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Isolation and pharmacological characterisation of papuantoxin-1, a postsynaptic neurotoxin from the venom of the Papuan black snake (*Pseudechis papuanus*)

Sanjaya Kuruppu [a], Shane Reeve [b], A. Ian Smith [b], Wayne C. Hodgson [a,*]

[a] *Monash Venom Group, Department of Pharmacology, Monash University, Vic. 3800, Australia*
[b] *Department of Biochemistry and Molecular Biology, Monash University, Vic. 3800, Australia*

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Abstract

The Papuan black snake (*Pseudechis papuanus*) is found throughout the southern coastal regions of Papua New Guinea and is thought to occur in the adjacent region of Iriyan Jaya. Neurotoxicity is a major symptom of envenomation by this species. This study describes the isolation of the first neurotoxin papuantoxin-1 from the venom of *P. papuanus*. Papuantoxin-1 (6738 Da), which accounts for approximately 5% of the whole venom, was purified to homogeneity using successive steps of RP-HPLC. The toxin (0.3–1.0 μM) caused concentration dependent inhibition of indirect twitches (0.1 Hz, 0.2 ms and supramaximal V) and inhibited the responses to nicotinic agonists in the chick biventer cervicis nerve-muscle preparation, indicating a postsynaptic mode of action. However, papuantoxin-1 displayed no signs of myotoxicity. Papuantoxin-1 displayed pseudo-irreversible antagonism of cumulative concentration-response curves to carbachol at the skeletal muscle nicotinic receptors with an estimated pA_2 value of 6.9 ± 0.3. CSL black snake antivenom, which is raised against the venom of the Australian black snake *Pseudechis australis*, appears to be effective in reversing the effects of papuantoxin-1. Thus, black snake antivenom should be considered for the treatment of the neurotoxic effects following envenomation by the Papaun black snake.

Keywords: Neurotoxin; Snake; Venom; Antivenom; Skeletal muscle; Chick biventer

1. Introduction

The Papuan black snake (*Pseudechis papuanus*) is found throughout the southern coastal regions of Papua New Guinea and is thought to occur in the adjacent region of Iriyan Jaya [1]. The primary symptom of envenoming by the Papuan black snake is neurotoxicity. Other symptoms include haemostatic disturbances, tender lympadenopathy, vomiting and abdominal pain [2]. CSL polyvalent snake antivenom is effective in neutralising the life-threatening symptoms of envenoming in experimental animals [3] and humans [2]. Although the presence of dark urine, possibly due to myoglobin or haemoglobin, occurs in mice following venom administration [3], myotoxicity has not been reported as a major feature following envenoming.

Previous research on the venom from the Papuan black snake (*P. papuanus*) has identified postsynaptic neurotoxic, as well as myotoxic activity, and the ability of CSL tiger and black snake antivenoms to neutralise these effects in vitro [4,5]. In addition, an anticoagulant with PLA_2 activity has been isolated from the venom [6]. Interestingly, neurotoxicity is rarely seen following systemic envenoming in humans by Australian black snakes, with myotoxicity being the major indication. In contrast, neurotoxicity is a life-threatening effect following envenomation by *P. papuanus* [2]. Despite this, no neurotoxins have been isolated from this venom. This study describes the isolation of papuantoxin-1 and its pharmacological characterisation at skeletal muscle nicotinic receptors.

2. Materials and methods

2.1. Venom preparation and storage

P. papuanus venom was purchased from Venom Supplies Pty. Ltd., South Australia. Freeze-dried venom and

* Corresponding author. Tel.: +61 3 9905 4861; fax: +61 3 9905 5851.
E-mail address: wayne.hodgson@med.monash.edu.au (W.C. Hodgson).

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stock solutions of venom prepared in 0.1% bovine serum albumin (BSA) in 0.9% saline were stored at −20 °C until required.

