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A PHARMACOLOGICAL CHARACTERISATION OF DEATH ADDER (*Acanthophis* Spp.) VENOMS AND TOXINS

A Thesis Submitted to the

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ADDENDUM

Page 3, Table 1.1: Since certain snakes from the Colubridae family such as *Boiga irregularis* have been shown to cause significant clinical neurotoxicity (Fritts *et al.*, 1990, *Am. J. Trop. Med. Hyg.*, **42**, 607-611) the common name should read "Colubrid snakes" and not "Harmless snakes".

Page 11, paragraph 1, line 4 and elsewhere (page 17, 18 & 19): Substitute "Wüster *et al.*, unpublished" with "Wüster, W., pers. comm., 2003".

Page 23, line 2: Substitute "*Naia*" with "*Naja*".

Page 26, first sentence: Add reference "(Tan & Ponnudurai, 1990)".

Page 26, end of last line: Delete "pancreatic loop" and add "loop similar to PLA_2s from mammalian pancreatic juices".

Page 29, paragraph 2, line 9: Add reference "(Schmidt & Middlebrook, 1989)".

SCHMIDT, J.J. & MIDDLEBROOK, J.L. (1989). Purification, sequencing and characterization of pseudexin phospholipases A_2 from *Pseudechis porphyriacus* (Australian red-bellied black snake). *Toxicon*, **27**, 805-818.

Page 31, paragraph 2, line 1: Add "snake" before "α-neurotoxin".

Page 33, paragraph 1, line 1: Add "snake" before "β-neurotoxin".

Page 46, third last line: Delete "in" before "*in vivo*".

Page 48, line 3, substitute "immunised" with "hyper-immunised".

Page 49, add at start of paragraph 2: "Anticholinesterase therapy work by inhibiting acetylcholinesterase and thereby increasing the availability of acetylcholine in the neuromuscular junction."

Page 63, Lalloo *et al.* (1994) reference: Delete (*Papuanus*) and add (*papuanus*).

Page 65, Loveridge (1948) reference: Abbreviate journal name as "*Bull. Mus. Comp. Zool.*".

Page 81, Table 1: Delete "rug 6657" from the table.

Page 96, section 2: Should read "...blocked contractile responses to exogenous nicotinic agonists, but not to exogenous KCl, ...".

Page 111, paragraph 2, line 3: While it is unusual to have unbonded half-cysteines in snake toxins it is not the case with death adder venoms. As stated in the General Introduction, such a toxin (i.e. Toxin Aa c) has been previously isolated from *Acanthophis antarcticus* venom (Kim & Tamiya, 1981a).

Page 124, line 1: Should read "Discrete concentration-response curves to (±)-epibatidine were repeatable in the guinea-pig isolated ileum after an incubation period ...".

Page 136, paragraph 1, last line: Should read "While this phenomenon requires further investigation, this study has shown for the first time that a short-chain neurotoxin (only about 1 kD smaller than α-bungarotoxin) is capable of fully inhibiting specific [^{3}H]-MLA binding in hippocampus homogenate, albeit at concentrations greater than 10^{-4} M."

Page 139, Blanchard *et al.*, (1979) reference: Delete "[125I]-alpha-bungarotoxin" and add "[^{125}I]-alpha-bungarotoxin".

Page 182, Lalloo *et al.* (1996) reference: Substitute "*QJM*" with "*Q. J. Med.*".

This thesis is dedicated to my wonderful parents

Sunanda and Vidura Wickramaratna

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DECLARATION

In accordance with Doctor of Philosophy Regulations of Monash University the following declarations are made:

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any University or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

All of the experiments described herein were performed in accordance with the guidelines of the National Health and Medical Research Council Code of Practice for the Care and Use of Animals for Experimental Purposes in Australia, and were approved by the Animal Ethics Committee (Department of Pharmacology, Monash University).

In accordance with Doctorate Regulation 17 of Monash University this thesis is a combination of original papers published in peer reviewed journals and unpublished chapters. The ideas, development and writing up of all the papers in the main body of the thesis were the principal responsibility of the candidate, working within the Department of Pharmacology under the supervision of Dr. Wayne Hodgson.

The inclusion of co-authors in published papers reflects the fact that the work came from active collaboration between researchers and acknowledges input from collaborators especially in the provision of venoms or access to specialised equipment.

[redacted]

Janith C. Wickramaratna

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(Venom Supplies Pty. Ltd., Australia) for providing data on death adder venom yields. My thanks also go out to Mr. Duncan McRae for kindly providing the death adder pictures.

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To my brother, thanks very much for all your support and love. You have been an excellent role model in my life. Lastly, but most of all I would like to thank my parents for all that they have done for me. Your endless love and encouragement have made me what I am today, and to you I dedicate this thesis.

SUMMARY

The current study examined venoms from *Acanthophis hawkei*, *A. praelongus*, *A. pyrrhus*, *A. rugosus*, *A.* sp. Seram, *A. wellsi* and four geographic variants of *A. antarcticus* (New South Wales (NSW); Queensland (Qld); South Australia (SA); Western Australia (WA)) for both *in vitro* neurotoxicity and myotoxicity. In addition, the efficacy of CSL death adder antivenom against the *in vitro* neurotoxicity and myotoxicity of these venoms was studied. The first ever myotoxin from a death adder venom, and two neurotoxins from *A. rugosus* and *A.* sp. Seram venoms were isolated and characterised.

Clinically, the most important symptoms of death adder envenomations are those relating to neurotoxicity. Therefore, the venoms were examined for *in vitro* neurotoxicity using the chick biventer cervicis nerve-muscle preparation. All venoms (0.3 – 10 μg/ml) caused concentration-dependent inhibition of nerve-mediated twitches, and blocked contractile responses to exogenous acetylcholine and carbachol, suggesting postsynaptic neurotoxicity. CSL death adder antivenom, which has been raised against *A. antarcticus* venom, remains the principal therapy for death adder envenomation. Hence, the efficacy of CSL death adder antivenom against the *in vitro* neurotoxicity of death adder venoms was examined. Antivenom (1 unit/ml) inhibited the neurotoxicity produced by *A. hawkei*, *A. praelongus* and *A. pyrrhus* venoms (10 μg/ml), but was markedly less effective against *A. antarcticus* (NSW; SA; WA), *A. rugosus* and *A.* sp. Seram venoms (10 μg/ml). However, a higher concentration of antivenom (5 units/ml) was effective against all venoms tested. The anticholinesterase neostigmine (10 μM) caused a transient recovery of death adder venom (3 μg/ml) induced neurotoxicity. However, neostigmine had no significant effect on the overall neurotoxicity of any death adder venom as determined by the t_{90} values (i.e. time taken to cause 90% inhibition of nerve-mediated twitches).

Following the studies on whole venoms, two neurotoxins (i.e. acantoxin IVa, MW 6815; and acantoxin Va, MW 7991), were isolated from *A.* sp. Seram and *A. rugosus* death adder venoms, respectively. Acantoxin IVa and acantoxin Va caused pseudo-irreversible antagonism at skeletal muscle nicotinic acetylcholine receptors (nAChR). 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 acantoxin Va was several fold less potent than acantoxin IVa at skeletal muscle nAChR, it was about 350 fold more potent than acantoxin IVa at α7-type neuronal nAChR. In contrast to long-chain neurotoxins, the short-chain neurotoxin acantoxin IVa completely inhibited specific [^{3}H]-methyllycaconitine binding in rat hippocampus homogenate. Neither acantoxin IVa nor acantoxin Va displayed activity at α4β2 subtype neuronal nAChR or cytisine-resistant [^{3}H]-epibatidine binding sites.

Based on earlier studies on *A. antarcticus* venom it was thought that death adder venoms were devoid of myotoxic activity. However, guided by LC-MS profiles the first myotoxic PLA_2 component from death adder venom was isolated. Acanmyotoxin-1 (MW 13811), isolated from *A. rugosus* venom, caused dose-dependent myotoxicity in the chick biventer preparation. Antivenom studies showed that CSL death adder antivenom (5 units/ml) was effective in neutralising the myotoxic activity of acanmyotoxin-1 (1 μM). It was found that PLA_2 activity is essential for the myotoxic activity of acanmyotoxin-1. Following the isolation of acanmyotoxin-1, all death adder whole venoms (10 – 50 μg/ml) were examined for *in vitro* myotoxicity. Studies showed that *A.* sp. Seram, *A. praelongus*, *A. rugosus* and *A. wellsi* venoms were myotoxic, while *A. antarcticus* (NSW; Qld; SA; WA), *A. hawkei* and *A. pyrrhus* venoms were devoid of myotoxic activity. CSL death adder antivenom (5 units/ml) was effective in neutralising the activity of those myotoxic death adder venoms.

It is anticipated that the neurotoxicity and myotoxicity studies on death adder venoms will be of potential clinical relevance and that the isolated neurotoxins will be of use in studying neuronal nicotinic acetylcholine receptors.

PUBLICATIONS

The work in this thesis has resulted in the following publications:

Original Articles

Wickramaratna, J.C., Fry, B.G., Loiacono, R.E., Aguilar, M.I., Alewood, P.F. & Hodgson, W.C. Isolation and pharmacological characterization of a neurotoxin from the venom of the *Acanthophis*. sp. Seram death adder. *J. Pharmacol. Exp. Ther.* (in submission)

Wickramaratna, J.C., Fry, B.G. & Hodgson, W.C. (2003). Species-dependent variations in the *in vitro* myotoxicity of death adder (*Acanthophis*) venoms. *Toxicol. Sci.*, **74**, 352-360.

Wickramaratna, J.C., Fry, B.G., Aguilar, M.I., Kini, R.M. & Hodgson, W.C. (2003). Isolation and pharmacological characterization of a phospholipase A_2 myotoxin from the venom of the Irian Jayan death adder (*Acanthophis rugosus*). *Br. J. Pharmacol.*, **138**, 333-342.

Fry, B.G., **Wickramaratna, J.C.**, Hodgson, W.C., Alewood, P.F., Kini, R.M., Ho, H. & Wüster, W. (2002). Electrospray liquid chromatography/mass spectrometry fingerprinting of *Acanthophis* (death adder) venoms: taxonomic and toxinological implications. *Rapid Commun. Mass Spectrom.*, **16**, 600-608.

Fry, B.G., **Wickramaratna, J.C. (80%)**, Jones, A., Alewood, P.F. & Hodgson, W.C. (2001). Species and regional variations in the effectiveness of antivenom against the *in vitro* neurotoxicity of death adder (*Acanthophis*) venoms. *Toxicol. Appl. Pharmacol.*, **175**, 140-148.

Review Articles

Fry, B.G., Winkel, K.D., **Wickramaratna, J.C.**, Hodgson, W.C. & Wüster, W. (2003). Effectiveness of snake antivenom: species and regional venom variation and its clinical impact. *J. Toxicol.-Toxin Rev.*, **22**, 23-34.

Hodgson, W.C. & **Wickramaratna, J.C.** (2002). *In vitro* neuromuscular activity of snake venoms. *Clin. Exp. Pharmacol. Physiol.*, **29**, 807-814.

Communications

Wickramaratna, J.C., Fry, B.G., Loiacono, R.E., Aguilar, M.I., Alewood, P.F. & Hodgson W.C. (2003). Neuropharmacology of a short-chain neurotoxin isolated

from the venom of the *Acanthophis* sp. Seram death adder. *Proceedings of the 5th Australian Peptide Conference*, Session C, 25.

Wickramaratna, J.C., Fry, B.G., Aguilar, M.I., Kini, R.M. & Hodgson, W.C. (2002). Isolation and pharmacological characterisation of a myotoxic PLA_2 from a death adder venom. *Proceedings of the 6th Asia-Pacific Congress on Animal, Plant and Microbial Toxins & 11th Annual Scientific Meeting of the Australasian College of Tropical Medicine*, 23.

