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Isolation and characterization at cholinergic nicotinic receptors of a neurotoxin from the venom of the *Acanthophis* sp. Seram death adder

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Abstract

The present study describes the isolation of the first neurotoxin (acantoxin IVa) from *Acanthophis* sp. Seram death adder venom and an examination of its activity at nicotinic acetylcholine receptor (nAChR) subtypes. Acantoxin IVa (MW 6815; 0.1–1.0 μM) caused concentration-dependent inhibition of indirect twitches (0.1 Hz, 0.2 ms, supramaximal V) and inhibited contractile responses to exogenous nicotinic agonists in the chick biventer cervicis nerve-muscle, confirming that this toxin is a postsynaptic neurotoxin. Acantoxin IVa (1–10 nM) caused pseudo-irreversible antagonism at skeletal muscle nAChR with an estimated pA_2 of 8.36 ± 0.17. Acantoxin IVa was approximately two-fold less potent than the long-chain (Type II) neurotoxin, α-bungarotoxin. With a pK_i value of 4.48, acantoxin IVa was approximately 25,000 times less potent than α-bungarotoxin at α7-type neuronal nAChR. However, in contrast to α-bungarotoxin, acantoxin IVa completely inhibited specific [^{3}H]-methyllycaconitine (MLA) binding in rat hippocampus homogenate. Acantoxin IVa had no activity at ganglionic nAChR, α4β2 subtype neuronal nAChR or cytisine-resistant [^{3}H]-epibatidine binding sites. While long-chain neurotoxin resistant [^{3}H]-MLA binding in hippocampus homogenate requires further investigation, we have shown that a short-chain (Type I) neurotoxin is capable of fully inhibiting specific [^{3}H]-MLA binding.

Keywords: Acantoxin IVa; Alpha7; Death adder; Neurotoxin; Nicotinic; Venom

1. Introduction

Death adders (genus *Acanthophis*) are the widest ranging of the Australian elapids being found not only in continental Australia, but North throughout the Torres Straight Islands, Papua New Guinea, Irian Jaya and the Indonesian islands of Seram, Halmahera, Obi and Tanimbar. Although up to 12 species and 3 subspecies of death adders have been described thus far [1], considerable debate remains about species identification [2]. Pharmacologically, only the venom of the common (*Acanthophis antarcticus*) death adder has been studied in detail but the venoms of the major lineages have been analyzed by liquid chromatography/mass spectrometry [3].

Previously, using the chick biventer cervicis nerve-muscle (CBCNM) preparation, we have shown that *A.* sp. Seram death adder venom causes concentration-dependent inhibition of indirect twitches and blocks contractile responses to exogenous nicotinic agonists [4], suggesting the presence of postsynaptic neurotoxins. The rank order of neurotoxicity showed that *A.* sp. Seram venom was one of the most neurotoxic of the ten death adder venoms tested [4,5].

Snake α-neurotoxins, also called curaremimetic or postsynaptic neurotoxins, are members of the functionally and structurally diverse class of toxins called three-finger

Abbreviations: CBCNM, chick biventer cervicis nerve-muscle; ACh, acetylcholine; CCh, carbachol; dTC, d-tubocurarine; nAChR, nicotinic acetylcholine receptor; MLA, methyllycaconitine

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toxins. They are generally classified as short-chain (Type I) or long-chain (Type II) neurotoxins based on their amino acid sequence and phylogenetic grouping [6–8]. Short-chain neurotoxins consist of 60–62 amino acid residues and four disulfide bridges. Long-chain neurotoxins have 66–74 amino acid residues and usually five disulfide bridges [7]. Until recently, the major functional difference between short-chain and long-chain α-neurotoxins was thought to be in the kinetics of association and dissociation with the skeletal muscle nicotinic receptor [9]. However, it has now been shown that long-chain neurotoxins containing a fifth disulfide bridge bind to neuronal α7-type nicotinic acetylcholine receptors (nAChR) with higher affinity than short-chain neurotoxins [10]. Therefore, it should be possible to classify snake postsynaptic neurotoxins on the basis of pharmacological activity on nicotinic acetylcholine receptors.

The skeletal muscle nAChR is a heteropentameric protein consisting of five membrane-spanning subunits with the stoichiometry of 2α1, 1β1, 1γ, and 1δ or 1ε, depending on adult or fetal forms of the receptor, respectively [11]. While lacking in γ, δ and ε subunits, neuronal nicotinic acetylcholine receptors (neuronal nAChR) are pentameric assemblies of various complements of α and β subunits [12]. To date, eight neuronal nAChR α (α2–7, α9, α10) subunits and three β (β2–4) subunits have been identified in the mammal, and an additional α subunit, α8, has been identified in the chick optic lobe [13,14]. Although this allows for a large number of possible neuronal nAChR subtypes, only the heteropentamer α4β2 and homopentamer α7 subtypes from the mammalian brain have been studied extensively [15,16]. While it is well known that most snake α-neurotoxins bind with extremely high affinity to skeletal muscle nAChR, long-chain neurotoxins such as α-bungarotoxin have also been valuable probes for studying neuronal nAChR containing α7, α8 or α9 subunits [10,17]. On the other hand, κ-neurotoxins such as neuronal bungarotoxin are selective for α3β2 neuronal nAChR [18,19].

To date, two short-chain neurotoxins (acanthophin a and toxin Aa c) and three long-chain neurotoxins (toxin Aa b, acanthophin d and Aa e) have been isolated from *A. antarcticus* venom [20–24]. While acanthophin a and acanthophin d have been shown to block skeletal muscle nAChR, no such studies were performed for toxin Aa b, toxin Aa c and Aa e. These latter toxins were classified as postsynaptic neurotoxins on the basis of lethality in mice and sequence homology to other α-neurotoxins. Furthermore, none of these toxins have been studied at neuronal nAChR. No postsynaptic neurotoxins have been isolated from any other death adder venom.

The aim of this study was to isolate a neurotoxin from the venom of death adders found on the island of Seram (Indonesia) and to examine its pharmacological activity on both skeletal muscle and neuronal nAChR.

2. Methods

2.1. Venom preparation and storage

A. sp. Seram venom was purchased from Venom Supplies Pty. Ltd., South Australia. Freeze dried venom and stock solutions of venom prepared in 0.1% bovine serum albumin (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 and passed through a 0.45 μm Millipore (Bedford, MA, USA) filter. Reversed-phase high performance liquid chromatography (RP-HPLC) separations were performed on a Waters 600 HPLC system (Waters Corporation, MA, USA) using Vydac preparative (250 mm × 20 mm, 10 μm, 300 Å) and Phenomenex Jupiter semi-preparative (250 mm × 10 mm, 5 μ, 300 Å) C18 columns. The column was equilibrated with solvent A (0.1% trifluoroacetic acid—TFA), and the sample then eluted with the following gradient conditions of solvent B (90% acetonitrile in 0.09% TFA) and solvent A at a flow rate of 10 ml/min: 0–60% over sixty min (1% gradient) and then 60–80% in 5 min (4% gradient). The eluant was monitored at 214 and 280 nm.

