NeuroToxicology 31 (2010) 239–243

Contents lists available at ScienceDirect

NeuroToxicology

Variations in the pharmacological profile of post-synaptic neurotoxins isolated from the venoms of the Papuan (*Oxyuranus scutellatus canni*) and coastal (*Oxyuranus scutellatus scutellatus*) taipans

Rachelle Kornhauser [a], Andrew J. Hart [a], Shane Reeve [b], A. Ian Smith [b], Bryan G. Fry [c], Wayne C. Hodgson [a,*]

[a] Monash Venom Group, Department of Pharmacology, Monash University, Victoria 3800, Australia
[b] Department of Biochemistry and Molecular Biology, Monash University, Victoria 3800, Australia
[c] Department of Biochemistry, Bio21 Institute, University of Melbourne, Parkville, Victoria 3010, Australia

ARTICLE INFO

Article history:
Received 26 October 2009
Accepted 18 December 2009
Available online 29 December 2009

Keywords:
Taipan
Neurotoxin
Post-synaptic
Snake
Venom
Competitive antagonism
Pseudo-irreversible

ABSTRACT

Based on murine LD_{50} values, the taipans (i.e. *Oxyuranus microlepidotus*, *Oxyuranus scutellatus* and *Oxyuranus scutellatus canni*) are the most venomous snake genus in the world. Despite this, little is known about the toxins contained in their venoms. The aim of the present study was to isolate and characterise post-synaptic neurotoxins from the venoms of the Papuan taipan (*O. s. canni*) and coastal taipan (*O. scutellatus*), and to compare their pharmacology. A 6770 Da toxin (i.e. α-oxytoxin 1) and a 6781 Da toxin (i.e. α-scutoxin 1) were isolated from the venoms of *O. s. canni* and *O. scutellatus*, respectively, using reverse-phase high performance liquid chromatography. Both α-oxytoxin 1 (0.3–1 μM) and α-scutoxin 1 (0.1–1 μM) caused concentration-dependent inhibition of indirect twitches in the chick biventer cervicis nerve-muscle preparation. Contractile responses to exogenous carbachol (CCh), but not potassium chloride (KCl), were inhibited by both toxins, suggesting a post-synaptic mode of action. The inhibitory effect of α-oxytoxin 1 was reversed by washing. Cumulative concentration–response curves to CCh were obtained in the presence and absence of the toxins with the subsequently determined pA_2 of α-scutoxin 1 being 44.7-fold higher than α-oxytoxin 1 (i.e. 8.38 ± 0.59 versus 7.62 ± 0.04). The current study shows that Papuan taipan and coastal taipan venom both contain potent post-synaptic neurotoxins which exhibit different pharmacological profiles. The effect of α-oxytoxin 1 is atypical of most snake venom post-synaptic neurotoxins displaying a 'competitive' mode of action, whereas α-scutoxin 1 possesses pseudo-irreversible or non-competitive activity.

1. Introduction

The inland (*Oxyuranus microlepidotus*), coastal (*Oxyuranus scutellatus*) and Papuan (*Oxyuranus scutellatus canni*) taipans are elapids from the genus *Oxyuranus*. They are considered to be three of the most venomous snakes in the world with murine LD_{50} values of 0.025, 0.099 and 0.0505 mg/kg (s.c.), respectively (Broad et al., 1979; Sutherland, 1983). The clinical features of systemic taipan envenoming include neuromuscular paralysis, lymphadenopathy, coagulopathy and spontaneous bleeding (Trevett et al., 1995a). Treatment for systemic envenoming by any of the three taipans involves administration of CSL taipan antivenom, which is raised against the coastal taipan (Trevett et al., 1995b). Crachi et al. (1999) carried out a comparative study investigating the effects of antivenom on the *in vitro* neurotoxicity of venoms from the three taipan snakes, and found that CSL taipan antivenom is effective in inhibiting the neurotoxic effects of the whole venom from the Papuan and coastal taipans. However, the antivenom was less effective against whole venom of the inland taipan (Crachi et al., 1999).