2.2. Fractionation of venom

Freeze-dried venom was dissolved in distilled water prior to reverse-phase high performance liquid chromatography (RP-HPLC) separation using a Shimadzu HPLC system (LC-10ATvp pump and SPD-10AVP detector). Venom was initially fractionated using a Phenomenex Jupiter semi-preparative (250 mm × 10 mm, 5 μm, 300 Å) C18 column. The column was equilibrated with solvent A (0.1% trifluoroacetic acid (TFA)) and the sample eluted with the following gradient conditions of solvent B (90% acetonitrile in 0.09% TFA) at a flow rate of 2 ml/min: 0–60% over 60 min and 60–80% in 5 min [7]. The eluant was monitored at 280 and 214 nm. Eleven major peaks were identified and screened for neurotoxic activity using the chick biventer nerve-muscle preparation (see below). Only the peak with a retention time of 27 min displayed activity and was chosen for further purification.

The purified component was re-run on a Phenomenex Jupiter analytical (150 mm × 2 mm, 5 μm, 300 Å) C18 column after equilibrating with solvent A (0.1% TFA). The sample was eluted with the following gradient conditions of solvent B at a flow rate of 0.2 ml/min: 0–20% over 5 min, 20–60% in 40 min and then 60–80% over 5 min [7]. The eluant was monitored at 280 and 214 nm.

2.3. Molecular mass determination by electrospray mass spectrometry

The sample was dissolved in 50% acetonitrile and analysed using a Micromass ZMD (Micromass UK Ltd., Manchester, UK) with electrospray probe. The capillary voltage was set to 3000 V and the cone voltage to 30 V. Nitrogen gas was used as a curtain gas with a flow rate of 3.3 L/min. The sample was delivered by continuous infusion with a syringe pump at 8 μL/min into the LC–MS system and analysed using positive ion mode. Data processing was performed using the software package MassLynx version 3.5 (Micromass UK Ltd.).

2.4. Amino acid sequence determination

Purified peptides were loaded into the sequencing chamber of a Procise N-Terminal amino acid sequencer (Applied Biosystems, Foster City, CA, USA) and the amino acid sequence determined (Edman degradation, PTH derivatisation chemistry and the separation of derivatised amino acids by RP-HPLC), using the manufacturers recommended methods and reagents.

2.5. Chick isolated biventer cervicis nerve-muscle (CBCNM) preparation

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

Motor nerves were stimulated every 10 s (0.2 ms duration) at supramaximal voltage using a Grass S88 stimulator [8]. d-Tubocurarine (dTC; 10 μM) was added with the subsequent abolition of twitches confirming 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 [8]. The preparations were then equilibrated for at least 30 min with nerve stimulation (as above) before addition of toxin. In all experiments, toxin (0.3–1 μM) was left in contact with the preparations until responses to nerve stimulation were abolished, or for a maximum of 3 h if total twitch blockade did not occur. At the conclusion of the experiment, responses to ACh, CCh and KCl were obtained as described above. The time taken to reduce the amplitude of the twitches by 90% (t_{90}) was calculated in order to provide a quantitative measure of neurotoxicity [9].

In experiments examining the potential myotoxic effects of the toxin, the biventer cervicis muscle was directly stimulated every 10 s with pulses of 2 ms duration at supramaximal voltage. In these experiments, the electrodes were placed around the belly of the muscle and d-tubocurarine (10 μM) remained in the organ bath for the duration of the experiment. Toxin was left in contact with the preparation until twitch blockade occurred, or for a 3 h period (as above). Myotoxicity is indicated by the inhibition of direct twitches or a contracture of the skeletal muscle [10].

In additional experiments, the reversible nature of the binding of the toxin to the skeletal muscle nicotinic receptor (nAChR) was examined by adding CSL black snake antivenom (BSAV; 5 units/ml) at t_{50} (i.e. the time at which the initial twitch height was reduced by 50 %) after the addition of toxin (0.3 μM). In some experiments, neostigmine (6 μM) was added at t_{50} following the addition of toxin (0.3 μM) or dTC (8 μM).