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

2.3. Molecular mass determination by electrospray mass spectrometry

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

2.4. Amino acid sequence determination

Pure peptide (400 μg) was dissolved in 400 μl of 6 M guanidinium hydrochloride and then 8 μl of 2-mercap-

toethanaol was added. The sample was then vortexed and briefly centrifuged. Subsequently, 80 μl of 4-vinylpyridine was added, nitrogen gas passed over the sample for 2 min, the sample sealed airtight and then incubated at 37 °C for 2 h. The reduced/alkylated peptide was N-terminally sequenced using Edman degradation chemistry on an Applied Biosystems 477A Protein Sequencer (Applied Biosystems, CA, USA).

2.5. Chick isolated biventer cervicis nerve-muscle preparation

All experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the US National Institutes of Health and approved by the Monash University Pharmacology Animal Ethics Committee, Australia. Male chicks aged between 4 and 9 days were killed with CO_2 and both biventer cervicis nerve-muscle preparations were removed. These were mounted under 1 g resting tension in organ baths (5 ml) containing Krebs solution of the following composition (mM): NaCl, 118.4; KCl, 4.7; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $CaCl_2$, 2.5; $NaHCO_3$, 25; glucose, 11.1. The Krebs solution was bubbled with carbogen (95% O_2 and 5% CO_2) and maintained at 34°C.

Indirect twitches were evoked by stimulating the motor nerve every 10 s with pulses of 0.2 ms duration at a supramaximal voltage [25] using a Grass S88 stimulator. After a 30 min equilibration period, d-tubocurarine (dTC; 10 μM) was added. Subsequent abolition of twitches confirmed selective stimulation of nerves. Twitches were then re-established by thorough washing (30 min). Contractile responses to acetylcholine ACh (1 mM for 30 s), carbachol (CCh) (20 μM for 60 s) and KCl (40 mM for 30 s) were obtained in the absence of stimulation [25]. Electrical stimulation was then recommenced and the preparations were allowed to equilibrate for a further 30 min period, with frequent washing, before commencement of the experiment. Toxin (0.1–1.0 μM) was left in contact with the preparations until complete twitch blockade occurred, or for a 1 h period. Contractile responses to ACh, CCh or KCl were then obtained as described above. In additional experiments, the reversibility of the toxin (0.3 μM) was determined by washing the preparation at 10 min intervals for a 3 h period after full twitch inhibition had occurred. In some experiments, neostigmine (10 μM) was added at t_{50} (i.e. time taken to cause 50% inhibition of the initial twitch height) after the addition of toxin (0.3 μM) or dTC (8 μM).

To further study the activity of the toxin at skeletal muscle nAChR, a cumulative concentration–response curve to CCh (0.6–80 μM) was obtained in the chick unstimulated biventer cervicis nerve-muscle preparation. After completion of the curve the tissue was thoroughly washed. Then dTC (1–10 μM), α-bungarotoxin (2–7 nM) or toxin (1–10 nM) was added and allowed to equilibrate for a 1 h period. The cumulative concentration–response curve to CCh was then repeated in the presence of toxin. Responses to CCh were expressed as a percentage of the maximum CCh response prior to the addition of toxin.

2.6. Guinea-pig isolated ileum

Dunkin–Hartley guinea-pigs (0.5–1.1 kg) were gassed with CO_2 (80% in 20% O_2) and killed by exsanguination, and then approximately 2 cm long segments of ileum dissected out. These were attached to wire tissue holders and mounted under 1 g resting tension in organ baths (5 ml) containing Krebs solution maintained at 34 °C. Preparations were equilibrated for 30 min and a maximal response to histamine (10 μM) was obtained. To study the activity of toxin at ganglionic nAChR, a discrete (i.e. individual additions of drug with washes in between additions) concentration–response curve to (±)-epibatidine (1 nM–3 μM) was obtained. To avoid receptor desensitization a 15 min interval was allowed between additions of (±)-epibatidine and responses were terminated by washing after 30 s. After completion of the curve, the tissue was thoroughly washed. Then mecamylamine (0.3–10 μM) or toxin (1 μM) was added and allowed to equilibrate for a 1 h period. The discrete concentration–response curve to (±)-epibatidine was then repeated in the presence of mecamylamine or toxin. Contractions to (±)-epibatidine were expressed as a percentage of the maximal histamine (10 μM) response.

2.7. Membrane preparation for radio-ligand binding assays

Female Sprague–Dawley rats weighing 180–220 g were gassed by CO_2 (80% in 20% O_2) and killed by decapitation. The brains were removed and either the hippocampus or midbrain was dissected out. These were then homogenized in 50 volumes of ice-cold homogenizing buffer, made up of (in mM): NaCl, 14.4; KCl, 0.2; $CaCl_2$, 0.2; $MgSO_4$, 0.1; HEPES, 2; phenylmethyl-sulfonyl fluoride (PMSF), 1; pH 7.5, using a Polytron (model CH-6010 Kinematica, Switzerland). Homogenized membranes were centrifuged (20,000 × *g*, 15 min, 4 °C) and pellets were resuspended in fresh homogenizing buffer [26]. These were incubated at 37 °C for 10 min and then centrifuged as before. Pellets were washed another two times by resuspension and centrifugation, without the incubation step. Pellets were stored at −80 °C until required. Protein content was quantified by the BCA protein assay (Pierce, IL, USA) according to manufacturer's instructions.

2.8. [^{3}H]-methyllycaconitine binding assays

Competition binding studies with [^{3}H]-methyllycaconitine ([^{3}H]-MLA) were done according to a previously described method [26]. Hippocampus membrane pellets were resuspended in binding buffer made up of (in mM): NaCl, 144; KCl, 1.5; $CaCl_2$, 2; $MgSO_4$, 1; Tris–HCl, 200;

HEPES, 20; bovine serum albumin, 0.1% (w/v); pH 7.5. Membrane samples (400 μg of protein) were pipetted into 96-well plates and incubated for 2 h in a 100 μl final volume containing 2 nM [^{3}H]-MLA ($K_D = 2.2 \pm 0.6$ nM, from previous unpublished studies in the lab), binding buffer and various concentrations of α-bungarotoxin, cold MLA or toxin. Non-specific binding was determined in the presence of 1 mM (−)-nicotine. Experiments were done in duplicate, except for total and non-specific binding which were done in quadruplicate. The incubations were terminated by harvesting membranes onto polyethyleneimine-soaked (0.5% w/v, for 2 h) GF/C filters (UniFilter-96, Perkin-Elmer Life Sciences, MA, USA) using a Filtermate 196 Harvester (Packard, CT, USA). Following this, the filters were washed five times with ice-cold binding buffer. Filters were then dried overnight and counted on a Packard TopCount (Packard, CT, USA).