The neurotoxic components of snake venoms can be divided into two main groups: pre-synaptic (β)-neurotoxins and post-synaptic (α)-neurotoxins. Pre-synaptic neurotoxins exert their effect by inhibiting vesicle recycling within the somatic nerve terminal which, in turn, leads to a decrease in transmitter release (Hodgson and Wickramaratna, 2002). The pre-synaptic β-neurotoxins isolated from the inland, coastal and Papuan taipans are paradoxin (Fohlman, 1979; Hodgson et al., 2007), taipoxin (Chang et al., 1977) and cannitoxin (Kuruppu et al., 2005), respectively. Each consists of three subunits (α, β and γ), with molecular weights ranging from 45 to 46 kDa (Fohlman, 1979; Kuruppu et al., 2005).

* Corresponding author. Tel.: +61 3 9905 4861; fax: +61 3 9905 2547.
E-mail address: wayne.hodgson@med.monash.edu.au (W.C. Hodgson).

0161-813X/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.neuro.2009.12.009

α-Neurotoxins are antagonists of post-synaptic skeletal muscle nicotinic receptors. Short-chain post-synaptic neurotoxins consist of 60–62 amino acid residues and four disulphide bridges, whereas long-chain post-synaptic neurotoxins consist of 66–74 amino acid residues and five disulphide bridges (Fry, 1999; Hodgson and Wickramaratna, 2002). Post-synaptic neurotoxins have been isolated from two of the three taipans: oxylepitoxin-1 (6789 Da) from the inland taipan (Clarke et al., 2006), and taipan toxins 1 and 2 (6726 and 6781 Da, respectively) from the coastal taipan (Zamudio et al., 1996). However, no post-synaptic toxins have been isolated from the venom of the Papuan taipan and the pharmacological activity of the post-synaptic neurotoxins from the coastal taipan was not determined.

The aim of the present study was to isolate and characterise post-synaptic neurotoxins from the venoms of the Papuan and coastal taipans. This work will provide insight into the mechanisms behind the high lethality of taipan venoms.

2. Materials and methods

2.1. Venom

Freeze dried *O. s. canni* and *O. s. scutellatus* venom was purchased from Venom Supplies (Tanunda, South Australia).

2.2. Reverse-phase high performance liquid chromatography (RP-HPLC)

O. s. canni and *O. s. scutellatus* venoms were fractionated using a Shimadzu high performance liquid chromatography system. Venom (20 μl of a 1 mg/ml stock solution) was loaded onto a Phenomenex Jupiter analytical (150 mm × 2 mm, 5 μM, 300 Å) C18 column equilibrated with solvent A (0.1% trifluoroacetic acid) and eluted with solvent B (90% acetonitrile, 0.1% trifluoroacetic acid and water), 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. The eluant was monitored at 214 nm.

2.3. Chick biventer cervicis nerve-muscle preparation

Chickens (4–10 days old) were killed by CO_2 inhalation and exsanguination, and the biventer muscles were removed. Each muscle was attached to a wire tissue holder and placed in a 5 ml organ bath filled with physiological salt solution of the following composition (mM): NaCl, 118.4; $NaHCO_3$, 25; glucose, 11.1; KCl, 4.7; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $CaCl_2$, 2.5. The organ baths were bubbled with carbogen (95% O_2, 5% CO_2) and maintained at a temperature of 34 °C under a resting tension of 1 g. The preparation was stimulated using a Grass stimulator (0.1 Hz, 0.2 ms, supramaximal voltage) and the twitches recorded on a MacLab system via a Grass FT03 transducer. The tissues were equilibrated for 10–15 min after which *d*-tubocurarine (10 μM) was added. The subsequent abolition of twitches indicated the selective stimulation of the motor nerves. The tissues were washed repeatedly until twitch height was restored. In the absence of nerve stimulation, submaximal responses to CCh (20 μM; 60 s) and KCl (40 mM; 30 s) were obtained. Varying concentrations of α-oxytoxin 1 (0.3–1 μM) and α-scutoxin 1 (0.1–1 μM) were added to separate organ baths and left in contact for 60 min or until twitches were abolished. After this time period, the stimulator was switched off and responses to exogenous CCh and KCl were obtained (as above).

To test for reversibility, α-oxytoxin 1 (1 μM) was added to the organ bath and left in contact for 60 min (as above). After twitch blockade, the tissue was then washed at 5 min intervals until twitch height was restored.

Cumulative concentration–response curves to CCh (0.6–80 μM) were obtained in unstimulated chick biventer cervicis nerve-muscle preparations in order to determine the potency (i.e. pA_2 values) of the toxins. Tissues were set up as before, and each toxin (30–300 nM) was added to separate organ baths and allowed to equilibrate for a 1 h period. A cumulative concentration–response curve to CCh was then carried out in the presence of the toxin (or vehicle).