Wickramaratna, J.C., Fry, B.G., Loiacono, R.E., Aguilar, M.I., Alewood, P.F. & Hodgson, W.C. (2001). Pharmacology of novel components from death adder venoms. *Proc. Aust. Soc. Clin. Exp. Pharmacol. Toxicol.*, **9**, 50.

Wickramaratna, J.C., Fry, B.G., Jones, A., Alewood, P. & Hodgson, W.C. (2000). *In vitro* neurotoxicity of three novel postsynaptic neurotoxins from death adder venoms. *Proc. Aust. Soc. Clin. Exp. Pharmacol. Toxicol.*, **8**, 34.

Wickramaratna, J.C., Hodgson, W.C., Jones, A., Alewood, P. & Fry, B.G. (2000). Regional and species variation in the effectiveness of antivenom against the *in vitro* neurotoxicity of death adder venoms. *Proceedings of the XIIIth World Congress of the International Society on Toxinology*, P209.

Other publications by the author:

Original Articles

Fry, B.G., Lumsden, N.G., Wüster, W., **Wickramaratna, J.C.**, Hodgson, W.C. & Kini, R.M. Isolation of a neurotoxin (alpha-colubritoxin) from a 'non-venomous' colubrid: evidence for early origin of venom in snakes. *J. Mol. Evol.* (in press)

Wickramaratna, J.C. & Hodgson, W.C. (2001). A pharmacological examination of venom from three species of death adder (*Acanthophis antarcticus*, *Acanthophis praelongus* and *Acanthophis pyrrhus*). *Toxicon*, **39**, 209-216.

Communications

Wickramaratna, J.C. & Hodgson, W.C. (1999). The neuromuscular activity of venom from three species of death adder. *Proc. Aust. Soc. Clin. Exp. Pharmacol. Toxicol.*, **6**, 34.

CHAPTER 1

General Introduction

Snakes

The first snakes probably appeared during the Cretaceous age, which dates back 100 - 120 million years. Although their fossil record is poor it is now accepted that snakes arose from lizards (Goin & Goin, 1971; Parker & Grandison, 1977). The earliest fossils recognisable as belonging to a snake are those of *Lapparentophis defrenne* in the Sahara from the Lower Cretaceous age (Parker & Grandison, 1977). Today, there are approximately 3200 species of snakes belonging to 11 - 15 major families (Table 1.1) forming the suborder Serpentes of the order Squamata (Harris, 1991; Hider *et al.*, 1991; Shine, 1998). Of the 15 recognised families, 8 are found in Australia. These are Typhlopidae, Madtsoiidae, Pythonidae, Acrochordidae, Laticaudidae, Hydrophiidae, Elapidae and Colubridae (Shine, 1998). Even at the level of families the systematic classification of snakes is unclear (Cogger, 2000).

Snakes have undergone extensive adaptation to occupy most of the major habitat (Goin & Goin, 1971). Some have adapted to burrowing, while others have taken to the trees, and one (or two) family has become entirely marine. The abundant diversity of snakes is further reflected by their size, ranging from the smallest of species of 100 mm in length to the Anacondas and Reticulated Pythons that may grow to more than 9 m. However, there are some characteristic features that are found common to most members of the suborder Serpentes.

The most obvious features of snakes are the elongated flexible body and the absence, or apparent absence, of limbs. Although all snakes have no traces of forelimbs a few families of snakes, especially boas and pythons, have evolutionary vestiges of hind limbs (Shine, 1998; Cohn & Tickle, 1999). These appear externally as small horn-sheathed claws

(Parker & Grandison, 1977). However, no snakes have any trace of a shoulder girdle or of a breast bone (Parker & Grandison, 1977; Shine, 1998).

Table 1.1 Major Snake Families*

Scientific Name	Common Name
Typhlopidae	Blindsnakes
Leptotyphlopidae	Blindsnakes
Anomalepidae	Blindsnakes
Uropeltidae	Shield-tailed snakes
Aniliidae	Pipe snakes
Madtsoiidae	Madtsoiids
Pythonidae	Pythons
Boidae	Boas
Xenopeltidae	Sunbeam snakes
Acrochordidae	Filesnakes
Viperidae	Vipers
Laticaudidae	Sea kraits
Hydrophiidae	Viviparous seasnakes
Elapidae	Elapids
Colubridae	Harmless snakes

* Adapted from Shine (1998).

Elongation of the body has meant modification and reorganisation of the internal organs to fit the long narrow body. Most of the major internal organs, such as lungs,

kidneys, liver and sex organs are themselves elongated, and paired organs lie in a staggered arrangement (Parker & Grandison, 1977; Shine, 1998). The right kidney lies farther forward towards the head than the left, with the testes and ovaries also staggered in a similar manner. In most snakes, the left lung is considerably smaller than the right or entirely absent (Parker & Grandison, 1977). However, this is compensated by the increased capacity of the remaining lung. In some sea snakes the lung extends back to the vent and, in addition, acts as a hydrostatic organ. Given that the urinary bladder has completely disappeared through evolution, all snakes excrete nitrogenous waste in a semi-solid form (Parker & Grandison, 1977). Although hard to imagine, all the basic systems found in other vertebrates are present inside snakes. However, they are modified and reorganised to a such an extent that recognition is difficult (Shine, 1998).

Venomous Snakes

It is thought that the evolution of venomous snakes occurred more recently, possibly around 30 million years ago in the Miocene age. There are approximately 1300 species of venomous snakes found in 3 to 6 families (Parker & Grandison, 1977; Harris, 1991), although classification of these families is unclear. However, most authorities do accept Colubridae, Elapidae and Viperidae as valid venomous families. In the past Hydrophiinae and Laticaudinae have been considered as subfamilies of Elapidae (Parker & Grandison, 1977). More recently, authors have considered these as separate families, named Hydrophiidae and Laticaudidae (Shine, 1998).

Colubridae

Colubridae is the largest dominant family of snakes and members are found in all parts of the world except the coldest regions (Savitzky, 1980). However, they do not enjoy such status in Australia being confined to the northern and eastern regions (Shine, 1998). Most colubrids are harmless but a few possessing grooved fangs are venomous (White, 1998b). In these species the fangs are mounted at the rear of the upper jaw, hence they are referred to as opisthoglyphous (Cogger, 2000). Given the position of the fangs, most venomous colubrids do not pose a danger to humans. The brown tree snake (*Boiga irregularis*) is the only Australian venomous colubrid (White, 1998b). Although aggressive at times the bite is not known to be fatal or dangerous to adult humans. This is thought to be due to an inability of the snake to inflict a serious bite and deliver high quantities of venom (Weinstein *et al.*, 1991; White, 1998b). However, this venom has recently been shown to cause neurotoxicity in *in vitro* studies (Lumsden *et al.*, 2003). In South Africa, Boomslang (*Dispholidus typus*) tree snakes are notorious for causing dangerous bites (Harris, 1991). In these snakes the fangs are located further forward compared to those of other colubrids.

Elapidae

Species belonging to the Elapidae family are found in Asia, Africa, America and Australia, and include cobras, mambas, kraits and taipans. Almost all venomous terrestrial snakes in Australia belong to the Elapidae family (Sutherland, 1994; White, 1998b). Australian elapids are thought to be closely related to the viviparous sea snakes (Cogger, 2000). Hence, suggestions have been made to include these elapids under Hydrophiidae. However, for the moment they are treated as belonging to the Elapidae family (Cogger,

2000). Australian elapids are thought to be the world's most venomous snakes (i.e. based on murine LD_{50} values), with nine of the top ten living exclusively on this continent (Broad *et al.*, 1979; Lalloo *et al.*, 1995a). Elapids probably arrived in Australia from Asia 15 - 20 million years ago (Shine, 1998). They typically have a small head with short to medium sized (3 – 5 mm) fixed fangs at the front of the upper jaw (Fairley, 1929a,b). Due to this fang arrangement these snakes are referred to as proteroglyphous (Cogger, 2000). In elapids the fang consists of an enclosed venom canal as opposed to an open groove as in vipers (Parker & Grandison, 1977). With this effective biting mechanism, combined with a highly lethal venom, it is of no wonder that these snakes command a reputation for being highly dangerous.

Hydrophiidae and Laticaudidae

The families Hydrophiidae and Laticaudidae contain the sea snakes. Although most sea snakes are found in coastal waters of warmer regions, some species are distributed throughout the Indian and Pacific oceans (Shine, 1998). However, low temperatures and high salinity restrict migration by these snakes to other waters. Hydrophiidae include all viviparous sea snakes, while Laticaudidae include those sea snakes that return to land to lay eggs (oviparous). Therefore, it is thought that Laticaudidae is the more primitive group of sea snakes, also more specifically called sea kraits (Parker & Grandison, 1977). The majority of sea snake species belong to the Hydrophiidae family (Heatwole, 1999). As discussed previously, sea snakes are closely related to the elapids. Like the elapids, sea snakes are proteroglyphous and have highly lethal venoms. However, they have adapted to the aquatic life through some evolutionary modifications. The nostrils are equipped with a closing mechanism and the tail is paddle-shaped to enhance movement (Parker &

Grandison, 1977). All sea snakes have developed a salt gland beneath the tongue to excrete excess salt (Greene, 1997).

Viperidae

Old World vipers (Viperinae) and pit-vipers (Crotalinae) belong to the Viperidae family. Vipers are well represented in Africa, Europe, Asia, North America and South America (Harris, 1991). Since vipers are absent in Australia it has been suggested that this group may have evolved later than the Elapidae family – the dominant group in Australia (Parker & Grandison, 1977). Vipers in general tend to be built heavier than elapids and are generally sluggish in movement (Shine, 1980). Unlike elapids, vipers tend to have large flattened triangular heads. They also posses the most effective biting apparatus with long fangs that can move forwards and upwards of the maxillary bone to strike the prey at a perpendicular angle (Fairley, 1929a). This allows deeper penetration and greater venom deposition. The classification of snakes into Crotalinae as opposed to Viperinae, is based on the presence of infrared-sensing pits (Greene, 1997). These are located on each side of the head between the nostril and the eye (Parker & Grandison, 1977). These pits allow some pit-vipers to detect temperature differences of 1 to 2 °C from a distance of 1 to 2 feet (Harris, 1991). The value of heat-sensing organs for detecting warm-blooded prey and possible predators at night is quite apparent. This venomous family includes the well-known Western diamond-backed rattlesnake (*Crotalus atrox*), Malaysian pit-viper (*Calloselasma rhodostoma*) and Russell's viper (*Daboia russelii*).

Acanthophis Genus

Also called death adders, *Acanthophis* spp. were first described by Shaw in 1794 (Storr, 1981). 'Acanthophis' means spiny snake, perhaps referring to the spiky tail or the ridged scales (Sutherland & Tibballs, 2001). Early settlers called these snakes "death" adders due to their hideous aspect and their deadly bite (Campbell, 1966). Although it is an elapid, the death adder is unique in having a viper-like appearance and behaviour (Shine, 1980). In fact, the death adder was once wrongly classified as the only viperid in Australia (McCoy, 1885 cited in Shine, 1980). Although having doubts, Krefft (1869) correctly classified this snake as an elapid. The death adder has a broad, almost triangular head, a thin neck and a short, stout body that terminates abruptly to a thin, short tail (Campbell, 1966; Cogger, 2000). Death adders have 19 – 23 mid-body scale rows, 110 – 170 ventrals, a single anal and 35 – 65 subcaudal scales (Hoser, 1995; Cogger, 2000). These scales may be somewhat keeled and, unlike any other Australian elapid, death adders have a series of subocular scales under the eyes (Cogger, 2000). The eyes are small with the pupil of each being a vertical ellipse. Although a thorough study has not been undertaken, *A. antarcticus* and *A. hawkei* are considered to be the largest of the death adders growing to a maximum length of 1.1 – 1.2 m (Covacevich, 1981; Hoser, 2002). In contrast, *A. pyrrhus* is among the smallest of the species being less than 0.75 m in length (Gow & Swanson, 1977; White, 1998a). While death adders are comparatively smaller in length than other Australian elapids, they tend to be heavy bodied like the viperids (Shine, 1980). The colour of death adders vary from one habitat to another and even within the same locality (Hoser, 1985; Johnston, 1996). They may be light brown to a reddish brown, or even black (Campbell, 1966). Their unique tail tip is usually a distinctive cream, white or yellow colour. The

cryptic colour pattern of these snakes is most brilliant in young specimens but fades with age.