2.9. [^{3}H]-epibatidine binding assays

Binding studies with [^{3}H]-epibatidine were done by modifying a method previously described [27]. Midbrain membrane pellets were resuspended in binding buffer. For saturation binding studies, membrane samples (300 μg of protein) were pipetted into 96-well plates and incubated for 2 h in a 100 μl final volume containing binding buffer and [^{3}H]-epibatidine (1–800 pM). For competition binding studies, membrane samples (300 μg of protein) were incubated for 2 h in a 100 μl final volume containing [^{3}H]-epibatidine (0.4 nM), binding buffer and various concentrations of (−)-nicotine or toxin. Non-specific binding was determined in the presence of 300 μM (−)-nicotine. As previously described, incubations were terminated by harvesting membranes onto polyethyleneimine-soaked GF/C filters, and these were then washed six times with ice-cold binding buffer. Cytisine-resistant [^{3}H]-epibatidine sites were determined by adding cytisine (100 nM) to a 200 μl final volume incubation containing membrane samples (600 μg of protein), binding buffer and 0.4 nM [^{3}H]-epibatidine [28].

2.10. Chemicals and drugs

The following drugs and chemicals were used: acetonitrile (Fisher Scientific, UK); α-bungarotoxin, acetylcholine chloride, bovine serum albumin, carbamylcholine chloride (carbachol), cytisine, d-tubocurarine chloride, HEPES, histamine dihydrochloride, mecamylamine hydrochloride, neostigmine methyl sulfate, (−)-nicotine di-(+)-tartrate, phenylmethyl-sulfonyl fluoride, polyethyleneimine (PEI; 50% w/v solution), Trizma HCl, Trizma Base (Sigma Chemical Co., St. Louis, MO, USA); trifluoroacetic acid, 4-vinylpyridine (Fluka Chemika-Biochemika, Buchs, Switzerland); (±)-epibatidine dihydrochloride (Research Biochemicals International, Natick, MA, USA); [^{3}H]-methyllycaconitine (26.5 Ci/mmol), methyllycaconitine citrate (Tocris Cookson Ltd., Avonmouth, Bristol, UK); [^{3}H]-epibatidine (66.6 Ci/mmol; DuPont NEN, Boston, MA, USA). Sequencing grade chemicals were obtained from Applied Biosystems (Australia). [^{3}H]-MLA and [^{3}H]-epibatidine were diluted in binding buffer. Except where indicated, stock solutions were made up in distilled water. All reagents were of analytical grade.

2.11. Analysis of results and statistics

In isolated tissue experiments, responses were measured via a Grass Force–Displacement Transducer (FT03 C; Grass Instrument Co., Quincy, MA, USA) and recorded on a MacLab System (ADInstruments, USA). In neurotoxicity studies involving the CBCNM preparation, twitch height was expressed as a percentage of the initial twitch height prior to the addition of toxin. Where indicated, statistical significance was determined by one-way analysis of variance (ANOVA) or Student's unpaired *t*-tests on the t_{90} values. Contractile responses to ACh, CCh and KCl were expressed as a percentage of the respective initial response. These were compared against the vehicle control response via a one-way ANOVA. Dose dependent effect of mecamylamine at ganglionic nAChR was determined by a one-way ANOVA of the (±)-epibatidine (0.3 μM) response in the absence and presence of various concentrations of mecamylamine. The reduction in maximal response to CCh in the presence of varying concentrations of α-bungarotoxin or toxin was analyzed by a one-way ANOVA of the CCh (0.1 mM) response. All ANOVAs were followed by a Bonferroni-corrected multiple t-test. Statistical significance was indicated when $P < 0.05$. All statistical tests were carried out using the SigmaStat (Version 1.0; Jandel Corporation, CA, USA) software package.

Cumulative concentration–response curves to CCh in the CBCNM preparation in the absence and presence of various concentrations of dTC, α-bungarotoxin or toxin were analysed by using one or more of the following methods: Schild plot analysis, Lew and Angus method [29] or modified Lew and Angus method [30,31]. Concentration–response curves to CCh were fitted by non-linear regression to the standard sigmoidal dose–response (variable slope) equation using PRISM 3.02 (GraphPad Software, San Diego, CA). To determine antagonist potency by the Lew and Angus method the negative logarithm of EC_{50} values (i.e. pEC_{50} values) obtained from the above curve fittings were fitted to the following equations [29,30]:

$$pEC_{50} = -\log([B] + 10^{-pK_b}) - \log c \quad (1)$$

or

$$pEC_{50} = -\log([B]s + 10^{-pK}) - \log c \quad (2)$$

where [*B*] represents antagonist concentration, *s* is equivalent to Schild slope factor, log *c* is a fitting constant and pK_b is equivalent to pA_2. In Eq. (1) the pK_b is given directly as a fitted parameter. However, in Eq. (2) when *s* is not

significantly different from unity (NSDU) then it is constrained to be as such and hence the pK value is equivalent to pK_b [30,31]. When s is significantly different from unity the pA_2 value can be estimated by pA_2 = pK/s. Deviations from competitive antagonism were determined by comparing the goodness-of-fit of Eq. (1) with Eq. (2) using an F test [29].

Where under non-equilibrium conditions the agonist concentration–response curves in the presence of an antagonist display varying maximal responses, the pA_2 value of this antagonist can be estimated by using the modified Lew and Angus method [30,31]. Here the dependent variable (i.e. $p\mathrm{EC}_{50}$) in the equations was replaced with $p\mathrm{EC}_{25\%}$ (i.e. equieffective agonist concentrations based on the level representing the 25% maximal response of the control agonist concentration–response curve) as described in Christopoulos et al. [30]. This was done since it is not possible to compare EC_{50} values under conditions of varying maximal responses using Schild plot analysis.

Data from saturation and competition binding studies were analyzed by non-linear curve fitting using PRISM. Saturation binding data were fitted to a single-site ligand binding model to determine the dissociation constant (K_D) and maximum binding (B_{max}) values. Competition binding data were fitted to both one-site and two-site binding models, and the best fit determined by an F test using PRISM. IC_{50} values were converted to K_i values using the following equation [32]:

$$K_i = \frac{\mathrm{IC}_{50}}{1} + \left(\frac{[D]}{K_D}\right)$$

where $[D]$ represents radio-ligand concentration. Data are expressed as mean ± S.E.M.

