibre contracture responses to exogenous agonists after complete inhibition by toxin or, in the case of subunits and recombinations that did not result in complete blockade, at 300 min. Data represent the mean ± SEM of 4 experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, significantly different from control response, one-way ANOVA. For clarity, only data points recorded every 4 min are displayed in panels A and B.

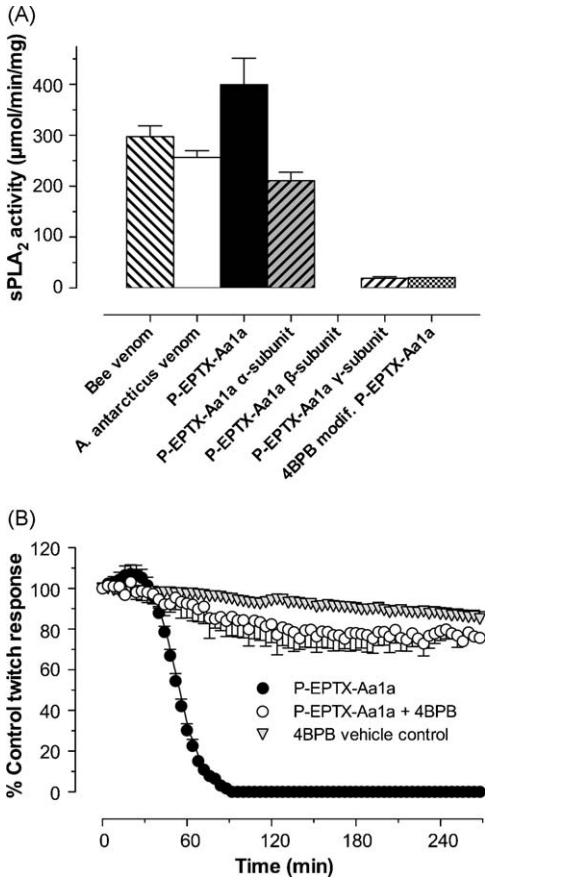

Fig. 8. PLA_2 activity of whole venom, toxin and subunits and neurotoxicity of chemically modified toxin. (A) PLA_2 activity of whole *Acanthophis antarcticus* (NSW) venom (open bar, $n = 6$), P-EPTX-Aa1a (closed bar, $n = 4$) and its three subunits (striped bars, $n = 3$–6), and 4BPB-modified P-EPTX-Aa1a (stippled bar, $n = 1$) as determined by the $sPLA_2$ assay kit. Bee (*A. mellifera*) venom (left hand bar $n = 18$) was used as a positive control. (B) Alkylated P-EPTX-Aa1a (open circles, $n = 4$), produced by chemical modification with 4-bromophenacyl bromide (4BPB), failed to inhibit indirectly stimulated fast twitch contractions in the CBCNM preparation when compared to 4BPB vehicle controls (open triangles, $n = 4$). 222 nM P-EPTX-Aa1a (closed circles, $n = 4$) is included for comparison. Data represent the mean ± SEM. For clarity, only data points recorded every 4 min are displayed in panel B.

significant toxicity, as β- and γ-subunits (150 nM) combined only caused incomplete inhibition of twitch contractions with only 51 ± 7% neuromuscular blockade after 270 min (Fig. 7B). The equimolar recombination of the α- and γ-subunits (150 nM) caused some inhibition of twitch responses with 73 ± 13% neuromuscular blockade at 270 min ($n = 4$) while the recombination of the α- and β-subunits (150 nM) resulted in complete neuromuscular blockade with a t_{90} value of 149 ± 6 min ($n = 4$; Fig. 7B). The equimolar recombination of all 3 subunits (150 nM) produced the most rapid inhibition of twitches ($t_{90} = 114 \pm 7$ min, $n = 4$; Fig. 7B), which was slower in comparison to the whole P-EPTX-Aa1a complex (one-way ANOVA, $p < 0.05$) but significantly more rapid than with the α-subunit alone. Finally, no individual P-EPTX-Aa1a subunit or recombined subunits inhibited agonist responses (Fig. 7C).

3.9. Secretory phospholipase A_2 ($sPLA_2$) activity

The $sPLA_2$ activity of whole *A. antarcticus* venom and P-EPTX-Aa1a was determined to be 257 ± 14 and 400 ± 52 μmol/min/mg, respectively ($n = 4$; Fig. 8A). In comparison, the $sPLA_2$ activity of the positive control, *A. mellifera* bee venom, was 287 ± 10 μmol/min/mg ($n = 18$). The α-subunit also displayed appreciable PLA_2 activity of 211 ± 17 μmol/min/mg ($n = 6$) while the β- and γ-subunits did not exhibit any significant PLA_2 activity ($n = 3$).