Classification and Distribution

Taxonomically, it is a substantial problem identifying species within the genus *Acanthophis* due to the high degree of species variability (Hoser, 1998; Aplin & Donnellan, 1999). The identifying characteristics such as colour, number and texture of scales, used in separating species are shared to varying degrees by multiple species (Hoser, 1998). However, between different populations there remains considerable morphologicai differences, and until recently these were classified under very few species (Wüster *et al.*, 1999).

Four species were described prior to the 20th century and three of these are widely accepted as valid species (Cogger, 2000). They are *Acanthophis antarcticus* described by Shaw in 1794, *Acanthophis praelongus* by Ramsay in 1877 and *Acanthophis pyrrhus* by Boulenger (1898, cited in Storr, 1981). While *Acanthophis laevis* was described by Macleay (1877, cited in Hoser, 1998) it is not recognised by some taxonomists as a valid species (Cogger, 2000). Other *Acanthophis* species and subspecies described are:

Acanthophis antarcticus schistos (Wells & Wellington, 1985)

Acanthophis barnetti (Hoser, 1998)

Acanthophis crotalusei (Hoser, 1998)

Acanthophis cummingi (Hoser, 1998)

Acanthophis hawkei (Wells & Wellington, 1985)

Acanthophis lancasteri bottomi (Hoser, 1998)

Acanthophis lancasteri lancasteri (Wells & Wellington, 1985)

Acanthophis pyrrhus armstrongi (Wells & Wellington, 1985)

Acanthophis rugosus (Loveridge, 1948)

Acanthophis wellsei (Hoser, 1998)

Acanthophis woolfi (Hoser, 1998)

Although descriptions of these species are found in Zoological and Herpetological publications there remains considerable debate on their validity (Wüster *et al.*, 1999). This is especially the case with the recent descriptions by Hoser (1998). Many respected taxonomists have strongly disputed Hoser's descriptions and naming of these species (Aplin & Donnellan, 1999; Wüster *et al.*, 1999). More recently, Hoser (2002) described the populations of death adders found in Seram, Tanimbar and Kei Islands as *A. groenveldi*, *A. macgregori* and *A. yuwoni*, respectively. Hoser also classified the *A. antarcticus* population from South Australia as a new subspecies of *A. antarcticus*, namely *A. antarcticus cliffrosswellingtoni* (Hoser, 2002). In the same publication, an *A. wellsei* subspecies, *A. wellsei donnellani*, was described. However, given that these descriptions are presented in a relatively unknown amateur publication it is unlikely that these species names will be accepted by mainstream taxonomists (Aplin, 1999). Hoser (2002) himself acknowledges that many of his previous descriptions (Hoser, 1998), except for *A. wellsei*, are not accepted by many taxonomists and relevant governmental departments in Australia. Although an unfortunate situation, such problematic zoological nomenclature is part and parcel of venom research. In future, it is possible that some of these names might change as our understanding of the systematics of the *Acanthophis* genus increases (Wüster *et al.*, 1999).

As *A. wellsei* was inadequately described by Hoser (1998), it has been re-examined more thoroughly by Aplin and Donnellan (1999). They confirmed this death adder to be a

valid species and it is now named more appropriately as *A. wellsi* (Aplin & Donnellan, 1999). More recently, based on phylogenetic analysis of mitochondrial DNA sequences, *A. rugosus*, *A. hawkei*, *A. wellsi*, *A. laevis*, and the population of death adders on the island of Seram have been shown to be distinct species (Wüster *et al.*, unpublished). Although Hoser (2002) named the Seram death adders as *A. groenveldi*, based on specimens nearly 100 years old, other taxonomists have strongly advised against the use of this name (Wüster, W., pers. comm., 2003). Therefore, for the current work, it will be treated as an unnamed species, namely *A.* sp. Seram. For the purpose of this thesis the following species of death adders were of interest: *A. antarcticus*, *A. hawkei*, *A. praelongus*, *A. pyrrhus*, *A. rugosus*, *A. wellsi* and *A.* sp. Seram. These snakes were chosen since it was possible to obtain a sufficient quantity of their venom from commercial or collaborative sources to complete the vast majority of the experiments. As suggested by highly regarded taxonomists (Aplin & Donnellan, 1999; Wüster *et al.*, 1999), the species names documented by Hoser were not utilised whenever possible.

A. antarcticus (Fig. 1.1a), also called the common death adder, is found in Western Australia, South Australia, along the east coast of New South Wales and much of inland Queensland (Fig. 1.1b). *A. praelongus* (Fig. 1.2a), commonly called the northern death adder, is found in the Cape York region of Queensland and adjacent areas (Fig. 1.2b). *A. pyrrhus* (Fig. 1.3a) inhabits arid regions of central Australia and Western Australia (Fig. 1.3b), giving rise to its common name, the desert death adder (Sutherland, 1988). *A. wellsi* (Fig. 1.4a) is distributed in the Pilbarra region of Western Australia (Fig. 1.4b; Aplin & Donnellan, 1999). This gives rise to its common name, the Pilbarra death adder. Similarly, the Barkly Tableland death adder (*A. hawkei*; Fig. 1.5a) is found on the black-soil plains of the Barkly Tableland region of the Northern Territory (Fig. 1.5b)(Wells & Wellington, 1985). *A. rugosus* (Fig. 1.6a) is found in the Merauke region of Irian Jaya (Fig. 1.6b) hence

it is commonly called the Irian Jayan death adder. *A.* sp. Seram (Fig. 1.7a) death adder is found on the island of Seram, Indonesia (Fig. 1.7b).

Diet and Habits

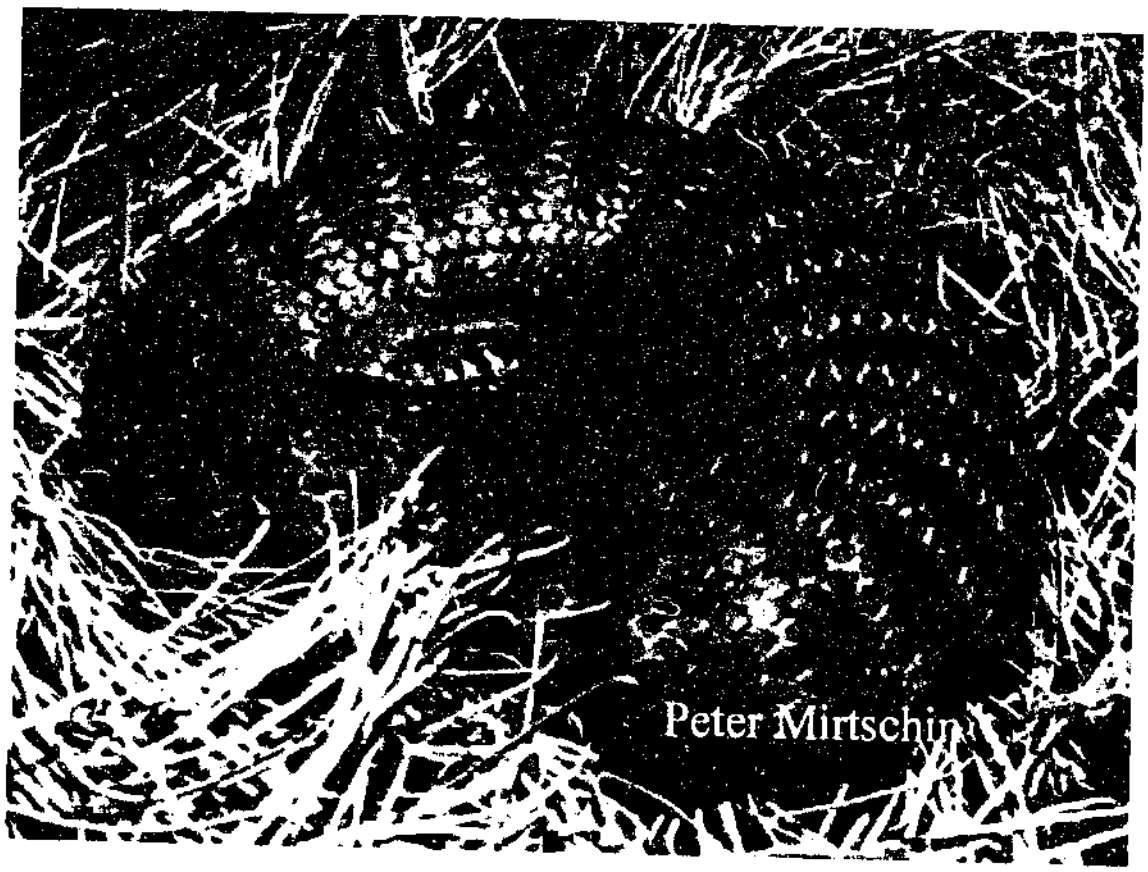
Death adders are usually active only at night. During daylight they use their broad head to burrow beneath sand, gravel or leaves, and lie partially buried. Only part of the snakes back and the tail may be visible offering excellent camouflage for both protection and ambush (Campbell, 1966). Death adders lure their prey by twitching their worm-like tail in front of their head (Chiszar *et al.*, 1990). They feed on lizards, birds, frogs, mice and rats (Shine, 1980). Unlike other elapids, female death adders sexually mature at about 42 months of age. They reproduce in alternate years and give birth to some 12 to 24 live young at a time (Shine, 1980; Sutherland, 1994).

Generally, death adders are very sluggish in movement and, unlike other Australian snakes, tend not to escape when approached by people (Campbell, 1966). This behaviour has given rise to their other name, "deaf" adders. It has been observed that death adders do not strike until touched (Kellaway & Eades, 1929; Campbell, 1966). However, when provoked they flatten their body and strike with amazing speed, and tend to hang on. In captivity, death adders tend not to strike at inanimate objects and hence deplete their venom supply (Sutherland & Tibballs, 2001). However, some species such as *A. pyrrhus* have been observed to engage in cannibalism in captivity (Hoser, 1998).

Biting Apparatus and Envenomation

The death adder possesses a most effective and unique biting apparatus for an elapid, with fangs averaging 6.2 mm in length, and to a maximum of 8.3 mm (Fairley, 1929a).