2.4. Matrix associated laser desorption time of flight (MALDI-TOF).

To obtain the molecular mass of each toxin, MALDI-TOF analysis was performed with an Applied Biosystems Voyager-DE STR BioSpectrometry Workstation operated in positive polarity in linear mode using sinapinic acid matrix (Agilent Technologies) for low resolution protein analysis. Matrix (1 μl) was spotted on the sample plate and air dried; sample (1 μl) diluted in acetonitrile/water (1:1) containing 0.1% (v/v) formic acid was then spotted on dried matrix and allowed to dry. Data was collected from 500 laser shots (337 nm nitrogen laser). These were then signal averaged and processed with Data Explorer software.

A Micromass ZMD Electrospray mass spectrometer (Micromass UK Ltd., Manchester, UK) was used to perform the analysis of isolated toxin under the following conditions: 3.0 kV capillary, 30, 60 or 90 V cones, in positive ion mode. Nitrogen gas was used as a curtain gas with a flow rate of 3.3 l/min. Samples were injected by direct infusion at 8 μl/min. Data processing was performed using MassLynx version 3.5 (Micromass UK Ltd., Manchester, UK).

2.5. Partial N-terminal amino acid sequencing

The N-terminal amino acid sequences of α-oxytoxin 1 and α-scutoxin 1 were obtained using Edman degradation chemistry on an Applied Biosystems 492/492C Procise Protein Sequencer. The liquid sample was loaded onto a pre-treated filter on the cartridge (Biobrene Plus). A blot sample of stained PVDF membrane was cut into bands and washed three times, alternating between water and 50% methanol/water. The bands were then cut into smaller pieces and loaded onto the sequencer cartridge for sequencing. Data was processed using Procise 610 software.

2.6. Chemicals and drugs

The following were used: carbamylcholine chloride (carbachol, Sigma), *d*-tubocurarine (Sigma), trifluoroacetic acid and acetonitrile. Unless otherwise indicated, all drugs were made up in distilled water as were subsequent dilutions.

2.7. Data analysis

Twitch height, measured at regular time intervals, was expressed as a percentage of the original twitch height (i.e. before the addition of the toxin). Contractile responses to exogenous CCh and KCl were expressed as a percentage of their original responses. For the neurotoxicity studies, a one-way analysis of variance (ANOVA) was used to analyse whether there was a statistically significant difference (measured at $P < 0.05$) between the results in each respective study, followed by a Bonferroni multiple comparison *t*-test (GraphPad Prism 5.0). For the reversibility experiment (i.e. washing of the organ bath after complete twitch inhibition), a paired *t*-test was carried out, with statistical significance indicated by $P < 0.05$. The pA_2 of each toxin was determined using the modified Lew and Angus method (GraphPad Prism 5.0). The quantity of each toxin in the venom, expressed as a percentage, was calculated using area-under-curve analysis from the elution profile of each whole venom.