In order to further study the activity of the toxin at the nAChR, a cumulative concentration-response curve to CCh (0.6–80 μM) was obtained in the CBCNM preparation in the absence of stimulation. The tissue was thoroughly washed after the completion of the curve. Then dTC (1–10 μM) or toxin (100–200 nM) was added and allowed to equilibrate for a 1 h period. The cumulative

concentration-response curve was then repeated in the presence of dTC or toxin. Responses to CCh were expressed as a percentage of the maximum CCh response prior to the addition of toxin.

2.6. Determination of PLA_2 activity

PLA_2 activity of the toxin was determined using a secretory colourimetric assay kit (Cayman Chemical, USA). 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 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB). Colour changes were monitored using a CER-ES900C micro-plate reader (Bio-Tek Instruments, USA) at 405 nm, sampling every minute for a 5 min period. PLA_2 activity was expressed as micromoles of phosphatidylcholine hydrolysed per minute per milligram of enzyme.

2.7. Chemicals and drugs

The following drugs were used: acetylcholine chloride, bovine serum albumin, carbamylcholine chloride (carbachol), d-tubocurarine chloride, neostigmine methyl sulfate (Sigma Chemical Co., St. Loius, MO, USA), potassium chloride (Ajax Chemicals, Sydney, Australia), trifluoroacetic acid (Auspep, Melbourne, Australia), acetonitrile (Ajax Finechem, Australia) and black snake antivenom (CSL Ltd., Melbourne, Australia). Except where indicated all stock solutions were made up in distilled water.

2.8. Analysis of results and statistics

In all experiments, responses were measured via a Grass Force-Displacement Transducer (FT03; Grass Instrument Co., Quincy, MA, USA) and recorded on a PowerLab system (ADInstruments, Australia). For neurotoxicity and myotoxicity studies in the CBCNM preparation, changes in twitch height were expressed as a percentage of the initial twitch height prior to the addition of toxin/vehicle. Contractile response to ACh, CCh and KCl were expressed as a percentage of their respective initial responses. Where indicated, statistical significance was determined by one-way analysis of variance (ANOVA), which was followed by a Bonferroni-corrected multiple *t*-test. All statistical analyses were performed using the SigmaStat (Version 2.0; Jandel Corporation, CA, USA) software package. In order to determine antagonist potency in the CBCNM preparation, cumulative concentration-response curves to CCh, in the absence or presence of dTC/toxin, were analysed by the Schild's method and/or the modified Lew Angus method as described previously [7,11,12]. The pEC_{25} values obtained from non-linear regression analysis were fitted to 'simple Lew Angus' or 'Lew Angus' equations [7,11,12] and the goodness-of-fit determined using an F-test [13]. Analyses were performed using PRISM 4.0 (GraphPad Software, San Diego, CA, USA) software package.

3. Results

3.1. Isolation and purification of papuantoxin-1

Papuantoxin-1 was isolated from *P. papuanus* venom by successive RP-HPLC separations. Initial fractionation of whole venom using a semi-preparative column produced more than 10 major peaks (Fig. 1a). The peak eluted by approximately 27% of solvent B, was subjected to further purification using an analytical column. In order to determine the homogeneity and location of papuantoxin-1 in relation to other venom components, the whole venom and papuantoxin-1 were run under the same conditions using the analytical column (Fig. 1a and b). Papuantoxin-1 elutes as a clean peak with a retention time of approximately 14 min.