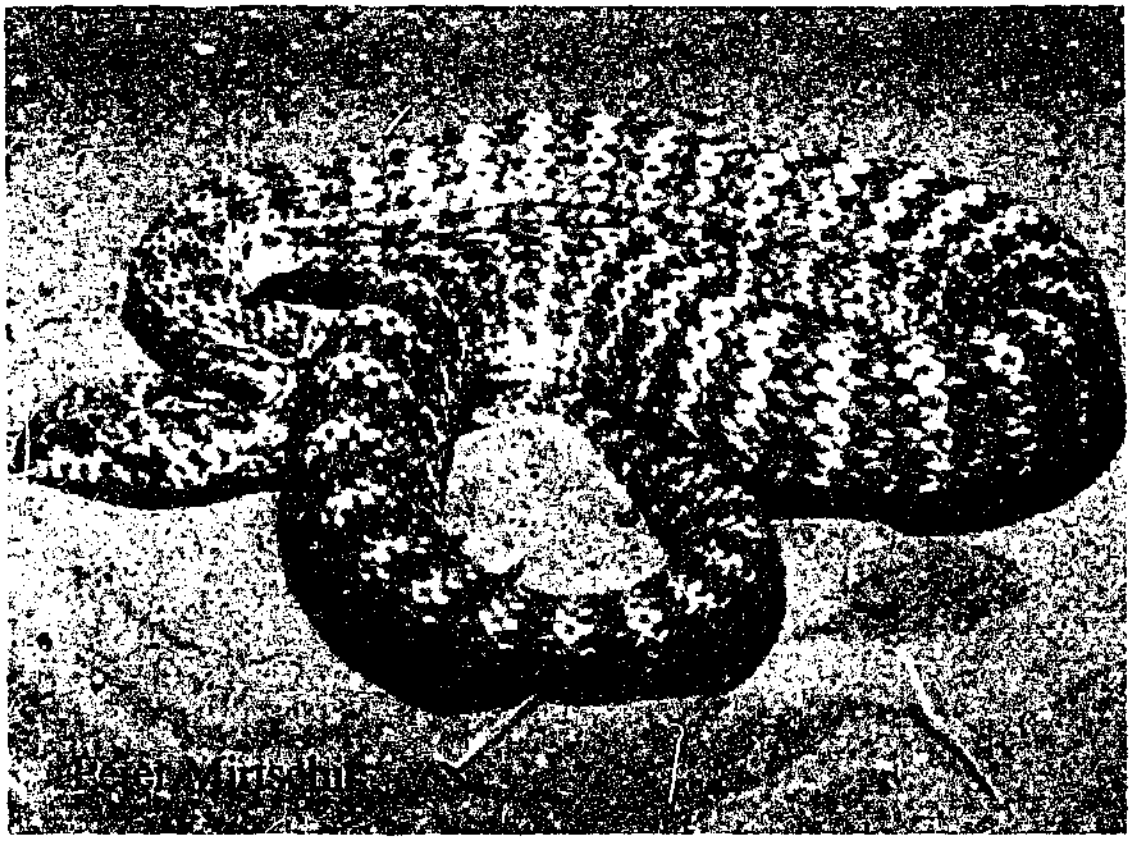
(a)



(b)

Fig. 1.1 (a) Photo of *A. antarcticus* (common death adder) and (b) a map of Australia showing its distribution (adapted from Cogger, 2000).

(a)



(b)

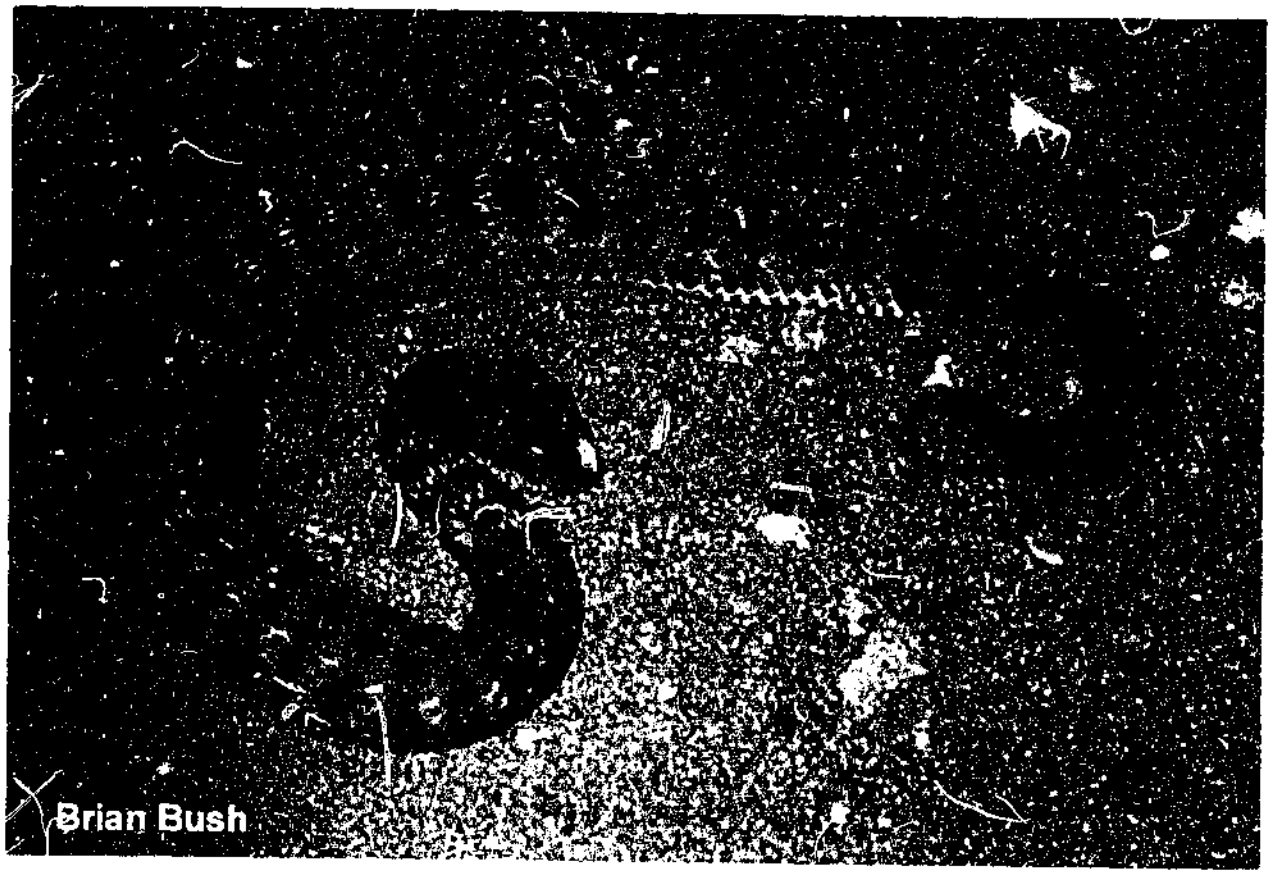
Fig. 1.3 (a) Photo of *A. pyrrhus* (desert death adder) and (b) a map of Australia showing its distribution (adapted from Cogger, 2000).

(a)

(b)

Fig. 1.2 (a) Photo of *A. praelongus* (northern death adder) and (b) a map of Australia showing its distribution (adapted from Cogger, 2000).

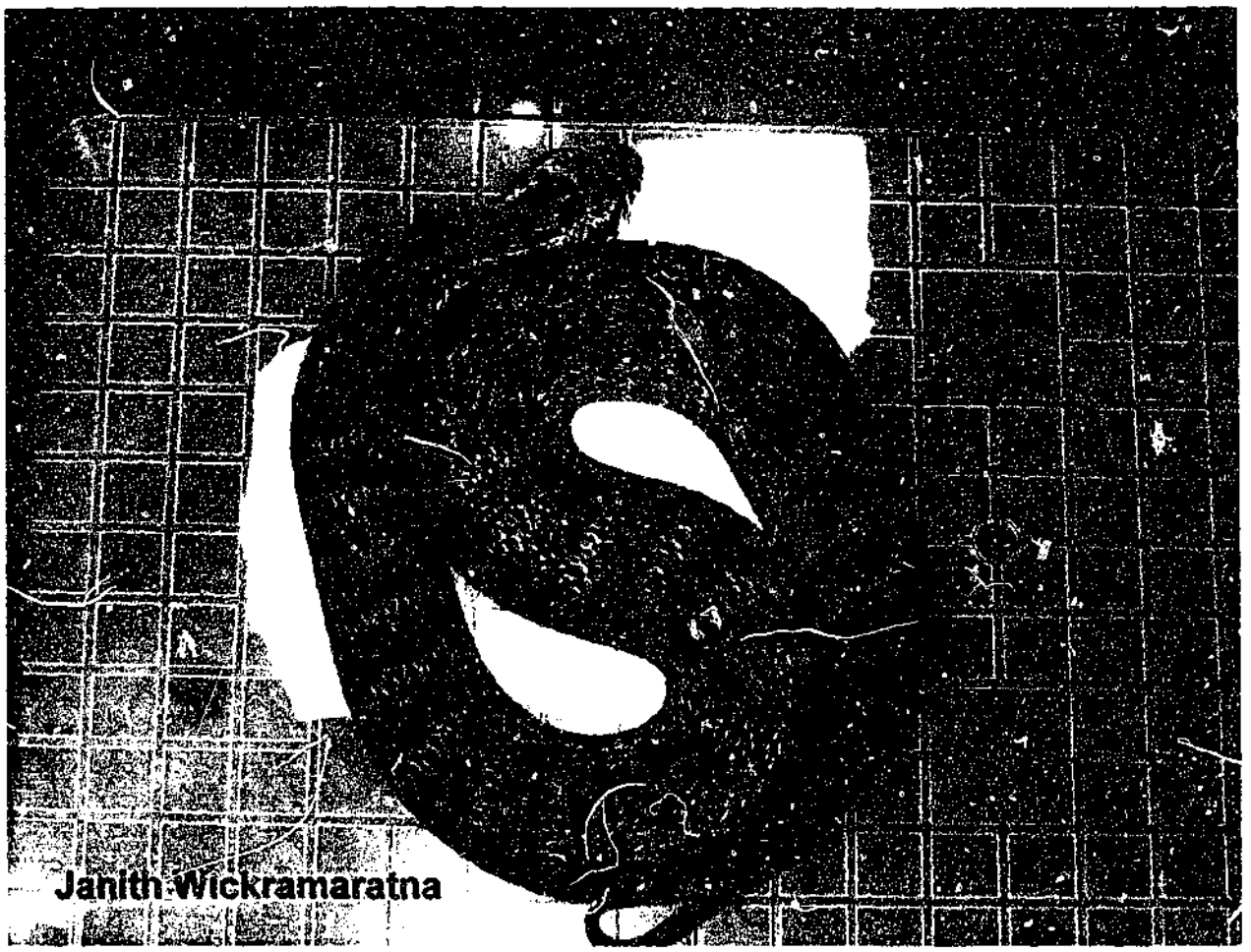
(a)



(b)

Fig. 1.4 (a) Photo of *A. wellsi* (Pilbarra death adder; also called black head death adder) and (b) a map of Australia showing its distribution (adapted from Aplin & Donnellan, 1999).

(a)



(b)

Fig. 1.5 (a) Photo of *A. hawkei* (Barkly Tableland death adder) and (b) a map of Australia showing its distribution (adapted from Wüster *et al.*, unpublished).

(a)

(b)