3. Results

3.1. Isolation and purification of acantoxin IVa

Acantoxin IVa was isolated from *A*. sp. Seram venom by successive RP-HPLC separations. The initial fractionation of *A*. sp. Seram venom using a preparative column produced ten major peaks. The second peak was subjected to further purification by RP-HPLC. In order to determine homogeneity and location of acantoxin IVa in relation to other peaks of the whole venom, both *A*. sp. Seram venom and acantoxin IVa were run on the same conditions using a Phenomenex Jupiter analytical column (Fig. 1a and b). Acantoxin IVa eluted as a clean peak separating away from minor contaminants at about 28% solvent B (i.e. at approximately 13 min).

3.2. Purity and molecular mass determination

Homogeneity and molecular mass of acantoxin IVa were determined by electrospray mass spectrometry (Fig. 2).

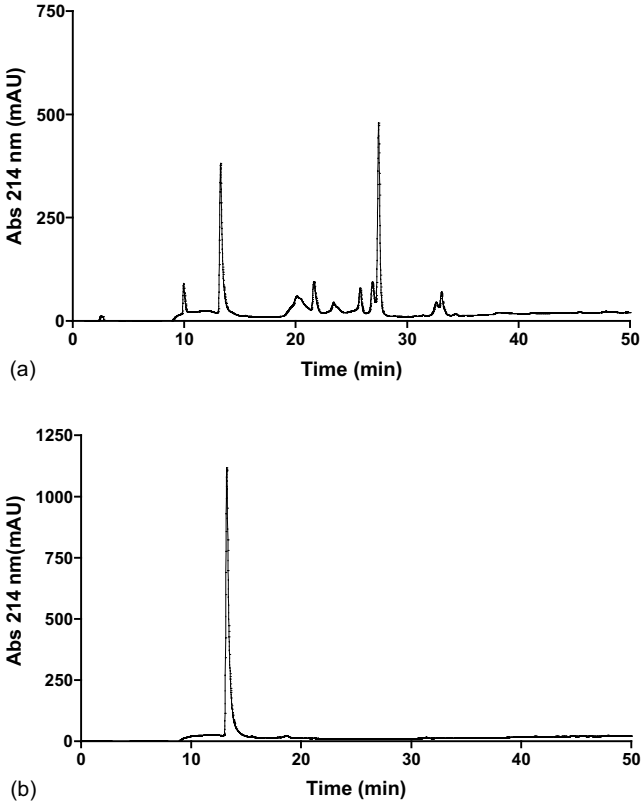

Fig. 1. RP-HPLC chromatograph of (a) *A*. sp. Seram venom or (b) acantoxin IVa run on a Jupiter analytical C18 column, equilibrated with solvent A (0.1% TFA) and eluted with the following gradient conditions of solvent B (90% acetonitrile in 0.09% TFA) and solvent A: 0–20% over 5 min, 20–60% in 40 min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

The mass spectra of purified acantoxin IVa displayed several charged states and these could be reconstructed into a single molecular mass of 6815.17 ± 0.55 Da. The molecular masses of previously isolated death adder neurotoxins are indicated in Table 1 for comparison.

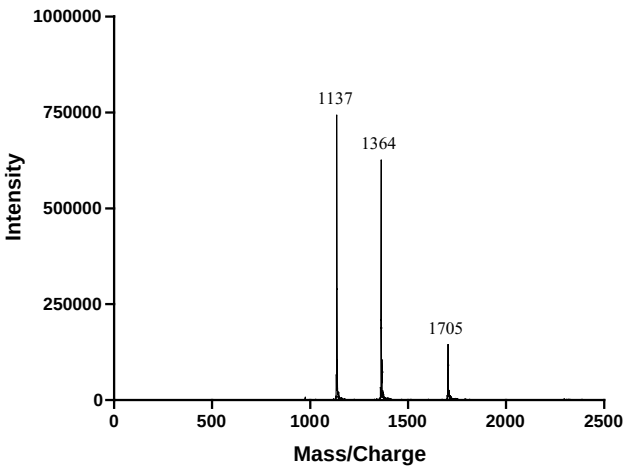

Fig. 2. Electrospray mass spectrometry of acantoxin IVa. The spectra shows a series of multiple-charged ions, related to molecules bearing four to six protons.

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

Species	Neurotoxin	Molecular mass (Da)	N-terminal sequence
A. sp. Seram	Acantoxin Iv[a]	6815	MQ**C**CNQQSSQ PKTTTT**C**PGG
A. antarcticus	Toxin Aa c[a]	6898	MQ**C**CNQQSSQ PKTTTT**C**PGG
Pseudechis australis	Toxin Pa a[b]	6758	MT**C**CNQQSSQ PKTTT**I**CAGG
Oxyuranus s. scutellatus	Taipan toxin 1[c]	6726	MT**C**YNQQSSE AKTTTT**C**SGG
A. antarcticus	Acanthophin a[d]	7155	Not determined
A. antarcticus	Toxin Aa b[e]	8135	VI**C**YRGYNNP QT**C**PPGENV**C**
A. antarcticus	Acanthophin d[f]	8387	VI**C**YRKYTNN VKT**C**PDGENV**C**
A. antarcticus	Aa e[g]	8752	VI**C**YVGYNNP QT**C**PPGGNV**C**

[a] [21].
[b] [42].
[c] [34].
[d] [20].
[e] [22].
[f] [23].
[g] [24].

3.3. N-terminal amino acid sequence

The N-terminal amino acid sequence of acantoxin IVa was determined (Table 1). Molecular weight gains after reduction/alkylation indicated that acantoxin IVa contains nine cysteines. Within the first 20 amino acid residues, the location of half-cystines was typical of short-chain neurotoxins. The N-terminal sequence of acantoxin IVa was compared with other protein sequences at the National Center for Biotechnology Information (NCBI) database using the BLAST service. Although acantoxin IVa was smaller in molecular mass than toxin Aa c isolated from *A. antarcticus* venom both toxins were identical in sequence to the first 20 amino acid residues. Acantoxin IVa also shared high identity with the short-chain neurotoxin toxin Pa a (85%) from Australian king brown snake (*Pseudechis australis*) and taipan toxin 1 (75%) from coastal taipan (*Oxyuranus s. scutellatus*). Acantoxin IVa shared much lower identity with other previously isolated death adder long-chain neurotoxins such as toxin Aa b and acanthophin d.