3.2. Purity and the molecular mass determination

Homogeneity and molecular mass of papuantoxin-1 was determined by electrospray mass spectrometry. Papuantoxin-1 produced a mass spectra with two charged states, which can be constructed into a single molecular mass of 6738 Da (data not shown). This result was confirmed by

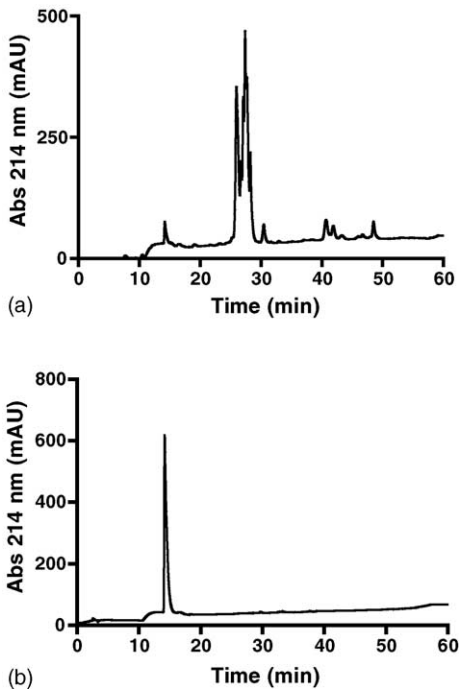

Fig. 1. RP-HPLC chromatograph of: (a) *P. papuanus* venom and (b) papuantoxin-1 run on a Jupiter analytical C18 column.

Table 1
Partial N-terminal sequence of neurotoxins from some Papua New Guinea and Northern Australian elapid venoms

Species	Neurotoxin	Molecular mass (Da)	N-terminal sequence	
P. papuanus	Papuantoxin-1	6738	MT**C**CNQQSS*Q	P**K***T**TTT
P. australis	Toxin Pa a[a]	6758	MT**C**CNQQSSQ	P**KT**TTI
A. antarcticus	Toxin Aa c[b]	6898	MQ**C**CNQQSSQ	P**KT**TTT
A. sp *seram*	Acantoxin IVa[c]	6815	MQ**C**CNQQSSQ	P**KT**TTT
Oxyuranus scutellatus scutellatus	Taipan toxin 1[d]	6726	MT**C**YNQQSSE	P**KT**TTT
M. ikaheka	Mikatoxin[e]	7775	DI**C**LSTPDVK	S**KT**CPP

Bold letters indicate identical amino acids present at the respective positions, in each of the toxins listed.
[a] [17].
[b] [25].
[c] [7].
[d] [14].
[e] [18].
* Most probable residue based on sequence homology.

MALDI-TOFF analysis of papuantoxin-1 (data not shown).

3.3. N-terminal amino acid sequence

The partial N-terminal amino acid sequence of papuantoxin-1 was determined. Comparison of the sequence with that of Toxin Pa a from the closely related *Pseudechis australis* species suggested the most probable outcomes for the undetected residues (see Table 1). The sequence was compared with other protein sequences at the National Center for Biotechnology Information (NCBI) database using the BLAST service. Papuantoxin-1 shared high sequence homology with other short-chain α neurotoxins Toxin Pa a, Toxin Aa c, acantoxin IVa (94%) and taipan toxin 1 (81%). Papuantoxin-1 shared much lower sequence homology with long-chain α neurotoxins such as Mikatoxin.

3.4. Neurotoxicity studies

Papuantoxin-1 (0.3–1.0 μM) caused concentration-dependent inhibition of twitches in the CBCNM preparation ($n = 4$; Fig. 2a). The t_{90} value for papuantoxin-1 (1.0 μM) was 34.8 ± 3.5 min. Vehicle (i.e. BSA) had no significant effect on the indirect twitches of the CBCNM preparation.