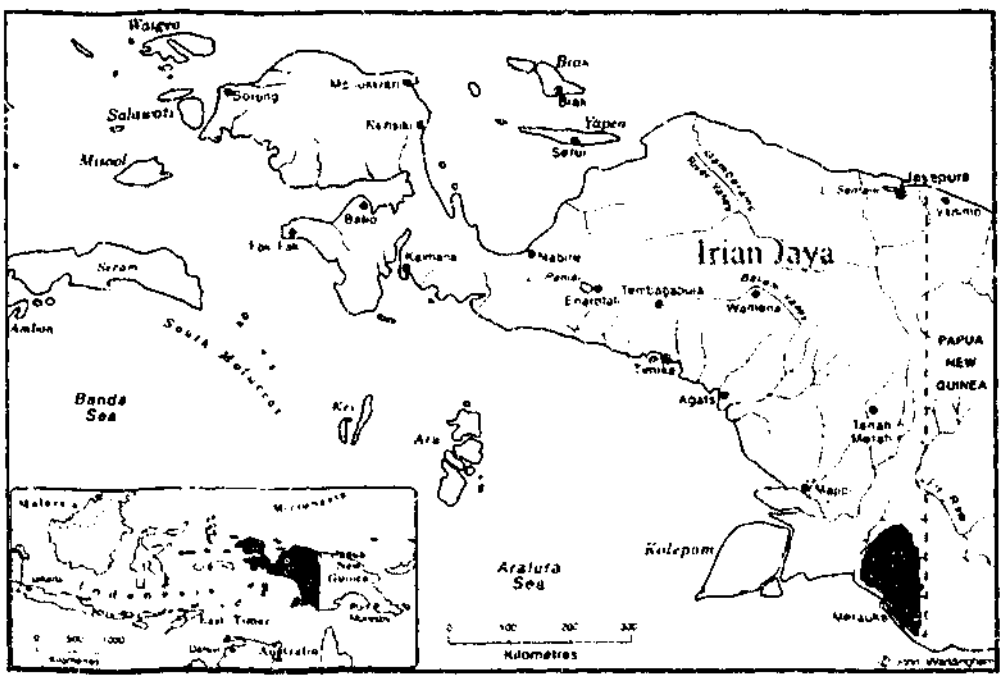
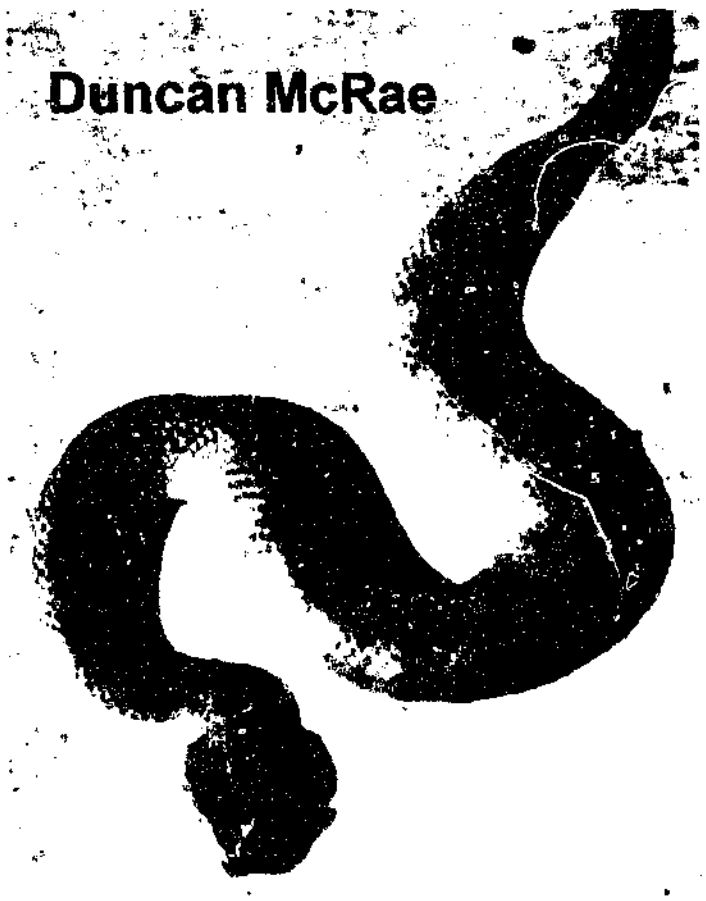


Fig. 1.6 (a) Photo of *A. rugosus* (Irian Jayan death adder) and (b) a map of Irian Jaya (formerly West Papua) showing its distribution (adapted from Wüster *et al.*, unpublished).

(a)



(b)

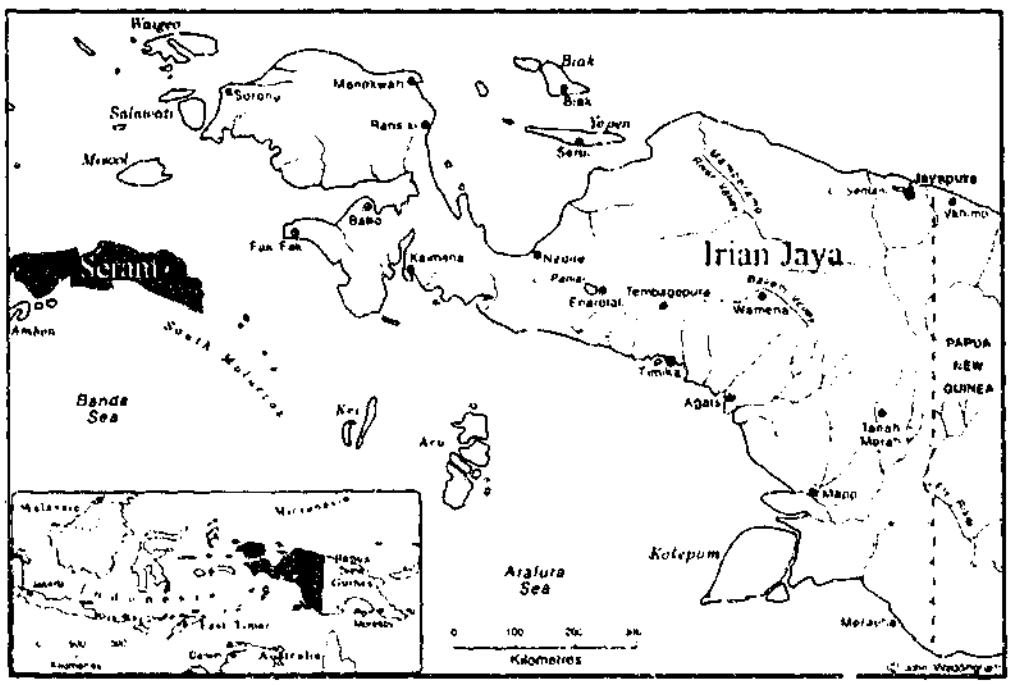


Fig. 1.7 (a) Photo of *A.* sp. Seram and (b) a map of Seram showing its distribution (adapted from Wüster *et al.*, unpublished).

These fangs can move forwards and upwards of the maxillary bone to strike the prey at a perpendicular angle. This allows deeper penetration and better venom deposition (Fairley, 1929a). Fairley (1929a) categorically states that the death adder ranks number one among Australian snakes for efficiency of biting and venom delivery.

Between 1910 and 1926, 195 people died from snake envenomations in Australia (Tidswell, 1906; Fairley, 1929b). Of the 190 cases of snake envenomations reported by Tidswell (1906), 10 were caused by death adders. With 5 of these victims dying, Fairley (1929a) estimated a 50% mortality rate for death adder envenomation. In an excellent study, 15 cases of death adder envenomations from Papua New Guinea were reported by Campbell (1966). He found that almost all bites were on the extremities of the victims, mainly on the foot and ankle. Due to excellent camouflage of the death adder only 1 of the victims saw the snake before being struck and 5 of them stepped on the snake. Eleven patients were given antivenom, and all survived (Campbell, 1966).

Presently, deaths due to snake bites are a rare occurrence in Australia. This is due to a number of factors including the availability of a range of monovalent antivenoms, increased awareness of correct first-aid procedures, better retrieval services and intensive-care medicine (White, 1998b). In the case of death adders, habitat destruction has markedly reduced their presence in populated areas decreasing the probability of a chance encounter (Covacevich *et al.*, 1998; Reed & Shine, 2002). Despite these changes, between 1981 and 1991 approximately 18 deaths due to snakebites were reported in a study carried out by the Commonwealth Serum Laboratories (Sutherland, 1992). However, only 1 of these deaths was due to death adder envenomation. Between the period 1992 to 1994 twelve deaths due to snake bite were reported with 1 death being due to a death adder bite (Sutherland & Leonard, 1995). Out of the 6 deaths recorded by the Australian Venom Research Unit in 1998, 1 death occurred in Western Australia due to a death adder bite (Sutherland &

Tibballs, 2001). Presently, it is estimated that up to 1000 – 3000 snake envenomations occur in Australia per year (White, 1998a; Currie, 2000). Of these about 100 – 500 cases require antivenom treatment and on average 2 deaths per year result (White, 1998a). Unlike in Australia, snake envenomations are still a serious medical problem in Papua New Guinea with an incidence as high as 526 per 100,000 people in some areas (Currie *et al.*, 1991; Lalloo *et al.*, 1995b). Between March 1990 and June 1992, 335 patients with snake bites presented to the Port Moresby General Hospital (Lalloo *et al.*, 1995a; Lalloo *et al.*, 1996). Other than Papuan taipans, death adders cause the most number of envenomations in the Central Province and National Capital District of Papua New Guinea, and are responsible for approximately 10% (i.e. 32 cases) of envenomed patients admitted to Port Moresby General Hospital (Lalloo *et al.*, 1994; Lalloo *et al.*, 1995a; Lalloo *et al.*, 1996). Of the 32 cases, 18 patients had signs of envenoming, and 17 of these patients were affected by neurotoxicity (Lalloo *et al.*, 1996). Thirteen patients were treated with death adder antivenom, and 5 patients required intubation and mechanical ventilation. All of the 32 patients survived following treatment.

Early clinical symptoms of envenomation by *Acanthophis* spp. include headache, vomiting, drowsiness and abdominal pain. In addition, there may be severe pain in the lymph nodes which drain the bite site (Campbell, 1966; Lalloo *et al.*, 1996). With paralysis of bulbar and occular muscles, swallowing becomes difficult and vision becomes blurry (Fairley, 1929b; Campbell, 1966). Ptosis indicates the earliest sign of the venom acting on the nervous system (Campbell, 1966). Death occurs through inhibition of respiration due to paralysis of the voluntary muscles. The neurological symptoms appear between 1 and 13 (median 3.5) hours after the bite (Lalloo *et al.*, 1996). Lalloo *et al.* (1996) reported that one patient developed renal failure after delayed presentation, 5 days after the bite. This patient had an elevated creatinine level of 1790 µmol/l on admission. Furthermore, two thirds of

death adder envenomed patients presenting to the Port Moresby General Hospital had significantly elevated creatine kinase levels (median of 411 IU/l, range of 164 - 4220 IU/l). In some envenomed patients, laboratory studies indicated mild prolongation of prothrombin and thromboplastin times (Lalloo *et al.*, 1996). In another clinical study, 2 out of 6 patients envenomed by death adders in Papua New Guinea had electrocardiographic abnormalities (Lalloo *et al.*, 1997). One patient developed second degree atrioventricular block (type II) and the other had septal T wave inversion. The patient with the second degree atrioventricular block also had elevated cardiac troponin T, which is thought to be indicative of myocardial damage.

Whole Venom Studies

A. antarcticus is known for producing large quantities of venom with an average yield upon milking of 84.7 mg and a maximum of 235.6 mg (Fairley & Splatt, 1929). It is capable of delivering 41.95 ± 16.13 mg of venom in the first feeding bite (Morrison *et al.*, 1983). Although no studies have been published on venom yields from other death adder species, Table 1.2 has been compiled from data kindly provided by Mr. Peter Mirtschin (Venom Supplies Pty. Ltd.). It should be noted that this data was generated for commercial record keeping purposes and may not be truly indicative of venom yields. Indeed, venom yields are also affected by the size of the snakes held in captivity (Fairley & Splatt, 1929). However, from the data available we can observe that *A. pyrrhus* yields a lot less venom upon milking compared to *A. antarcticus* geographic variants or *A. praelongus*. As previously mentioned, *A. pyrrhus* is among the smallest of the death adder species.

Table 1.2 Death Adder Venom Yield[†]

Species	Length of Snakes (cm)	Average Venom Yield (mg)	Maximum Venom Yield (mg)	Minimum Venom Yield (mg)
A. antarcticus (NSW)	30 – 50	38.0	119.6	2.4
A. antarcticus (Qld)	30 – 50	47.3	265.0	0.9
A. antarcticus (SA)	30 – 50	60.9	162.5	6.5
A. praelongus	30 – 50	35.4	311.0	1.4
A. pyrrhus	30 – 40	9.4	21.0	2.0

[†] Data from Mr. Peter Mirtschin (Venom Supplies Pty. Ltd.).