3.4. Chick isolated biventer cervicis nerve-muscle preparation

3.4.1. Neurotoxicity studies

While vehicle (i.e. BSA)/time had no significant inhibitory effect on twitch height, acantoxin IVa (0.1–1 μM) caused time-dependent inhibition of indirect twitches ($n = 5$–8; Fig. 3a). The t_{90} values for 0.1, 0.3 and 1.0 μM acantoxin IVa were 42.7 ± 3.1, 21.6 ± 3.6 and 9.7 ± 1.1 min, respectively ($n = 5$). Acantoxin IVa caused concentration-dependent inhibition of indirect twitches ($n = 5$; one-way ANOVA of t_{90} values, $P < 0.05$).

Acantoxin IVa (0.1−1 μM) significantly inhibited contractile responses to exogenous ACh (1 mM) and CCh (20 μM), but not KCl (40 mM), compared to vehicle ($n = 5$–8; one-way ANOVA, $P < 0.05$; Fig. 3b; data not shown for 0.1 and 1 μM acantoxin IVa).

3.4.2. Toxin reversibility studies

Washing of the preparation every 10 min for 3 h, after acantoxin IVa (0.3 μM) had caused complete twitch blockade, resulted in no recovery of indirect twitches ($n = 4$; data not shown). In addition, when neostigmine (10 μM) was added at t_{50}, i.e. after the addition of acantoxin IVa (0.3 μM), no sustained recovery of the twitch height was observed ($n = 4$; Fig. 3a). In fact, with a t_{90} value of 19.3 ± 0.7 min for acantoxin IVa (0.3 μM) plus neostigmine (10 μM) there was no significant difference between the t_{90} values when compared with acantoxin IVa (0.3 μM) alone ($n = 4$–5; Student's unpaired *t*-test). In contrast, when neostigmine (10 μM) was added at t_{50}, i.e. after the addition of dTC (8 μM), the twitch height recovered to $90.3 \pm 2.6\%$ of the initial height and was maintained at this level for the next 30 min ($n = 4$; data not shown). While contractile responses to exogenous ACh (1 mM) and CCh (20 μM) were unaffected by the combination of dTC (8 μM) and neostigmine (10 μM; $n = 4$; data not shown), contractile responses to exogenous nicotinic agonists were still abolished by the combination of acantoxin IVa (0.3 μM) and neostigmine (10 μM; $n = 4$; Fig. 3b).

3.4.3. Skeletal muscle nicotinic acetylcholine receptors

The positive control, dTC (1–10 μM), displayed classical competitive antagonism with parallel rightward shifts of the cumulative concentration–response curve to CCh, and no depression of the maximum response ($n = 5$–6; Fig. 4a). Using Schild plot analysis, the pA_2 for dTC was calculated to be 6.18 ± 0.15, and a Schild slope factor of 1.07 ± 0.1. Under the Lew and Angus method the pK_b was determined to be 6.29 ± 0.06. The slope factor was found to be not significantly different from unity since Eq. (1) was deemed to be a better fit by using an *F* test as stated in Section 2. Using the modified Lew and Angus method the pA_2 was estimated to be 6.28 ± 0.07, and the slope factor was NSDU. In contrast, acantoxin IVa (1–10 nM) caused a concentration-dependent depression of the maximum CCh response ($n = 4$–6; one-way

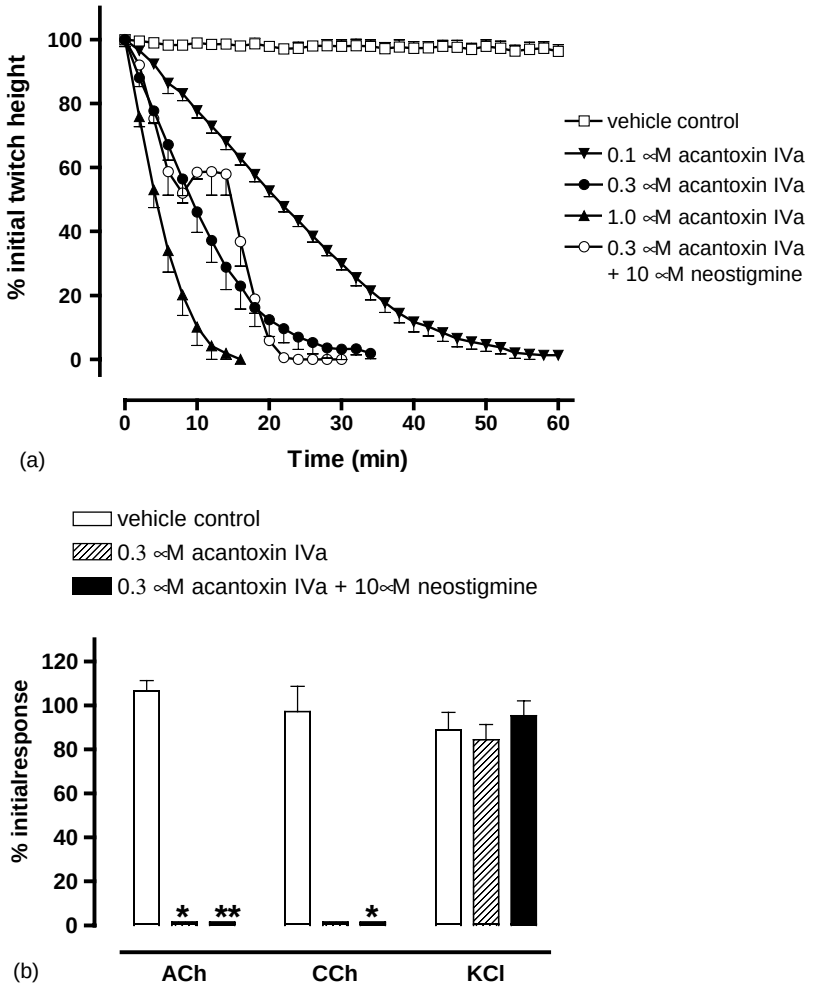

Fig. 3. The effect of acantoxin IVa (0.1–1 μM; $n = 5$), acantoxin IVa (0.3 μM; $n = 4$) with neostigmine (10 μM) at t_{50}, or vehicle ($n = 8$) on (a) indirect twitches or (b) contractile responses to exogenous ACh, CCh and KCl in the CBCNM preparation (data not shown for 0.1 and 1 μM acantoxin IVa). $^{*}P < 0.05$, significantly different from contractile responses in the presence of vehicle, one-way ANOVA.

ANOVA, $P < 0.05$; Fig. 4b). Using the modified Lew and Angus method the pA_2 for acantoxin IVa was estimated to be 8.36 ± 0.17, and the slope factor was NSDU. Like acantoxin IVa, α-bungarotoxin (2–7 nM) caused a concentration-dependent depression of the maximum cumulative CCh response ($n = 4$–5; data not shown). Using the modified Lew and Angus method the pA_2 for α-bungarotoxin was estimated to be 8.71 ± 0.06, and the slope factor was NSDU.