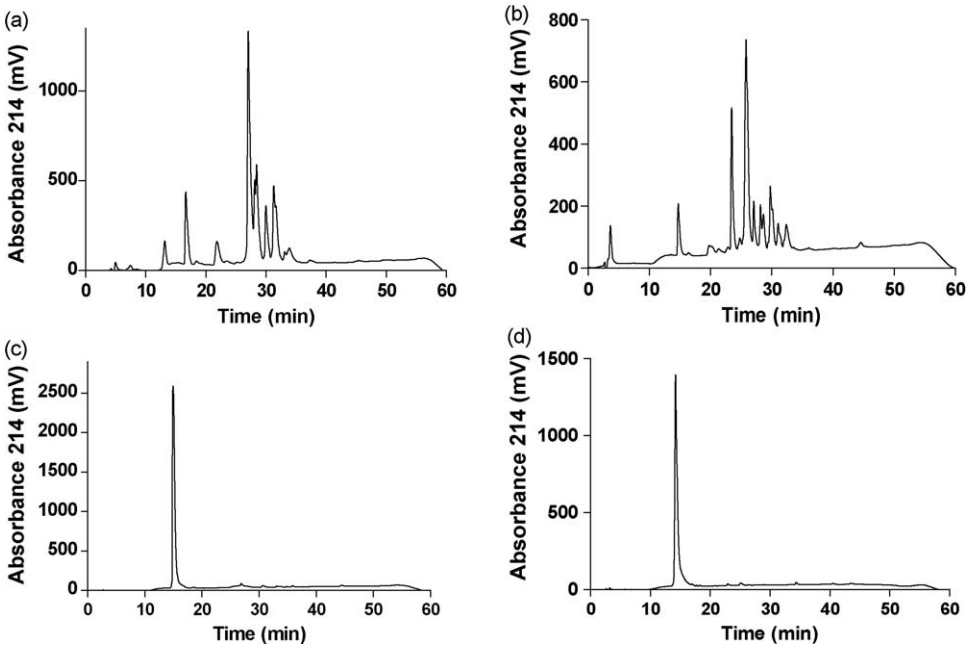

Fig. 1. RP-HPLC chromatograph of (a) *O. s. canni* venom, (b) *O. s. scutellatus* venom, (c) α-oxytoxin 1 or (d) α-scutoxin on a Jupiter analytical C18 column.

3. Results

3.1. RP-HPLC

HPLC profiles of *O. s. canni* and *O. s. scutellatus* venoms, obtained using a Jupiter analytical C18 column, indicated approximately 9 (Fig. 1a) and 11 major peaks (Fig. 1b), respectively, with α-oxytoxin 1 eluting at 15.0 min (Fig. 1c) and α-scutoxin 1 eluting at 13.8 min (Fig. 1d).

3.2. MALDI-TOF

MALDI-TOF analysis was performed to determine the molecular weights of each toxin. This analysis indicated the molecular weight of α-oxytoxin 1 to be 6770 Da, and the molecular weight of α-scutoxin 1 to be 6781 Da (data not shown).

3.3. Partial N-terminal amino acid sequencing

The partial N-terminal amino acid sequences of α-oxytoxin 1 and α-scutoxin 1 were obtained to enable a comparison of the sequences of the two toxins. This analysis indicated differences between the toxins at residues 27 and 36 (see Table 1).

3.4. Neurotoxicity studies

Both α-oxytoxin 1 (0.3–1 μM) and α-scutoxin 1 (0.1–1 μM) caused concentration-dependent inhibition of indirect twitches in the chick biventer cervicis nerve-muscle preparation (Fig. 2a and b, $P < 0.05$ one-way ANOVA). The toxins also significantly inhibited contractile responses to exogenous CCh, but not KCl (Fig. 2c and d, $P < 0.05$ Bonferroni multiple comparison *t*-test).

In unstimulated preparations, α-oxytoxin 1 (30–300 nM) produced parallel rightward shifts of concentration–response curves to carbachol without a significant depression of the maximal response (Fig. 3a). In contrast, increasing concentrations of α-scutoxin 1 (30–300 nM) caused non-parallel shifts in concentration–response curves to carbachol with a marked depression of the maximal response (Fig. 3b). The modified Lew and Angus method was used to determine pA_2 values of 7.62 ± 0.04 and 8.38 ± 0.59 for α-oxytoxin 1 and α-scutoxin 1, respectively.

The inhibition of indirect twitches by α-oxytoxin 1 (1 μM) was significantly reversed upon repeated washing returning to 77% of initial twitch height over a period of approximately 60 min (data not shown; $P < 0.05$, paired *t*-test).

4. Discussion

Venoms are an excellent source of highly potent toxins which have evolved over many millennia. These toxins target vital physiological processes and enable the host to immobilise or kill prey as well as, in some instances, play a defensive role against predators. Due to their high selectivity, snake venoms and toxins represent a greatly underutilized source of tools for biological research and potential lead compounds for pharmaceutical agents. Australia and South East Asia have an unparalleled collection of

Table 1
Partial N-terminal amino acid sequences of α-oxytoxin 1 and α-scutoxin 1, as well as partial N-terminal sequences of other post-synaptic neurotoxins from the taipan species.

Species	Neurotoxin	Estimated molecular mass (Da)	Partial N-terminal sequence
O. s. scutellatus	α-Scutoxin 1	6781	MTCYNQQSSE AKTTTTCSGG VSSCYKKTWY DGRGTRIERG (1–40)
O. s. canni	α-Oxytoxin 1	6770	MTCYNQQSSE AKTTTTCSGG VSSCYKETWY DGRGTT
O. s. scutellatus	Taipan toxin 1[a]	6726	MTCYNQQSSE AKTTTTCSGG VSSCYKKTWS DGRGTIIERG
O. s. scutellatus	Taipan toxin 2[a]	6781	MTCYNQQSSE AKTTTTCSGG VSSCYKKTWS DIRGTIIERG
O. microlepidotus	Oxylepitoxin-1[b]	6785	MTCYNQQSSE AKTTTTCSGG VSSCYKETWY

Amino acids underlined are deduced based on the sequences of the other toxins.
[a] Zamudio et al. (1996).
[b] Clarke et al. (2006).