Fairley (1929a) found that when tested milligram for milligram on sheep, *A. antarcticus* venom was 10 times more potent in causing death than Indian cobra (*Naia tripudians*) venom. Taking into account a "certainly lethal dose" of death adder venom of 0.025 mg/kg (s.c.) for sheep and the average venom yield, Fairley (1929a) estimated that a single milking would suffice to kill 84 sheep. Kellaway (1929a) studied the certainly lethal dose of *A. antarcticus* venom in a number of species (Table 1.3). He found that cats, rats and mice were more resistant than monkeys, rabbits and guinea-pigs to the venom (Kellaway, 1929b). Horses and sheep were the most susceptible to the toxicity of venom (Fairley, 1929b; Kellaway, 1929b). Post mortem findings found that haemorrhages were present in the lungs of horses, rabbits, guinea-pigs, cats, rats and mice, but not in monkeys. In addition, the venom was haemolytic in the horse and slightly haemolytic in the rat (Kellaway, 1929b). Kellaway (1929b) showed that intravenous injections of high concentrations of *A. antarcticus* venom (equivalent to 3 - 1000 times the certainly lethal dose) in rabbits, guinea-pigs and mice failed to produce thrombosis. In *in vitro* models the

venom was found to have no coagulant action and only feeble anticoagulant action (Kellaway, 1929a). Most animals, if not all, died from neurotoxicity causing respiratory failure. The certainly lethal dose for man was estimated to be between 0.025 mg/kg and 0.15 mg/kg (Kellaway, 1929b). More recently, in a comparative study of the toxicity of 25 snake venoms, Broad *et al.* (1979) ranked *A. antarcticus* venom as the ninth most potent venom with a murine LD_{50} of 0.338 mg/kg (s.c. in 0.1% bovine serum albumin in saline). Sutherland (1994) considered *A. antarcticus* to be the fifth most venomous Australian land snake after taking into account the toxicity and quantity of the venom produced.

Table 1.3 Certainly Lethal Dose of *A. antarcticus* Venom*

Species	Intravenous (mg/kg)	Subcutaneous (mg/kg)
Horse	–	0.04†
Monkey	–	0.15†
Cat	–	0.5†
Rabbit	0.12	0.15
Guinea-pig	0.06	0.15
Rat	0.2	0.4
Mouse	0.4	0.7

* Adapted from Kellaway (1929a).

† Approximate values.

Kellaway *et al.* (1932) found the neurotoxic action of *A. antarcticus* venom to be due to a peripheral curare-like neuromuscular block. More recently, *in vitro* studies using the

chick biventer cervicis nerve-muscle preparation showed that *A. antarcticus*, *A. praelongus* and *A. pyrrhus* venoms at bath concentrations of 3 – 10 μg/ml cause neurotoxicity through antagonist activity at postsynaptic nicotinic acetylcholine receptors (Wickramaratna & Hodgson, 2001). The same study ranked the three venoms in terms of neurotoxicity in both the chick biventer and mouse phrenic nerve-diaphragm preparations as following: *A. antarcticus* ≥ *A. pyrrhus* > *A. praelongus*. *A. antarcticus* venom displayed no myotoxic activity in monkeys and failed to produce myoglobinuria in mice (Mebs & Samejima, 1980; Sutherland *et al.*, 1981). However, in *in vitro* studies using the chick biventer cervicis nerve-muscle preparation, *A. praelongus* venom (30 μg/ml) initiated a significant direct contracture of muscle (i.e. rise in baseline) (Wickramaratna & Hodgson, 2001), which is indicative of myotoxic activity (Harvey *et al.*, 1994). In the same study, neither *A. antarcticus* nor *A. pyrrhus* venoms showed any myotoxic activity. Further studies based on histological analysis are required for definitive proof of the myotoxicity of *A. praelongus* venom.

A. antarcticus venom was found to be an incomplete prothrombin activator as Factor V was required to convert prothrombin to thrombin (Denson, 1969; Marshall & Herrmann, 1983). In a more recent *in vitro* study, it was found that *A. antarcticus*, *A. praelongus* and *A. pyrrhus* venoms possess anticoagulant activity (van der Weyden *et al.*, 2000). *A. praelongus* venom was found to have potent anticoagulant activity with this venom capable of prolonging the coagulation of the plasma for twice as long as *A. antarcticus* venom. Tan and Ponnudurai (1990) compared the enzymatic activity of *A. antarcticus* venom with other Australian elapid venoms. They found that *A. antarcticus* venom contains very low protease and phosphodiesterase activity. Although the L-amino acid oxidase activity of *A. antarcticus* venom was low compared to *Pseudechis australis* (King Brown snake) venom the acetylcholinesterase activity of *A. antarcticus* venom was higher (Tan & Ponnudurai,

1990). High phospholipase A activity was detected in this venom but was comparable to other Australian elapid venoms. *A. antarcticus* venom also had high hyaluronidase activity compared to other Australian elapid venoms.

Phospholipase A_2

Phospholipases are found in many Australian elapid venoms (Fry, 1999). These esterolytic enzymes cleave phospholipids and, depending on the site at which they hydrolyse ester bonds of 3-*sn*-phosphoglycerides, are classified as either phospholipase A_1, A_2, C or D (Harris, 1991; Kini, 1997). Phospholipase B is thought to hydrolyse lysophosphatides (Harris, 1991). Almost all snake venom phospholipases belong to the phospholipase A_2 (PLA_2) class. PLA_2 enzymes specifically hydrolyse the *sn*-2 ester bond of phosphoglycerides (van Deenen & de Haas, 1963). PLA_2s are generally classified as either intracellular or extracellular forms (Arni & Ward, 1996). Intracellular PLA_2s are involved in membrane homeostasis, phospholipid metabolism and other physiological processes (Mukherjee *et al.*, 1994; Kini, 1997). Extracellular PLA_2s are rich in mammalian pancreatic juices and snake venoms.

Over 150 extracellular PLA_2s have been isolated and sequenced (Danse *et al.*, 1997). These are further divided into four groups based on their primary structure (Kini, 1997). However, all members of these groups require Ca^{2+} as a cofactor for their enzymatic activity (Scott, 1997). Group I PLA_2 enzymes are found in mammalian pancreatic juices, elapid and sea snake venoms. These enzymes usually have 115 – 120 amino acid residues with 7 disulfide bonds (Kini, 1997). Group I PLA_2 enzymes are further divided into group IA and group IB. Group IA PLA_2 enzymes are from elapid and sea snake venoms, while group IB PLA_2 enzymes contain an additional pancreatic loop. Group II PLA_2 enzymes are

found in viperid and crotalid snake venoms (Heinrikson *et al.*, 1977; Kini, 1997). These PLA_2s contain 120 – 125 amino acid residues and 7 disulfide bonds. While these PLA_2s lack the elapid and pancreatic loops they have an additional C-terminal tail (Heinrikson *et al.*, 1977). Group II PLA_2 enzymes are also further divided into group IIA and group IIB. Group IIA PLA_2s contain an aspartate residue at the 49th position of their amino acid sequence. In contrast, group IIB PLA_2s contain Lys-49 instead of Asp-49 (Dhillon *et al.*, 1987; de Sousa *et al.*, 1998). It is thought that the group IIB PLA_2s do not bind to Ca^{2+} as effectively as Asp-49 containing PLA_2s, as Asp-49 is critically involved in Ca^{2+} binding (Maraganore *et al.*, 1984; Li *et al.*, 1994). However, the isolation of a group II PLA_2 containing a Ser-49 from *Echis carinatus sochureki* suggests that Asp-49 is not an absolute requirement for enzymatic activity (Polgar *et al.*, 1996). Group III PLA_2s are found in bee, Gila monster and Mexican bearded lizard venoms (Scott, 1997). These PLA_2s are glycoproteins containing 130 – 135 amino acid residues and do not share high identity with group I or II PLA_2s (Kini, 1997). Group IV PLA_2s include the conodipine-M from cone snail (*Conus magus*) venom (McIntosh *et al.*, 1995). This PLA_2 is made up of two chains, containing 77 and 42 amino acid residues, respectively (McIntosh *et al.*, 1995). Although the PLA_2 activity is Ca^{2+} dependent, conodipine-M does not share high identity in terms of primary structure with PLA_2s from other groups.

Elapid venom PLA_2 enzymes can induce many pharmacological activities including neurotoxicity, myotoxicity, cardiotoxicity, anticoagulant effects, initiation or inhibition of platelet aggregation, hypotension and haemolytic activity (Kini & Evans, 1989; Gutierrez & Lomonte, 1995; Kini, 1997). Studies have been done to determine whether the enzymatic activity correlates with the pharmacological potency. One problem is that the conditions and assays used to determine the enzymatic activity vary greatly between laboratories. However, when enzymatic activity of PLA_2s was compared with

pharmacological potency no significant correlation was found (Rosenberg, 1997a). Although it was generally considered that basic PLA_2s were more lethal than acidic or neutral PLA_2s this was found to be untrue when the isoelectric points were compared with mouse LD_{50} values (Dhillon *et al.*, 1987; Rosenberg, 1997a). In addition to the enzymatic activity, it was postulated that PLA_2 toxins may bind to specific target proteins to affect their pharmacological activity (Kini & Evans, 1989). Recently, two receptors were identified that bind secretory PLA_2s such as OS_1 and OS_2 from coastal taipan (*Oxyuranus scutellatus scutellatus*) venom (Lambeau *et al.*, 1989; Lambeau *et al.*, 1990; Lambeau *et al.*, 1991a,b; Lambeau *et al.*, 1994; Lambeau *et al.*, 1995). These were the N-type receptor in neurons, that bound neurotoxic PLA_2s, and the M-type receptor first identified in rabbit skeletal muscle cells using ^{125}I-labelled OS_2 (Lambeau *et al.*, 1989; Lambeau *et al.*, 1991a,b). Studies have also shown that some PLA_2s (eg. taipoxin) bind to both receptors with similar affinity while others (eg. the non-neurotoxic OS_1) bind preferentially to the M-type receptor (Lambeau *et al.*, 1989; Lambeau *et al.*, 1990). Notexin and *Naja mossambica mossambica* (CM-III) bind preferentially to the N-type receptor.

Death adders were thought to be unique among elapids in lacking PLA_2 components in their venoms (Fry, 1999). However, several PLA_2 components have been isolated from *Acanthophis* venoms. Acanthin I and II are PLA_2 components isolated from *A. antarcticus* venom (Chow *et al.*, 1998). Acanthin I has a PLA_2 activity of 51.57 ± 1.30 μmol phosphatidylcholine hydrolysed/min/mg and acanthin II has a specific activity of 46.85 ± 2.90 μmol/min/mg. Both acanthin I and II are basic with p*I*s of 10.2 ± 0.1 and 10.4 ± 0.1, respectively. Acanthin I and II have molecular masses of 12844.6 ± 0.6 and 12895.6 ± 0.5 daltons by mass spectrometry. These components have 118 – 119 amino acid residues and 7 disulfide bridges (Table 1.4). Pharmacologically, both acanthin I and II are potent inhibitors of collagen-induced platelet aggregation with IC_{50} values of 7 nM and 4 nM,

respectively (Chow *et al.*, 1998). Three additional antiplatelet components were isolated from *A. praelongus* venom. Praelongin 2bIII, 2cII and 2cIV have PLA_2 activities of 31.4 ± 0.4, 326.1 ± 10.2 and 362.5 ± 12.0 μmol/min/mg, respectively (Sim, 1998). The size of these components is similar to the acanthins with molecular masses of 12782.9 ± 2.6, 12971.4 ± 4.5 and 12971.9 ± 3.6 daltons for praelongin 2bIII, 2cII and 2cIV, respectively. Praelongin 2bIII, 2cII and 2cIV are also basic components with p*I*s of 10.3 ± 0.3, 9.4 ± 0.6 and 9.6 ± 0.6, respectively. Interestingly, PLA_2 activities of these components did not correlate with the antiplatelet activity. However, the IC_{50} values of 0.65, 180 and 55 μM for the antiplatelet activities of praelongin 2bIII, 2cII and 2cIV, respectively, correlated well with their respective p*I* values (Sim, 1998).