3.5. Ganglionic nicotinic acetylcholine receptors

Discrete concentration–response curves to (±)-epibatidine were repeatable after an incubation period of 1 h with no desensitization being observed ($n = 8$; Fig. 5a). Mecamylamine (0.3−10 μM) caused a concentration-dependent depression of the maximum response ($n = 4$–8; one-way ANOVA, $P < 0.05$; Fig. 5a). However, acantoxin IVa (1 μM) had no significant effect on the discrete concentration–response curve to (±)-epibatidine ($n = 4$; two-way repeated measures ANOVA; Fig. 5b).

3.6. α7-Type neuronal nicotinic acetylcholine receptors

In hippocampus homogenate, both unlabelled MLA and α-bungarotoxin were potent inhibitors of specific [^{3}H]-MLA binding ($n = 4$; Fig. 6). A one-site binding model was deemed to be a better fit by using an F test as stated in Section 2. While α-bungarotoxin ($pK_i = 8.89 \pm 0.13$) seemed to be slightly more potent than unlabelled MLA ($pK_i = 8.22 \pm 0.12$) there was no significant difference in potency ($n = 4$; one-way ANOVA, $P < 0.05$). Acantoxin IVa ($n = 4$), with a pK_i value of 4.48 ± 0.13, was approximately 25,000 times less potent than α-bungarotoxin at α7-type neuronal nAChR. While both unlabelled MLA and acantoxin IVa completely inhibited specific binding, this was not the case with α-bungarotoxin (Fig. 6). Approximately 8% of specific binding was resistant to high concentrations of α-bungarotoxin. Non-specific binding was close to 30% of total binding, which was comparable to previous studies [26,33].

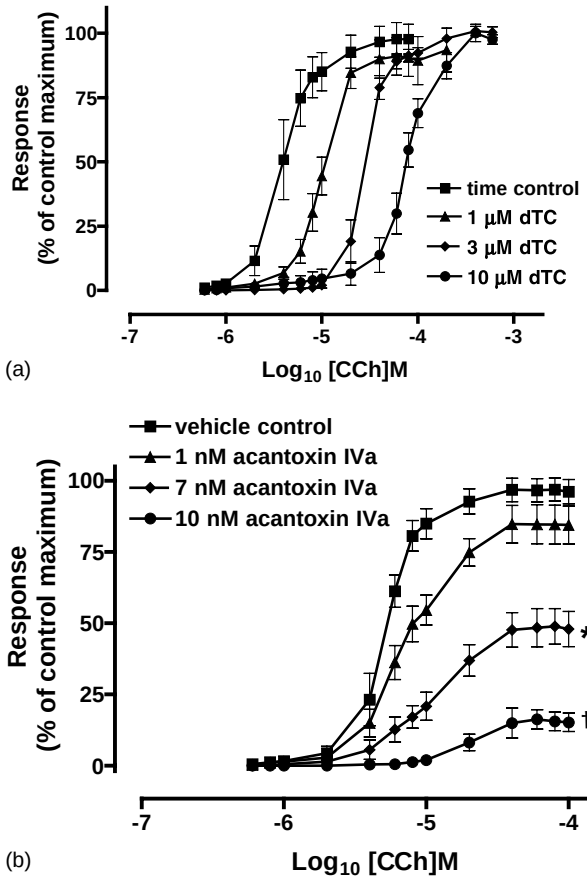

Fig. 4. The effect of (a) dTC (1–10 μM; $n = 5$–6) or (b) acantoxin IVa (1–10 nM; $n = 4$–6) on responses to cumulative additions of carbachol in the CBCNM preparation. $^{*}P < 0.05$, significantly different from 1 nM acantoxin IVa, one-way ANOVA. $^{\dagger}P < 0.05$, significantly different from 7 nM acantoxin IVa, one-way ANOVA.

3.7. α4β2-Subtype neuronal nicotinic acetylcholine receptors

Saturable specific binding of [^{3}H]-epibatidine in midbrain homogenate was fitted to a one-site binding model as it was deemed to be a better fit by using an F test as stated in Section 2 (data not shown). High affinity binding was demonstrated at this receptor site with a K_D of 113.3 ± 4.8 pM and a B_{max} of 41.7 ± 0.5 fmol/mg protein ($n = 4$). (−)-Nicotine caused full inhibition of specific [^{3}H]-epibatidine binding ($n = 4$; Fig. 7a). A two-site binding model was deemed to be a better fit by using an F test as stated in Section 2. (−)-Nicotine bound to two sites with pK_i values of 8.49 ± 0.50 and 7.27 ± 0.22, respectively. Acantoxin IVa caused no inhibition of specific [^{3}H]-epibatidine binding up to a concentration of 0.1 mM ($n = 4$; Fig. 7a). Non-specific binding was less than 6% of total binding.

3.8. Cytisine-resistant [^{3}H]-epibatidine binding sites

In midbrain homogenate, (−)-nicotine caused full inhibition of specific [^{3}H]-epibatidine binding to the cytisine-resistant [^{3}H]-epibatidine binding sites (Fig. 7b).

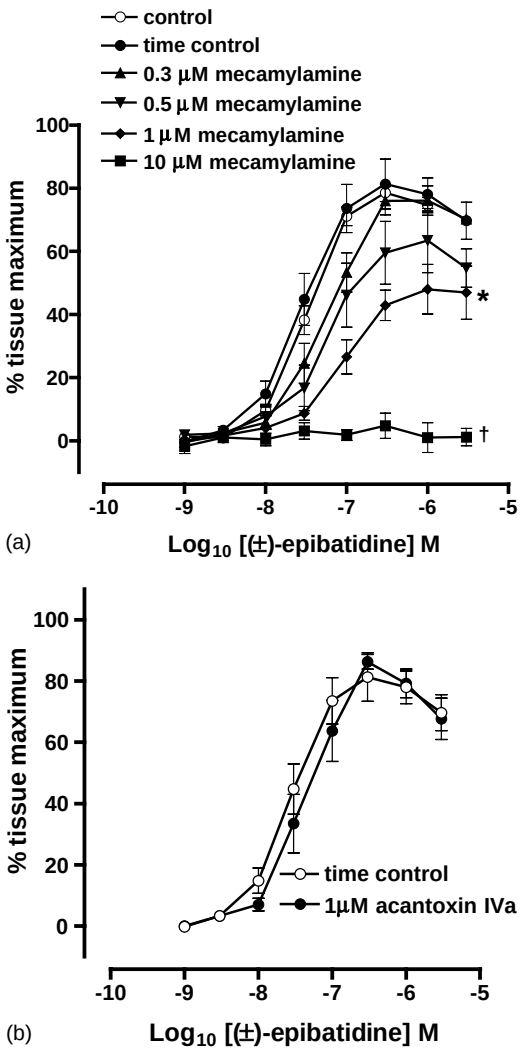

Fig. 5. The effect of (a) mecamylamine (0.3–10 μM; $n = 4$–8) or (b) acantoxin IVa (1 μM; $n = 4$) on responses to discrete additions of (±)-epibatidine in the guinea-pig ileum. $^{*}P < 0.05$, significantly different from 0.3 μM mecamylamine, one-way ANOVA. $\dagger P < 0.05$, significantly different from 1 μM mecamylamine, one-way ANOVA.