3.10. Chemical modification of histidine residues of P-EPTX-Aa1a using 4-bromophenacyl bromide

Modification of histidine residues in P-EPTX-Aa1a using 4BPB reduced the PLA_2 activity to 20 μmol/min/mg, equivalent to *ca.* 5% of the original P-EPTX-Aa1a complex (Fig. 8A). This is most likely the result of alkylation of a His^{48} residue previously identified at the active site of snake PLA_2 toxins [43]. Alkylated P-EPTX-Aa1a caused significantly reduced toxicity in the CBCNM preparation, with only a 13 ± 3.2% reduction in twitch responses at 240 min ($n = 4$; Fig. 8B). This occurred in the absence of any reduction in responses to exogenous agonists (data not shown). The vehicle (4BPB) had no effect on twitch tension or agonist responses (Fig. 8B).

4. Discussion

While the venom of *A. antarcticus* displays classical postjunctional neurotoxicity, and is known to contain a number of long- and short-chain postsynaptic α-neurotoxins [17], the present study clearly identifies the presence of a heterotrimeric SPAN complex. To our knowledge, P-EPTX-Aa1a represents the first SPAN complex to be biochemically and pharmacologically characterised from the venom of *A. antarcticus*. P-EPTX-Aa1a caused concentration-dependent inhibition of neuromuscular transmission, with no reduction of contracture responses to nicotinic cholinergic agonists, consistent with a prejunctional action to block ACh release. Some monomeric SPAN neurotoxins such as notexin also have myotoxic activity [44] but the absence of any effect on responses to KCl and direct muscle stimulation are consistent with P-EPTX-Aa1a acting as a presynaptic neurotoxin lacking myotoxicity. P-EPTX-Aa1a also produced a pronounced triphasic action, particularly under conditions of low quantal content, characterised by an initial brief depression, followed by a period of increased tension, and then a gradual decline to complete neuromuscular blockade. This is typical of SPANs including β-bungarotoxin [45], taipoxin [46], notexin [47] and crotoxin [48], especially under conditions of high $[Mg^{2+}]_o$ [25,26].

Under non-denaturing conditions the molecular mass of the P-EPTX-Aa1a complex was determined as *ca.* 43 kDa following separation of the SPAN on a size-exclusion column. Subsequent RP-HPLC of P-EPTX-Aa1a produced three major subunits whose combined masses, 44,698 Da, was in close agreement with the molecular mass of the SPAN complex estimated from size-exclusion chromatography. Masses and N-terminal sequences of all three P-EPTX-Aa1a subunits were similar to those found in the heterotrimeric SPAN complexes taipoxin, cannitoxin and to a lesser extent textilotoxin [35,42]. MALDI-TOF mass spectrometry revealed the γ-subunit of P-EPTX-Aa1a to be a glycoprotein as evidenced by the areas of heterogenous glycosylation observed in the MALDI spectra. Glycosylation was also described for γ-subunits of previously isolated heterotrimeric SPAN complexes such as taipoxin [41], cannitoxin [35], and paradoxin [49].

Similar to other heterotrimeric SPAN complexes, only the α-subunit of P-EPTX-Aa1a presented any significant PLA_2 or neurotoxic activity, likely contributing significantly to the overall toxicity of the complex. Unfortunately it is difficult to compare PLA_2 activity between studies as assay conditions vary considerably. However, using the same colourimetric assay, cannitoxin was found to possess a similar pattern of high PLA_2 activity in the whole complex and α-subunit, albeit at a lower activity than P-EPTX-Aa1a [35]. Furthermore the lack of PLA_2 activity with the β- and γ-subunits of P-EPTX-Aa1a was mirrored in the cannitoxin study. Despite lacking any significant neurotoxic or enzyme activity all three subunits of P-EPTX-Aa1a appear to be necessary for maximum neurotoxicity, with equimolar recombination of all subunits yielding close to the same neurotoxicity as the native P-EPTX-Aa1a complex. This duplication of homologous subunits possibly reflects an evolutionary approach to increase the affinity of the SPAN complex for its prejunctional target and hence its neurotoxicity [50]. This is perhaps because the affinity of the complex for the nerve terminal is the product of the affinities of its individual subunits [51], the additional subunits contribute to a better positioning of the PLA2 enzyme with respect to its substrate, and/or that they reduce non-specific binding of the active α-subunit. Regardless, the PLA_2 activity of the additional subunits is no longer important and therefore has been lost. As evidence, oligomeric SPANs such as the heterotrimeric taipoxin and paradoxin and heterohexameric textilotoxin are more potent than monomeric SPANs such as notexin, notechis II-5 and pseudexin-A in lethality studies (see [17]).