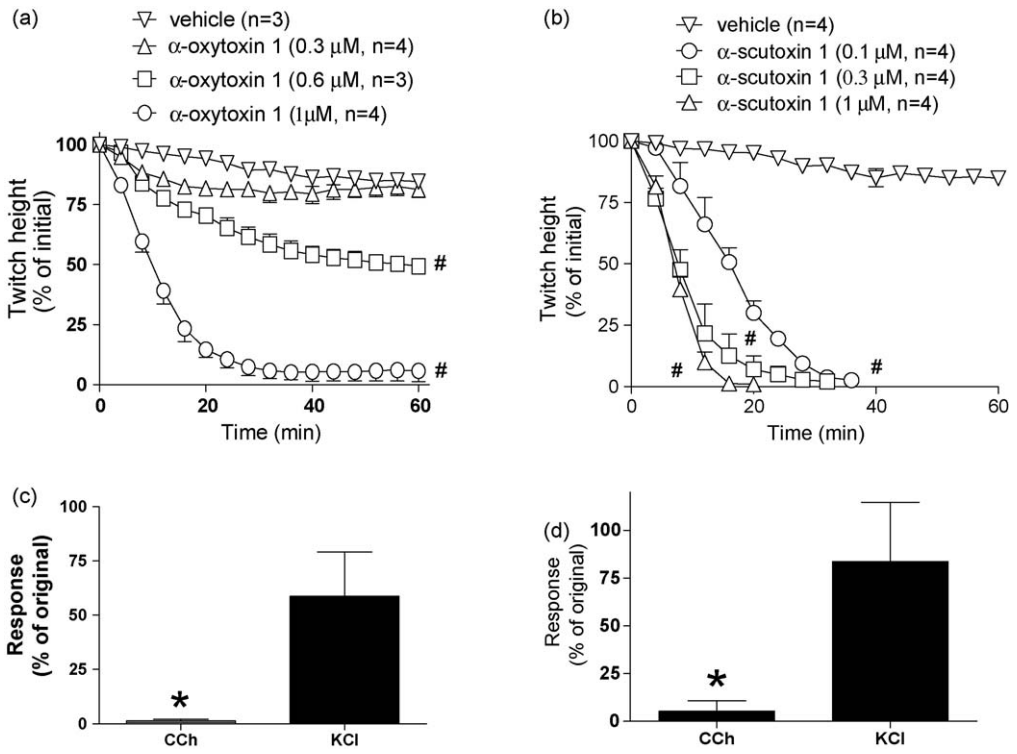

Fig. 2. The effect of varying concentrations of (a) α-oxytoxin 1 ($n = 3–4$) or (b) α-scutoxin 1 ($n = 4$) on indirect twitches. $^{\#}P < 0.05$, significantly different from vehicle, repeated measures ANOVA. The effect of (c) α-oxytoxin 1 (1 μM; $n = 4$) or (d) α-scutoxin 1 (0.3 μM; $n = 4$) on contractile responses to exogenous carbachol (CCh; 20 μM) or KCl (40 mM). $^{*}P < 0.05$, significantly different from original response, Bonferroni's corrected multiple comparison *t*-test.

highly venomous snakes and the venoms of many of these are still poorly understood. The Papuan taipan is an example of a species whose venom has not been extensively studied although we do know that it displays *in vitro* neurotoxicity which supports the symptomatic effects seen in envenomed humans. The aim of the current study was to isolate, pharmacologically characterise and compare post-synaptic neurotoxins from the venoms of the Papuan taipan and coastal taipan.