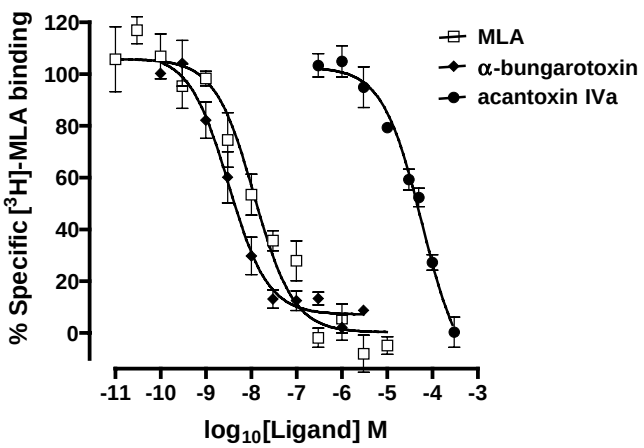

Fig. 6. Inhibition of specific [^{3}H]-MLA binding by unlabelled MLA ($n = 4$), α-bungarotoxin ($n = 4$) and acantoxin IVa ($n = 4$) in rat hippocampus homogenate. Data were fitted to a one-site binding model.

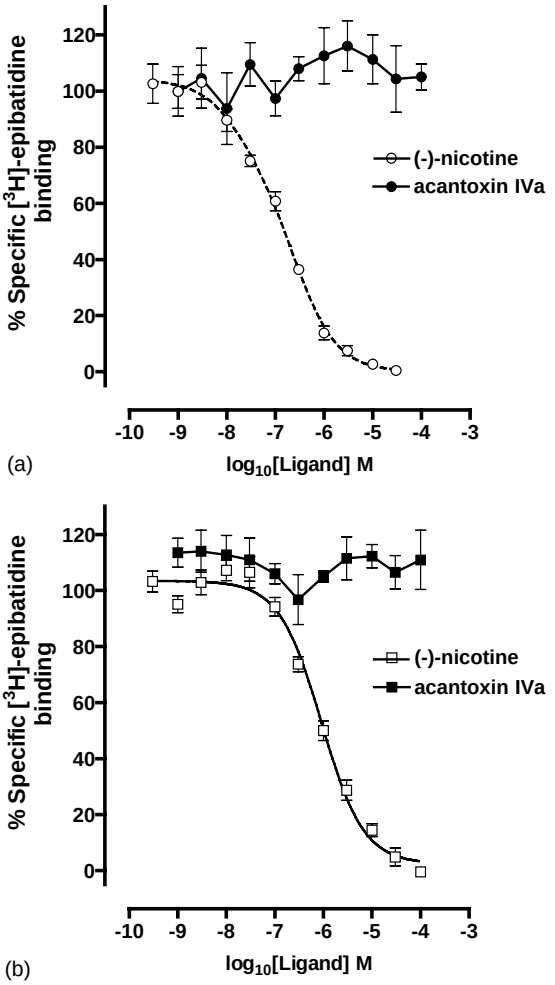

Fig. 7. Inhibition of specific [^{3}H]-epibatidine binding by (−)-nicotine ($n = 4$) and acantoxin IVa ($n = 4$) in rat midbrain homogenate with the (a) absence and (b) presence of cytisine (100 nM). Unbroken line represents data fitted to a one-site binding model. Dashed line represents data fitted to a two-site binding model.

This binding followed a one-site binding model with an IC_{50} of 0.96 ± 0.14 μM ($n = 4$). In contrast, acantoxin IVa caused no inhibition of binding up to a concentration of 0.1 mM ($n = 4$; Fig. 7b). Non-specific binding was about 18% of total binding.

4. Discussion

We have previously shown, based on in vitro studies, that death adder venoms are among the most neurotoxic [4,5]. Death adder venoms are thought to be rich in postsynaptic neurotoxins with five postsynaptic neurotoxins isolated from *A. antarcticus* venom. However, these neurotoxins have been poorly characterized in terms of nicotinic acetylcholine receptor pharmacology. In addition, neurotoxins from other species of death adder have not been isolated. Consequently, the present study describes the isolation of the first neurotoxin from *A.* sp. Seram death adder venom and the examination of its activity at a number of different nAChR subtypes.

Acantoxin IVa was isolated as a single peak from *A.* sp. Seram venom by successive RP-HPLC separations. As seen from the RP-HPLC chromatogram of the whole venom, acantoxin IVa elutes as the second peak and is a major component in the venom. Hence, the functional activity of this component is likely to be of importance in the overall context of envenomation. Using electrospray mass spectrometry, the molecular mass of acantoxin IVa was determined to be 6815 Da. It is well documented that elapid snake venom postsynaptic neurotoxins usually have molecular mass in the range of 6–9 kDa [24,34,35]. In fact, a molecular mass of 6815 Da is in the range expected for short-chain neurotoxins as opposed to long-chain neurotoxins [35]. The two short-chain neurotoxins isolated from *A. antarcticus* venom, acanthophin a and toxin Aa c, are larger than acantoxin IVa based on their minimum molecular mass of 7155 and 6898 Da, respectively, calculated from their amino acid composition [20,21]. Comparison of the N-terminal sequences also showed that acantoxin IVa shares very high identity with other short-chain neurotoxins from elapid snake venoms, in particular toxin Aa c.

Due to the sequence homology and molecular mass resemblance of acantoxin IVa to other elapid venom postsynaptic neurotoxins, this component was examined for in vitro neurotoxicity using the CBCNM preparation. Acantoxin IVa caused concentration-dependent inhibition of indirect twitches and blocked contractile responses to exogenous nicotinic agonists, confirming postsynaptic activity. However, unlike d-tubocurarine or the snake venom neurotoxin candoxin [36], neuromuscular blockade by acantoxin IVa was not readily reversible by washing. To further study the reversibility of the toxin, the anticholinesterase neostigmine was utilized. Although there was a partial recovery of the acantoxin IVa induced neuromuscular blockade this was not sustained. Similar observations were made when neostigmine (10 μM) was added after the addition of *A.* sp. Seram venom (3 μg/ml; unpublished data). However, clinicians have used anticholinesterases successfully to reduce the amount of antivenom administered following envenomation by death adders in Australia and Papua New Guinea [37]. The lack of a sustained effect of neostigmine on acantoxin IVa induced inhibition, observed in the current study, is likely to reflect the pseudo-irreversible nature of the antagonism (i.e. slow dissociation from the receptor) caused by acantoxin IVa (see below), and the short timeframe of the experiment. As previously documented, in association with artificial respiration and/or antivenom, anticholinesterases are likely to be a useful adjunct therapy for death adder envenomings given the longer time course of clinical management. However, we have previously indicated that caution should be observed when using anticholinesterase therapy to treat envenoming by some species of death adders given the

presence of myotoxic components which are unlikely to respond to anticholinesterase [38].