When 4BPB was used to chemically modify the active site of P-EPTX-Aa1a, both the enzymatic and neurotoxic activity were significantly inhibited, suggesting His^{48} at the catalytic site is important for neurotoxic activity at the neuromuscular junction. This loss of neurotoxicity following alkylation with 4BPB has been previously noted with other SPANs [35,52,53], although it may result from conformational changes in the toxin and an altered ability to form high affinity interactions with its specific binding protein target [54]. Nevertheless, recent studies using a single H48Q point mutation at the active site of the neurotoxic SPAN OS_2, unlikely to cause any structural perturbations at the interfacial surface, completely inhibited activity in the CBCNM preparation but only reduced lethality by icv injection by 16-fold [55]. This finding supports the idea that $sPLA_2$ activity is important for neurotoxicity at the neuromuscular junction but not for central neurotoxicity.

Further support that P-EPTX-Aa1a is a SPAN complex was provided by the ability of P-EPTX-Aa1a to induce fade in tetanic tension as has been previously reported for a number of SPAN complexes [36], but not postsynaptic snake α-neurotoxins [56], during the development of neuromuscular blockade. This is hypothesised to result from a block of prejunctional α3β2 cholinergic receptors that normally mediate autofacilitation of ACh release during high frequency stimulation at the neuromuscular junction [57]. Conversely, tetanic fade may also result from a block of prejunctional adenosine A_2 receptors previously shown to enhance neurotransmitter release [58]. Interestingly, agents that block A_2-mediated enhancement of ACh release have a very slow timecourse of action which may go some way to explain the enhanced degree of tetanic fade at lower concentrations of P-EPTX-Aa1a, presumably resulting from a slower timecourse of neuromuscular blockade. Of course tetanic fade may be the consequence of the external or internal mechanisms to interfere with synaptic vesicle release and recycling seen with SPANs (for a review see [59]). Regardless of the mechanism(s) that produces tetanic fade, it is a clinically important phenomenon that no doubt contributes to muscle fatigue in envenomed patients, particularly if the safety factor for neurotransmission has been compromised due to slowly developing neuromuscular blockade.

We investigated the efficacy of the polysulfonated naphthylurea anti-trypanosomal drug suramin to reverse neuromuscular blockade as it has been previously shown to inhibit the myotoxic and neurotoxic effect of bothropstoxin-I [37]. Being a strongly acidic polysulfonated substance, it may neutralise the $sPLA_2$ neurotoxic activity by forming acid-base complexes, as seen with other polyanionic compounds such as heparin [60]. Suramin has been proven effective in prolonging time to paralysis with the presynaptic neurotoxins β-bungarotoxin and crotoxin [38]. Preincubation with both monovalent death adder antivenom and suramin were effective in the prevention or significant delay of neurotoxicity, respectively. However, reversal of neurotoxicity with antivenom and suramin had varying degrees of effectiveness. Considering the partial effectiveness of suramin in slowing the onset of toxicity, this may be a complimentary treatment to antivenom to prevent delayed-onset toxicity in death adder envenomation. Importantly, however, suramin is ineffective against postsynaptic α-neurotoxins such as α-bungarotoxin [38], and is likely to have little effect against the postsynaptic α-neurotoxins previously identified in *A. antarcticus* venom (see [17] for a review).

In summary, P-EPTX-Aa1a from *A. antarcticus* (NSW) venom shares similar overall complex and subunit mass, degree of glycosylation, neurotoxicity, pattern of $sPLA_2$ activity and sequence homology to the subunits of other high molecular mass heterotrimeric SPAN complexes. In particular, P-EPTX-Aa1a displays the highest overall homology with taipoxin and cannitoxin,

from the coastal and Papuan taipan, respectively, belonging to same Acanthophiinae subfamily as *Acanthophis* spp. Importantly, while early preincubation of antivenom prevented the onset of neurotoxicity, late administration of antivenom (at t_{90}) failed to neutralize the *in vitro* neurotoxicity of P-EPTX-Aa1a. Therefore, since *A. antarcticus* (NSW) venom contains a potent irreversible SPAN, clinicians may need to be attentive of possible presynaptic neurotoxicity following envenomation by this snake and most likely many other *Acanthophis* spp. [21]. Therefore early intervention with antivenom is critically important in preventing severe prolonged envenomation from *A. antarcticus* and other Australo-Papuan death adders.

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