Papauantoxin-1 caused significant inhibition of contractile responses to exogenous ACh and CCh, but not KCl, compared to vehicle indicating a postsynaptic mode of action. ($n = 4$; one-way ANOVA, $P < 0.05$; Fig. 2b)

3.5. Reversibility studies

The addition of neostigmine (6 μM) at t_{50}, following the addition of papuantoxin 1 (0.3 μM) produced a transient increase in twitch height (data not shown). However, this was followed by the complete inhibition of indirect twitches within 38.5 ± 4.0 min ($n = 4$) of adding neostigmine. The maximum increase in twitch height following neostigmine addition was 94 ± 12% ($n = 4$). In contrast, the addition of neostigmine at t_{50}, following the addition of dTC (8 μM) resulted in the continued reversal of the inhibition of indirect twitches (84.9 ± 7.0% of initial height at 180 min; $n = 3$).

Prior to the addition of BSAV (5 units/ml) significantly attenuated the papuantoxin-1 (0.3 μM) induced inhibition of indirect twitches and contractile responses to exogenous ACh and CCh (data not shown). The addition of BSAV (5 units/ml) at t_{50}, after the addition of papuantoxin-1 (0.3 μM) prevented further inhibition of twitches but produced only a small reversal of the block over the next 2 h (Fig. 3a; $P < 0.05$; one-way ANOVA; $n = 4$). Addition of BSAV (5 units/ml) after the addition of papuantoxin-1 (0.3 μM) caused a significant increase in the response to exogenous ACh, but not to CCh and KCl (Fig. 4b; $P < 0.05$; one-way ANOVA; $n = 4$). BSAV (5 units/ml) alone had no significant effect on indirect twitches or contractile responses to exogenous agonists (data not shown).

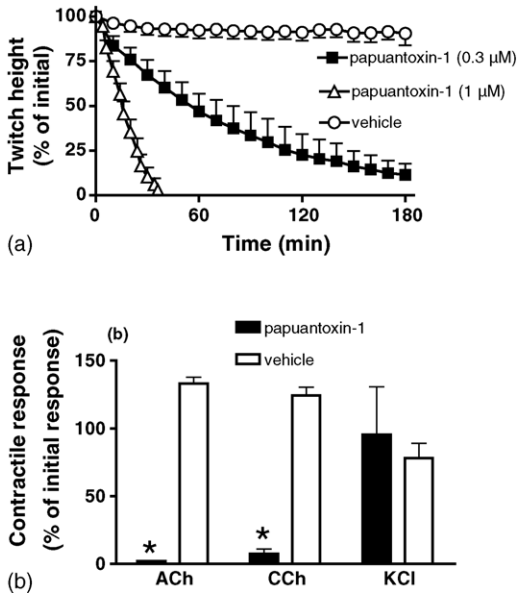

Fig. 2. The effect of papuantoxin-1 (0.3–1.0 μM) or vehicle (i.e. BSA) on: (a) indirect twitches and (b) contractile responses to exogenous ACh, CCh and KCl of the CBCNM preparation (data for effect of 1.0 μM on contractile responses not shown). $P < 0.05$, significantly different from vehicle, one-way ANOVA.

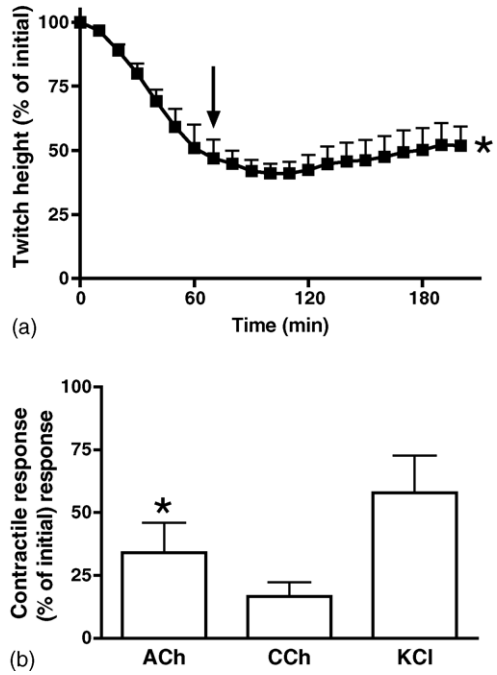

Fig. 3. The effect of BSAV (5 units/ml) added at t_{50} (indicated by arrow) following papuantoxin-1 (0.3 μM) on: (a) indirect twitches or (b) contractile responses to exogenous agonists. *$P < 0.05$, significantly different from papuantoxin-1 (0.3 μM) alone, one-way ANOVA; $n = 4$.