In order to determine the potency of acantoxin IVa at skeletal muscle nAChR, the CBCNM unstimulated preparation was utilized. Using dTC as a positive control we have shown that this preparation can be used successfully to determine the potency of an antagonist at skeletal muscle nAChR. While dTC caused classical competitive antagonism with a parallel rightward shift of the cumulative concentration–response curve to CCh this was not the case with acantoxin IVa or α-bungarotoxin. Both acantoxin IVa and α-bungarotoxin produced depression of the maximum CCh response. It is well known that α-bungarotoxin and most elapid venom postsynaptic neurotoxins dissociate very slowly from the skeletal muscle nAChR once they are bound [9]. Given the pseudo-irreversible antagonism caused by these toxins, the observed depression of the cumulative concentration–response curve to CCh is to be expected [39]. Pseudo-irreversible antagonism is a distinct pharmacological property of acantoxin IVa and is not caused by every elapid venom neurotoxin [36]. However, given that acantoxin IVa and α-bungarotoxin caused depression of the maximum CCh response, the classical Schild plot analysis cannot be utilized to determine the potency of these toxins. Previously, the modified Lew and Angus method has been used to provide estimates of pA_2 values that are in excellent agreement with those obtained from equilibrium binding assays [30,31]. In the current study, the pA_2 value of dTC determined via the modified Lew and Angus method was in excellent agreement with the pA_2 value and pK_b value determined by Schild plot analysis and Lew and Angus method, respectively. The Lew and Angus method is considered to provide a more accurate measure of potency than Schild plot analysis [29]. With an estimated pA_2 value of 8.36, acantoxin IVa is at least 100 times more potent than d-tubocurarine and only about two-fold less potent than α-bungarotoxin. Given that the slope factors for both α-bungarotoxin and acantoxin IVa are not significantly different from unity this suggests a competitive interaction with the skeletal muscle nAChR [29,30].

To determine the activity of acantoxin IVa at ganglionic nAChR, discrete concentration–response curves to (±)-epibatidine were obtained in the guinea-pig ileum. The positive control, mecamylamine, caused a gradual depression of the maximum response. This effect is in agreement with previous studies using nicotine as an agonist [40]. Acantoxin IVa had no activity at this receptor at a concentration of 1 μM. Previously it has been shown that neither α-bungarotoxin (long-chain neurotoxin) nor erabutoxin b (short-chain neurotoxin) have any activity at ganglionic nAChR [41].

Hippocampus homogenate was utilized to study the activity of acantoxin IVa at α7-type neuronal nAChR as it has been shown to be rich in this nicotinic receptor subtype [26,33]. Both positive controls, i.e. unlabelled MLA ($pK_i = 8.22$) and α-bungarotoxin ($pK_i = 8.89$), were potent inhibitors of the α7-type nicotinic radioligand [^{3}H]-MLA. In contrast, acantoxin IVa with a pK_i value of 4.48 was about 25,000 times less potent than the long-chain neurotoxin, α-bungarotoxin, at this receptor. Previously, it has been shown in both chick optic lobe homogenate and chimeric α7 receptors that long-chain neurotoxins are 300–20,000 times more potent at α7-type neuronal nAChR than short-chain neurotoxins [10,34]. Thus, in line with the molecular mass and N-terminal sequence, acantoxin IVa behaves like a short-chain neurotoxin pharmacologically. Interestingly, while both acantoxin IVa and unlabelled MLA completely inhibited specific binding, this was not the case with α-bungarotoxin. Furthermore, under the same conditions approximately 16% of specific [^{3}H]-MLA binding in hippocampus homogenate was resistant to binding by a long-chain neurotoxin isolated from *A. rugosus* death adder venom (unpublished data). Previous studies have also shown that approximately 15% of specific [^{3}H]-MLA binding in hippocampus homogenate is resistant to the long-chain neurotoxins α-cobratoxin and α-bungarotoxin even at high concentrations [23,30]. Given that detailed autoradiographical analysis has shown that the distribution of [^{125}I]-α-bungarotoxin and [^{3}H]-MLA binding sites correlate highly, it is unlikely that long-chain neurotoxin resistant [^{3}H]-MLA binding would represent another type of nAChR [26,33]. As suggested by these authors, explanations for this phenomenon are: (i) while small nicotinic ligands may occupy all five α7-subunits simultaneously the large size of α-bungarotoxin (MW = 7994) may hinder the binding to all five subunits simultaneously; (ii) due to the large size of α-bungarotoxin it may not have full access to receptors in all of the different membrane compartments preserved or created during membrane preparation. While this phenomenon requires further investigation, this study has shown that a short-chain neurotoxin (only about 1 kD smaller than α-bungarotoxin) is capable of fully inhibiting specific [^{3}H]-MLA binding.

In contrast to binding at α7-type nAChR, a short-chain neurotoxin as opposed to long-chain neurotoxins was capable of blocking nicotine-evoked release of dopamine in the rat striatum [42]. Hence, acantoxin IVa was examined for activity at other neuronal nAChR. Acantoxin IVa displayed no activity at α4β2 subtype neuronal nAChR or cytisine-resistant [^{3}H]-epibatidine binding sites. Evidence suggests that cytisine-resistant [^{3}H]-epibatidine binding sites are possibly that of α3β4 subtype neuronal nAChR [15].

In conclusion, the first neurotoxin from *A.* sp. Seram death adder venom was isolated. Although acantoxin IVa has a similar potency to α-bungarotoxin for skeletal muscle nAChR it is about 25,000 times less potent at α7-type nAChR. While long-chain neurotoxin resistant [^{3}H]-MLA binding requires further investigation this study has added another important piece of information in showing that a short-chain neurotoxin is capable of fully inhibiting specific [^{3}H]-MLA binding. Since acantoxin IVa had no

activity at ganglionic nAChR, α4β2 subtype neuronal nAChR and cytisine-resistant [^{3}H]-epibatidine binding sites further study is required to determine whether short-chain neurotoxins preferentially bind to another neuronal nAChR compared to long-chain neurotoxins.

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