Acanthoxin is a 13 kDa component that exists in two isoforms, acanthoxin A1 and A2 (van der Weyden *et al.*, 1997). It is a basic PLA_2 with a p*I* of 8.0 and specific activity of 23.93 ± 1.18 μmol of phospholipid hydrolysed/min/mg. While the full sequence of acanthoxin A1 (Hains *et al.*, 1999; van der Weyden *et al.*, 2001) has been determined, only a partial sequence of acanthoxin A2 is available (Table 1.4). While acanthoxin A1 is similar in molecular mass and sequence to acanthin II (i.e. 96% identity with acanthoxin A1) it is thought to be a neurotoxic PLA_2 component (van der Weyden *et al.*, 1997; van der Weyden *et al.*, 2001). While it has high sequence identity to pseudexin A (77%), a neurotoxic PLA_2 component from red-bellied black snake (*P. porphyriacus*), it has low toxicity with a LD_{100} value of 3.2 mg/kg (*s.c.*) in mice. Apart from lethality studies, no specific pharmacological studies have been done on acanthoxin A1 to confirm its activity at the neuromuscular junction.

Acanthoxin B and acanthoxin C are two additional PLA_2 components isolated from *A. praelongus* venom and *A. pyrrhus* venoms, respectively (van der Weyden *et al.*, 2000).

Both acanthoxin B and acanthoxin C have lower PLA_2 activity than acanthoxin A with specific activities of 4.0 ± 0.2 and 13.7 ± 0.7 μmol of phospholipid hydrolysed/min/mg, respectively. No functional studies were done on these partially sequenced PLA_2 components.

Table 1.4 Amino Acid Sequences of PLA_2 Components isolated from Death Adder Venoms

PLA_2 Components	**Amino Acid Sequence**
Acanthin I[a]	DLFQFGGMIG CANKGARSWL SYVNYGCYCG WGGSGTPVDE LDRCCQIHDN CYGEAEKKQC GPKMTSYSWK CANDVPVCND SKSACKGFVC DCDAAAAKCF AKAPYNKNNI GIGSKTRCQ
Acanthin II[a]	NLYQFGGMIQ CANKGARSWL SYVNYGCYCG WGGSGTPVDE LDRCCQIHDN CYGEAEKKRC GPKMTLYSWE CANDVPVCNS KSACEGFVCD CDAAAAKCFA KAPYNKNNIG IGSKTRCQ
Acanthoxin A1[b]	NLYQFGGMIQ CANKGARSWL SYVNYGCYCG WGGSGKPVDE LDRCCQIHDN CYGEAEKKGC GPKMTLYSWK CANDVPVCNS KSGCEGFVCD CDAAAAKCFA KAPYNKNNIG IGSKTRCQ
Acanthoxin A2[c]	DLFQFGGMIG CANKGARSWL SYVNYGCYCG WG
Acanthoxin B[d]	DLFQFGFMIQ CANKGSRPVF
Acanthoxin C[d]	NLFQFGGMIG CANKGTRSWL SYVNYGCYCG
Praelongin 2bIII[e]	Not determined
Praelongin 2cII[e]	Not determined
Praelongin 2cIV[e]	Not determined

[a] Chow *et al.* (1998); [b] van der Weyden *et al.* (2001); [c] van der Weyden *et al.* (1997); [d] van der Weyden *et al.* (2000); [e] Sim (1998)

Neurotoxins

Neurotoxins are extremely important components of snake venom as they are useful in both the capture of prey and in defence. They cause rapid paralysis of the voluntary muscles and, hence, inhibit movement and respiration. This ensures effective capture of prey with the least chance of injury to the snake. Snake neurotoxins act peripherally and do not pass the blood-brain barrier due to their relatively large size (Tu, 1996). Depending on the site of action snake neurotoxins are generally classified as either postsynaptic or presynaptic (Fig. 1.8).

Postsynaptic Neurotoxins

Postsynaptic, or α-neurotoxins, are antagonists of the nicotinic acetylcholine receptor on the skeletal muscle. They are widely referred to as 'curare-mimetic toxins' due to their similarity in action to the competitive nicotinic acetylcholine receptor antagonist d-tubocurarine (Endo & Tamiya, 1991). These neurotoxins bind with high affinity (K_D = 10^{-12} – 10^{-9} M) and specificity to acetylcholine binding sites on skeletal muscle nicotinic receptors (Changeux *et al.*, 1970). The skeletal muscle nicotinic receptor is a heteropentameric protein consisting of five membrane spanning subunits with the stoichiometry of 2α1, 1β1, 1γ and 1δ (Sáez-Briones *et al.*, 1999; Paterson & Nordberg, 2000). The receptor consists of two acetylcholine binding sites located at the interfaces between the α1 and γ, and the α1 and δ chains (Pedersen & Cohen, 1990; Sáez-Briones *et al.*, 1999). Given that these binding sites interact in a positively co-operative manner, by occupying one or both sites snake α-neurotoxins inhibit the opening of the ion channel associated with the receptor in response to cholinergic agonists (Paterson & Nordberg,

2000). This blocks nicotinic transmission in skeletal muscle and causes paralysis in envenomed prey.

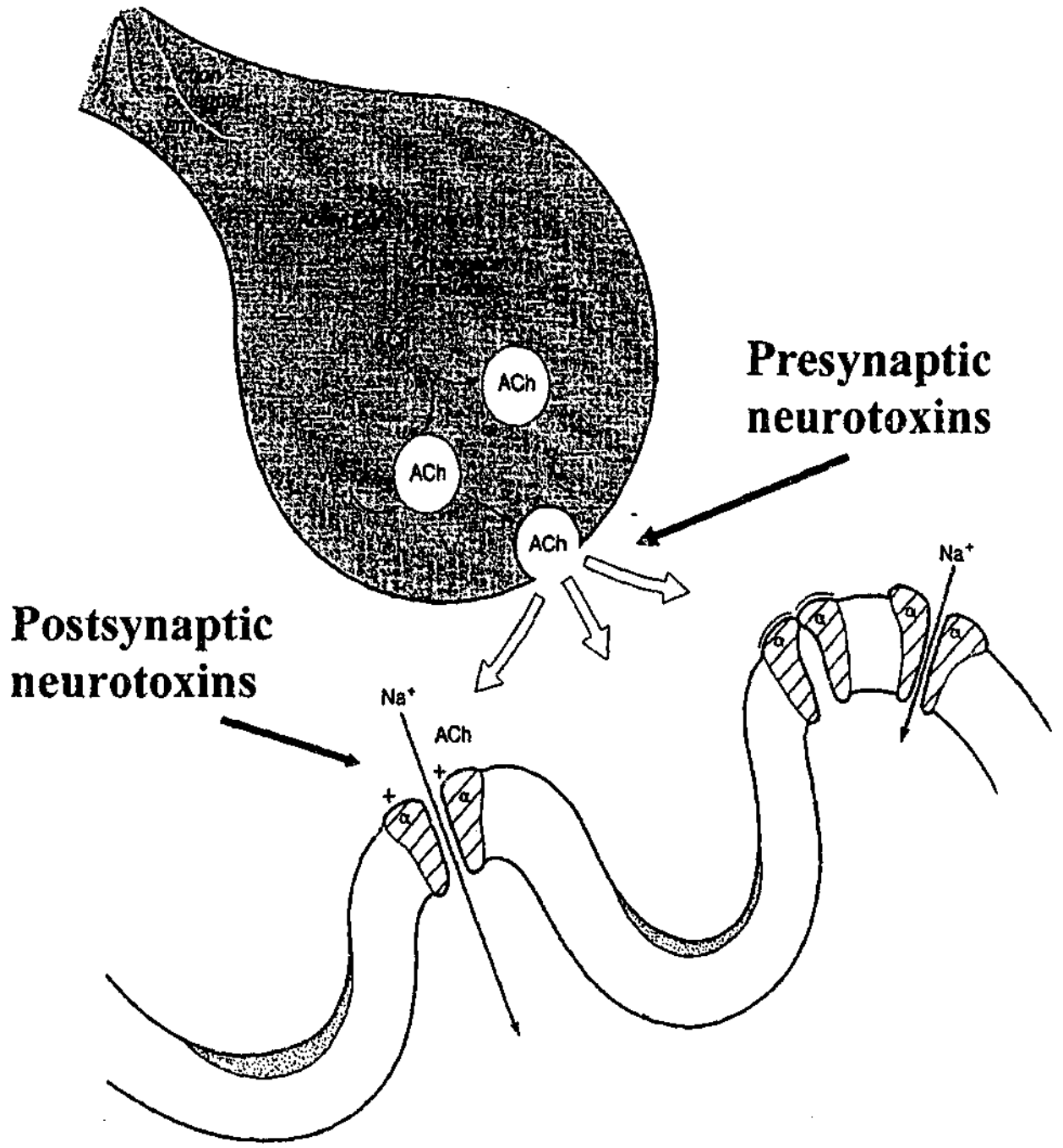


Fig. 1.8 Diagram illustrating the sites of action of postsynaptic and presynaptic neurotoxins at the neuromuscular junction (adapted from Neal, 1994).

In contrast to β-neurotoxins, α-neurotoxins are found predominately in the venoms of snakes from the families Elapidae and Hydrophiidae (Endo & Tamiya, 1991). However, two α-neurotoxins, namely α-AgTx (Jiang *et al.*, 1987) and DNTx I (Shelke *et al.*, 2002), have been isolated from pit viper *Agkistrodon halys* (Pallas) and viper *Daboia russelli russelli* venoms, respectively. Unlike other α-neurotoxins, DNTx I displays both postsynaptic neurotoxicity and cytotoxicity (Shelke *et al.*, 2002). A full amino acid sequence of DNTx I will be useful in determining the relationship of this toxin to other well known α-neurotoxins from elapid and sea snake venoms. Interestingly, an α-neurotoxin has recently been isolated and characterised from a colubrid (*Coelognathus radiatus*) venom (Fry *et al.*, in press).

To date, more than 100 postsynaptic neurotoxins have been isolated and sequenced from elapid and sea snake venoms (Endo & Tamiya, 1991). Depending on their sequence, postsynaptic neurotoxins are mainly subdivided into short- or long-chain neurotoxins (Dufton & Harvey, 1989). Short-chain neurotoxins consist of 60 to 62 amino acid residues and 4 disulfide bridges (Endo & Tamiya, 1991). Long-chain neurotoxins have 66 to 74 amino acid residues and usually 5 disulfide bridges. While the positions of 4 disulfide bridges are common to both long- and short-chain neurotoxins, the extra disulfide bridge found in long-chain neurotoxins is generally located between Cys-30 and Cys-34 (Endo & Tamiya, 1991). Until recently, the major functional difference between the two types of α-neurotoxins was thought to be in the kinetics of association and dissociation with the skeletal muscle nicotinic acetylcholine receptor (Tsetlin, 1999). It was shown that short-chain neurotoxins tend to associate with the receptor about 6 – 7 fold faster and dissociate 5 – 9 fold faster than long-chain neurotoxins (Chicheportiche *et al.*, 1975). However, it has also been shown that long-chain α-neurotoxins bind to α7 subtype neuronal nicotinic

acetylcholine receptors with higher affinity than short-chain neurotoxins (Servent *et al.*, 1997). While lacking the extra disulfide bridge, *Laticauda colubrina* toxin is considered to be a long-chain neurotoxin based on high sequence homology with other long-chain α-neurotoxins (Endo & Tamiya, 1991; Servent *et al.*, 1997). However, functionally it behaves as a short-chain neurotoxin at α7 subtype neuronal nicotinic acetylcholine receptors (Servent *et al.*, 1997). Therefore, functional classification of α-neurotoxins requires pharmacological characterisation of toxins at both skeletal muscle and neuronal nicotinic acetylcholine receptors.

Based on the amino acid sequence postsynaptic neurotoxins can be classified into a third class of α-neurotoxins called weak toxins or miscellaneous type (Shafqat *et al.*, 1991; Utkin *et al.*, 2001). Weak toxins are also called melanoleuca type since these toxins were first isolated from the *Naja melanoleuca* venom (Carlsson, 1975). These neurotoxins have 62 to 68 amino acid residues and 5 disulfide bridges (Nirthanan *et al.*, 2002). In contrast to long-chain neurotoxins, the fifth disulfide bridge is found in loop I of weak toxins (Utkin *et al.*, 2001). As the name suggests, weak toxins are typically much less toxic than either short- or long-chain neurotoxins (Nirthanan *et al.*, 2003). While the weak toxin (WTX) from *Naja kaouthia* venom was poorly reversible at skeletal muscle nicotinic acetylcholine receptors candoxin from *Bungarus candidus* venom was readily reversible at this receptor (Utkin *et al.*, 2001; Nirthanan *et al.*, 2002). The α-colubritoxin from *Coelognathus radiatus* venom is also a weak toxin that is readily reversible at skeletal muscle nicotinic receptors (Fry *et al.*, in press). Although they are currently a small class of postsynaptic neurotoxins many more weak toxins are likely to be isolated from colubrid venoms.