3.6. Myotoxicity studies

Papuantoxin-1 (1 μM) had no significant effect on direct twitches or baseline tension of the CBCNM preparation, compared to vehicle (i.e. BSA) (data not shown).

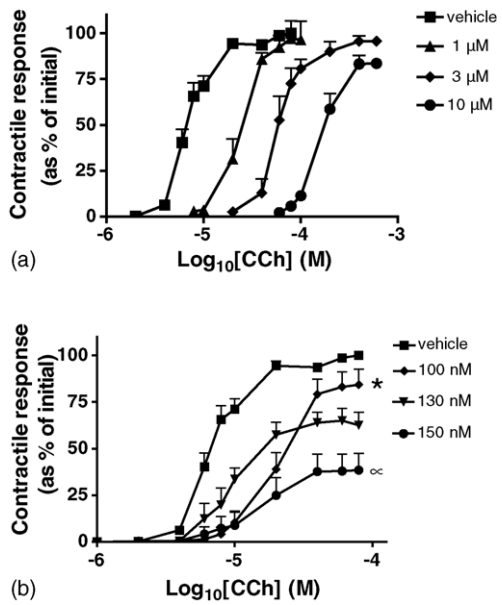

Fig. 4. The effect of (a) dTC (1–10 μM; $n = 4$–5) or (b) papuantoxin-1 (100–150 nM; $n = 4$–5) on responses to cumulative additions of carbachol in the CBCNM preparation. *$P < 0.05$, significantly different from vehicle, one-way ANOVA. $^{\alpha}P < 0.05$, significantly different from 100 nM, one-way ANOVA.

3.7. PLA_2 activity

Papuantoxin-1 had negligible PLA_2 activity of 1.9 ± 0.4 μmol/min/mg ($n = 3$) compared to that of bee venom (control) 312.5 ± 26.4 μmol/min/mg. This is in agreement with a previous study showing that the α-neurotoxin mikatoxin is devoid of PLA_2 activity [18].

3.8. Effects on skeletal muscle nicotinic receptors (nAChRs)

The nAChR antagonist dTC (1–10 μM) produced a parallel rightward shift of the concentration-response curve to CCh, without a significant depression of maximum, indicating competitive antagonism in this tissue (Fig. 4a; $n = 4$–5). Using Schild's analysis, the pA_2 value for dTC was calculated to be 6.3 ± 0.1 with a slope factor of 1.3 ± 0.3. Using the modified Lew Angus method the pA_2 was estimated to be 6.5 ± 0.2 and slope factor not significantly different to unity, since a simple Lew Angus equation was deemed a better fit by using an F-test [7]. In contrast, papuantoxin-1 caused a concentration-dependent depression of the maximal CCh response at the skeletal muscle nAChRs ($n = 4$–5; $P < 0.05$; one-way ANOVA; Fig. 4b). Following an F-test, the data fitted well into a 'simple Lew Angus' equation, producing an estimated pA_2 value for papuantoxin-1 of 6.9 ± 0.3.