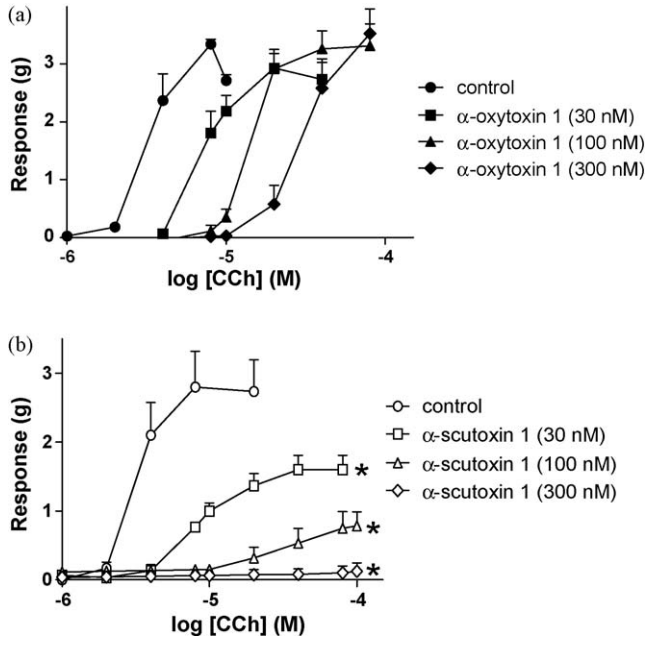

Fig. 3. The effect of increasing concentrations of (a) α-oxytoxin 1 ($n = 4–5$) or (b) α-scutoxin 1 ($n = 3$) on cumulative concentration–response curves to carbachol (CCh). $^{*}P < 0.05$ significantly different compared to control (modified Lew and Angus method of analysis).

A previous study in our laboratory, using the chick biventer cervicis nerve-muscle preparation, comparing the *in vitro* neurotoxic activity of inland, coastal and Papuan taipan venoms, found inland taipan venom to be markedly more potent than the other two (Crachi et al., 1999). However, a comparison of the activity of the pre-synaptic neurotoxins from the inland and coastal taipans (i.e. paradoxin and taipoxin) found that both abolished nerve-mediated twitches with no significant difference in neurotoxicity (Crachi et al., 1999). This indicated that the pre-synaptic neurotoxins are unlikely to be solely responsible for the high lethality of taipan venoms. On the other hand, a study involving a post-synaptic neurotoxin from the inland taipan venom (i.e. oxylepitoxin-1) indicated that it was highly potent, but no more than most other post-synaptic neurotoxins from Elapid venoms (Clarke et al., 2006).

In the current study, α-oxytoxin 1 and α-scutoxin 1 were isolated from the Papuan (*O. s. canni*) and coastal (*O. s. scutellatus*) taipans, respectively, and were found to comprise approximately 9% and 6% of the whole venoms. Their molecular masses, i.e. 6770 and 6781 Da, respectively, are consistent with those of other short-chain α-neurotoxins (Fry, 1999). α-Oxytoxin 1 and α-scutoxin 1 abolished indirect twitches, as well as inhibiting contractile responses to exogenous CCh, but not KCl, indicating a post-synaptic site of action. Additionally, concentration–response curves to carbachol obtained in the absence and presence of increasing concentrations of α-oxytoxin 1 displayed a parallel rightward shift with no depression of maximum response, indicating classical competitive antagonism (i.e. it is able to readily disassociate from the receptor). Interestingly, many snake post-synaptic neurotoxins are 'pseudo-irreversible', in that they dissociate from the receptor slowly and, therefore, prove resistant to washing. This type of activity was displayed by α-scutoxin 1

which produced a concentration-dependent decrease in the maximal response to carbachol. Using the modified Lew and Angus method of analysis, the pA_2 values of α-oxytoxin 1 and α-scutoxin 1 were found to be 7.62 and 8.38, respectively. In comparison to the 'classical' nicotinic receptor antagonist tubocurarine, which has a pA_2 value of 6.28 (Kuruppu et al., 2008), α-oxytoxin 1 and α-scutoxin 1 are approximately 20 times and 125 times, respectively, more potent at exerting their effects at the nicotinic acetylcholine receptor.