4. Discussion

Papuantoxin-1 was purified from the Papuan black snake venom following successive RP-HPLC separations. Although it appears to be a minor component (i.e. comprising approximately 5% of the venom), no other eluted venom components showed in vitro neurotoxic effects. Therefore, it seems to be solely responsible for the neurotoxic effects observed following human envenoming. Postsynaptic neurotoxins previously isolated from elapid snake venoms have molecular weights in the range of 6–9 kDa [14,15]. Specifically, the molecular mass of 6738 Da for papuantoxin-1 fits into the range expected for short-chain, as opposed to long-chain neurotoxins [16]. Toxin Pa a, isolated from the closely related Australian species *P. australis*, was the first postsynaptic neurotoxin isolated from the venom of a black snake (genus *Pseudechis*) [17]. It shares a similar molecular mass and very high sequence homology with papuantoxin-1. In contrast, Mikatoxin 1 [18], a long-chain neurotoxin isolated from *Micropechis ikaheka* (which inhabits the same geographical region as *P. papuanus*), shares very low sequence identity with Toxin Pa a or papuantoxin-1.

Papuantoxin-1 was characterised in the CBCNM preparation where it inhibited indirect twitches, and significantly inhibited contractile responses to exogenous ACh and CCh, confirming its action at nicotinic receptors.

Clinicians in Australia and Papua New Guinea have used anticholinesterases in order to reduce the amount of antivenom given to patients after envenoming by elapids such as death adders [19]. Therefore, the ability of neostigmine to reverse the neurotoxicity of papuantoxin-1 was examined. However, neostigmine only produced a transient increase in twitches indicating the pseudo-irreversible antagonism of skeletal muscle nicotinic receptors by papuantoxin-1. Similar effects have been reported for other elapid neurotoxins such as acantoxin IVa [7].

Venoms from black snakes (genus *Pseudechis*) are known to have strong myotoxic effects. Therefore, papuantoxin-1 was examined for myotoxic activity using the CBCNM preparation. Although papuantoxin-1 had no detectable in vitro myotoxic effects, the components eluted between 24 and 49 min (see Fig. 1a) displayed strong myotoxic effects (data not shown).

P. papuanus venom has high PLA_2 activity compared with other *Pseudechis* species [20]. Since basic snake venom PLA_2s is associated with presynaptic neurotoxins [21], it has been suggested that venom from *P. papuanus* may also contain presynaptic neurotoxins [3]. However, in the current study, no presynaptic neurotoxicity was detected in the in vitro screening of venom components eluted from the RP-HPLC column. The PLA_2 activity of this venom may be attributed to the myotoxins present, since they are commonly associated with high PLA_2 activity [22].

The CBCNM preparation was used to determine the potency of papuantoxin-1 at the skeletal muscle nAChR. Tubocurarine displayed classical competitive antagonism. However, papuantoxin-1 caused a concentration-dependent decrease in the maximum response to CCh. Elapid venom postsynaptic neurotoxins dissociate very slowly from the skeletal muscle nAChR [23,24]. Therefore, the observed decrease in the maximum response to CCh is to be expected. Although such pseudo-irreversible antagonism of nAChR by elapid venom neurotoxins has been reported previously [7], it is not a characteristic of every elapid venom neurotoxin [18]. Since papuantoxin-1 caused a depression of the CCh maximum response, Schild plot analysis could not be used to measure potency. The modified Lew Angus method has been used previously to determine pA_2 values of pseudo-irreversible neurotoxins [11,12]. Given a pA_2 value of 6.9, papuantoxin-1 appears to be at least 2-fold more potent than dTC, but 100-fold less potent that acantoxin IVa and α-bungarotoxin [7].

Further characterisation of papuantoxin-1 is necessary to determine its activity on other types of nicotinic receptors including ganglionic, α_7 subtype neuronal and $\alpha_4\beta_2$ subtype neuronal nAChRs [7]. In addition, components responsible for myotoxic effects of this venom require isolation and characterisation. In conclusion, papuantoxin-1 is the first neurotoxin to be isolated from the venom of *P. papuanus*. The toxin causes pseudo-irreversible antagonism of the skeletal muscle nAChRs, and appears to be the only venom component responsible for the neurotoxic effects observed after human envenoming by this species. Papuantoxin-1 appears to be neutralised by CSL black snake antivenom.

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