Further studies of α-oxytoxin 1 showed that its inhibitory effects were reversible upon washing in contrast to the results for oxylepitoxin-1, which was not reversible (Clarke et al., 2006). Short-chain α-neurotoxins associate and disassociate from nicotinic receptors at different rates and, depending on their amino acid sequence, display differing affinity for different subtypes of post-synaptic nicotinic acetylcholine receptors (Hodgson and Wickramaratna, 2002). In previous studies, it has been postulated that non-conservative substitutions, particularly at position 32, may affect the affinity and potency of a neurotoxin (Zamudio et al., 1996). This may be due to the fact that it is adjacent to position 33, which in many snake α-neurotoxins is a highly conserved arginine amino acid residue, and has been shown to be pertinent to the functional activity of the toxin (Endo and Tamiya, 1987). Nonetheless, there is still very little information known about the effect of amino acid substitutions on the pharmacological activity of post-synaptic neurotoxins. Oxylepitoxin-1, and taipan toxins 1 and 2 have almost identical N-terminal amino acid sequences (Clarke et al., 2006), and α-oxytoxin 1 and α-scutoxin 1 also have very similar sequences. The results of the current study indicate that it is likely that substitutions at other positions in the amino acid sequence also have the potential to affect the affinity and pharmacological characteristics of a neurotoxin on nicotinic acetylcholine receptors. For example, in the current study, non-conservative substitutions at positions 27 and 36 may account for the high potency of the toxins and their differences in pharmacological mode of action.

Conflict of interest statement

None.

References

Broad AJ, Sutherland SK, Coulter AR. The lethality in mice of dangerous Australian and other snake venoms. Toxicon 1979;17:661–4.

Chang CC, Lee JD, Eaker D, Fohlman J. The presynaptic neuromuscular blocking action of taipoxin. A comparison with β-bungarotoxin and crotoxin. Toxicon 1977;13:371–6.

Clarke C, Kuruppu S, Reeve S, Smith AI, Hodgson WC. Oxylepitoxin-1, a reversible neurotoxin from the venom of the inland taipan (*Oxyuranus microlepidotus*). Peptides 2006;27:2655–60.

Crachi MT, Hammer LW, Hodgson WC. The effects of antivenom on the *in vitro* neurotoxicity of venoms from the taipans *Oxyuranus scutellatus*, *Oxyuranus microlepidotus* and *Oxyuranus scutellatus canni*. Toxicon 1999;37:1771–8.

Endo T, Tamiya N. Current view on the structure function-relationship of post-synaptic neurotoxins from snake venoms. Pharmacol Ther 1987;34:403–51.

Fohlman J. Comparison of two highly toxic Australian snake venoms: the taipan (*Oxyuranus s. scutellatus*) and the fierce snake (*Parademansia microlepidotus*). Toxicon 1979;17:170–2.

Fry BG. Structure function properties of venom components from Australian elapids. Toxicon 1999;37:11–32.

Hodgson WC, Wickramaratna JC. *In vitro* neuromuscular activity of snake venoms. Clin Exp Pharmacol Physiol 2002;29:807–14.

Hodgson WC, Dal Belo CA, Rowan EG. The neuromuscular activity of paradoxin: a presynaptic neurotoxin from the venom of the inland taipan (*Oxyuranus microlepidotus*). Neuropharmacology 2007;52:1229–36.

Kuruppu S, Reeve S, Banjeree Y, Kini RM, Smith AI, Hodgson WC. Isolation and pharmacological characterisation of cannitoxin, a pre-synaptic neurotoxin from the venom of the Papuan taipan (*Oxyuranus scutellatus canni*). J Pharmacol Exp Ther 2005;315:1196–202.

Kuruppu S, Smith AI, Isbister GK, Hodgson WC. Neurotoxins from Australo-Papuan elapids: a biochemical and pharmacological perspective. Crit Rev Toxicol 2008;38:73–86.

Sutherland SK. Australian animal toxins. The creatures, their toxins, and care of the poisoned patient. Melbourne: Oxford University Press; 1983 p. 121–7.

Trevett AJ, Lalloo DG, Nwokolo NC, Naraqi S, Kevau IH, Theakston RDG, et al. Electrophysiological findings in patients envenomed following the bite of a Papuan taipan (*Oxyuranus scutellatus canni*). Trans R Soc Trop Med Hyg 1995a;89:415–7.

Trevett AJ, Lalloo DG, Nwoloko NC, Naraqi S, Kevau IH, Theakston RDG, et al. The efficacy of antivenom in the treatment of bites by the Papuan taipan (*Oxyuranus scutellatus canni*). Trans R Soc Trop Med Hyg 1995b;89:322–5.

Zamudio F, Wolf KM, Martin BM, Possani LD, Chiappinelli VA. Two novel alpha-neurotoxins isolated from the taipan snake, *Oxyuranus scutellatus*, exhibit reduced affinity for nicotinic acetylcholine receptors in brain and skeletal muscle. Biochemistry 1996;35:7910–6.