Many of the amino acid residues of erabutoxin a, a short-chain neurotoxin from the venom of the sea snake *Laticauda semifasciata*, necessary for high affinity binding to skeletal muscle nicotinic acetylcholine receptors have been identified. Using site-directed

mutagenesis, it has been shown that changing any one of Ser-8, Lys-27, Trp-29, Asp-31 or Arg-33 decreased the binding affinity of the toxin by several fold (Pillet *et al.*, 1993). In addition to these amino acid residues, Gln-7, Gln-10, Ile-36, Glu-38 and Lys-47 of erabutoxin a were shown to be important for high affinity binding to the skeletal muscle nicotinic receptor (Trémeau *et al.*, 1995). Therefore, it seems that specific amino acid residues from all three loops of erabutoxin a contribute to the "functional site", including both invariant and variant residues. It has been suggested that the invariant residues form a common "functional core", while the variant residues allow for prey specific high affinity binding (Trémeau *et al.*, 1995; Heatwole, 1999).

Although more than 100 neurotoxins have been isolated and sequenced only a few have undergone extensive pharmacological characterisation. Many toxins have been classified as postsynaptic neurotoxins on the basis of their amino acid sequence and/or observation of flaccid paralysis in mice. While in the past, neurotoxins have been tested in LD_{50} studies (tabulated in Mebs & Claus, 1991), these are becoming increasingly difficult to perform for obvious ethical and regulatory reasons. Other workers have utilised binding experiments using membrane bound nicotinic acetylcholine receptors from *Torpedo* electric organs (Chicheportiche *et al.*, 1975; Gong *et al.*, 1999). While this assay provides important information on postsynaptic neurotoxin binding, it is not possible to determine whether the toxin is an agonist or antagonist at the receptor. However, agonist or antagonist activity can be determined using *in vitro* neuromuscular preparations. As described in Hodgson & Wickramaratna (2002; refer appendix), both the mouse phrenic nerve hemidiaphragm and chick biventer cervicis nerve-muscle preparations have been used extensively to determine the neurotoxicity of snake venoms (Harvey *et al.*, 1994; Crachi *et al.*, 1999). The toad rectus abdominis and chick biventer cervicis preparations contain multiply innervated muscle fibres and, therefore, respond to exogenous nicotinic agonists.

Despite this, these or other such preparations have not been used to determine the pA_2 value of postsynaptic neurotoxins. This is probably due to the widely known fact that the majority of snake postsynaptic neurotoxins, especially the long-chain neurotoxins, undergo almost irreversible binding to the skeletal muscle nicotinic receptor (Chicheportiche *et al.*, 1975; Chang, 1979; Lee, 1979). Given the pseudo-irreversible antagonism displayed by most postsynaptic neurotoxins, Schild plot analysis of concentration response curves to nicotinic agonists in the presence of these neurotoxins is not possible. However, the "modified Lew and Angus" method may be used to generate a reliable estimate of the pA_2 value in these cases (Lew & Angus, 1995; Christopoulos *et al.*, 1999; Christopoulos *et al.*, 2001).

Previously, five postsynaptic neurotoxins have been isolated from *A. antarcticus* venom. Acanthophin a is a short-chain neurotoxin consisting of 8 cysteine residues and 4 disulfide bridges (Sheumack *et al.*, 1979). Although the amino acid sequence has not been determined, the amino acid composition indicates the presence of 63 amino acid residues. The molecular mass of acanthophin a was estimated to be 7700 ± 400 daltons by gel permeation chromatography. The minimum molecular mass was calculated to be 7155 daltons based on the amino acid composition of acanthophin a (Sheumack *et al.*, 1979). This toxin has an approximate LD_{50} of 0.16 mg/kg (*i.p.*) in mice, and blocked postsynaptic nicotinic receptors in the interdigital muscles of the mouse hind limb. The other short-chain neurotoxin isolated from *A. antarcticus* venom is the toxin Aa c. This toxin consists of 9 cysteine residues and 4 disulfide bridges (Kim & Tamiya, 1981a). The amino acid composition and sequence (Table 1.5) of toxin Aa c indicates the presence of 62 amino acid residues, and a molecular mass of 6898 daltons. The lethality (LD_{50}) of this toxin was determined to be 0.08 mg/kg (*i.m.*) in mice. However, no other pharmacological studies have been done on toxin Aa c to confirm its postsynaptic neurotoxicity.

Toxin Aa b, acanthophin d and Aa e are long-chain neurotoxins isolated from *A. antarcticus* venom. All three toxins consist of 10 cysteine residues and 5 disulfide bridges (Kim & Tamiya, 1981b; Sheumack *et al.*, 1990; Tyler *et al.*, 1997). The amino acid sequences (Table 1.5) indicate the presence of 73, 74 and 79 amino acid residues for toxin Aa b, acanthophin d and Aa e, respectively. With most long-chain neurotoxins consisting of 66 – 74 amino acid residues, Aa e has the longest amino acid sequence with 79 residues (Endo & Tamiya, 1991; Tyler *et al.*, 1997). The molecular mass of toxin Aa b was calculated from the amino acid composition to be 8135 daltons. While the molecular mass of acanthophin d was determined to be 8800 ± 200 daltons by gel permeation chromatography (Sheumack *et al.*, 1990), Fry (1999) calculated the molecular mass using the amino acid sequence to be 8387 daltons. Using mass spectrometry the molecular mass of Aa e was determined to be 8752 daltons (Tyler *et al.*, 1997). Once again the molecular mass of Aa e is among the largest for long-chain neurotoxins. Another interesting feature of Aa e is that it has two chromatographic isoforms, namely Aa e1 and Aa e2 (Tyler *et al.*, 1997). With reversed-phase high performance liquid chromatography (RP-HPLC), Aa e1 and Aa e2 elute at two different retention times. However, since both Aa e1 and Aa e2 have the same amino acid sequence they are the same toxin. Furthermore, mass spectrometric analysis showed that both Aa e1 and Aa e2 have the same molecular mass. This peculiar behaviour of Aa e was thought to be due to the presence of Pro-Pro residues in its long C-terminal tail causing it to adopt two different conformations (Tyler *et al.*, 1997). Hence, resulting in two different retention times for Aa e1 and Aa e2.

Out of the three long-chain neurotoxins isolated from *A. antarcticus* venom only acanthophin d has been shown to block postsynaptic nicotinic acetylcholine receptors (Sheumack *et al.*, 1990). It was also shown that the blockade of nicotinic receptors by acanthophin d is only very slowly reversible. In the case of toxin Aa b, lethality studies

indicated a LD_{50} value of 0.13 mg/kg (*i.m.*) in mice. While Aa e was found to be toxic in mice no LD_{50} value was obtained. No postsynaptic neurotoxins have been isolated from any other death adder venom.

Table 1.5 Amino Acid Sequences of Neurotoxins Isolated from Death Adder Venoms

Neurotoxin	Amino Acid Sequence
Acanthophin a	Not determined
Toxin Aa b[a]	VICYRGYNNP QTCPPGENVC FTRTWCDAFC SSRGKVVELG CAATCPIVKS YNEVKCCSTD KCNPFPVRPR RPP
Toxin Aa c[b]	MQCCNQQSSQ PKTTTTCPGG VSSCYKKTWR DHRGTIIERG CGCPRVKPGI RLICCKTDEC NN
Acanthophin d[c]	VICYRKYTNN VKTCPDGENV CYTKMWCDGF CTSRGKVVEL GCAATCPIRK PGNEVKCCST NKCNHPPKRK KRRP
Aa e[d]	VICYVGYNNP QTCPPGGNVC FTKTWCDARC HQLGKRVEMG CATTCPKVNR GVDIKCCSTD KCNPFPKTTP PWKRPRGKP

[a] Kim & Tamiya (1981b); [b] Kim & Tamiya (1981a); [c] Sheumack *et al.* (1990); [d] Tyler *et al.* (1997)

Presynaptic Neurotoxins

Presynaptic neurotoxins are also called β-neurotoxins as they act on the presynaptic terminal, as opposed to α-neurotoxins which act on the postsynaptic side of the neuromuscular junction. Presynaptic neurotoxins are commonly found in Elapidae, Hydrophiidae, Crotalidae and Viperidae venoms (Chang, 1985). It was previously thought that presynaptic neurotoxins are basic proteins, and that this was an important characteristic for presynaptic neurotoxicity (Gubenšek *et al.*, 1997). However, while most β-neurotoxins are basic, several non-basic presynaptic neurotoxins such as agkistrodotoxin from *Agkistrodon halys* (Pallas) venom (Kondo *et al.*, 1989), and Pa-1G and Pa-3 from *Pseudechis australis* venom (Takasaki *et al.*, 1990) have been isolated. Studies have also

shown that presynaptic neurotoxicity is not related directly to the enzymatic activity of these toxins. When mouse intravenous LD_{50} values of known β-neurotoxins were compared with their corresponding enzymatic activity, no significant correlation was observed (Rosenberg, 1997a; Montecucco & Rossetto, 2000). However, it is accepted that PLA_2 activity is of crucial importance for the neurotoxic activity of β-neurotoxins (Križaj & Gubenšek, 2000).

Presynaptic neurotoxins can be classified into subclasses based on their structural organisation (Yang, 1997). One subclass is the single-chain or monomeric neurotoxins, for example notexin from *Notechis scutatus* venom (Halpert & Eaker, 1975; Gubenšek *et al.*, 1997). Two-component neurotoxins include crotoxin from *Crotalus durissus terrificus* venom, and this toxin consists of a basic subunit, crotoxin B, non-covalently bound to the acidic non-toxic crotapotin (Delot & Bon, 1993; Yang, 1997). In contrast to crotoxin, β-bungarotoxin from *Bungarus multicinctus* venom is a two-chain neurotoxin made up of a PLA_2 subunit covalently linked to a non-enzymatic polypeptide (Kondo *et al.*, 1978a,b; Bon, 1997; Yang, 1997). Taipoxin from *Oxyuranus scutellatus scutellatus* venom on the other hand is a multi-chain toxin complex made up of three different subunits bound non-covalently (Fohlman *et al.*, 1976; Fohlman *et al.*, 1977; Yang, 1997). Although the above toxins are structurally different there was no direct relationship between chain structure and potency (Simpson *et al.*, 1993). However, a relationship between chain structure and binding of toxin was observed. While the binding of β-bungarotoxin, taipoxin and textilotoxin to receptors on the mouse hemidiaphragm was poorly reversible, crotoxin was found to be slightly reversible and notexin substantially reversible (Simpson *et al.*, 1993).

A characteristic feature of presynaptic neurotoxicity is that, even at high doses of β-neurotoxins, there is a interval of about 1 hour between injection and death (Fletcher & Rosenberg, 1997; Rosenberg, 1997a; Montecucco & Rossetto, 2000). This was confirmed

in *in vitro* when a 10-fold increase in concentration of paradoxin failed to significantly hasten the inhibition of indirect twitches in the mouse phrenic nerve-diaphragm muscle preparation (Hodgson & Rowan, 1997). In *in vitro* nerve-muscle preparations, presynaptic neurotoxins generally cause a triphasic effect on acetylcholine release (Su & Chang, 1984; Harris, 1991). The three phases are: an initial depression of acetylcholine release followed by enhanced transmitter release and, finally, a gradual failure of transmitter release to complete blockade (Fletcher & Rosenberg, 1997; Rowan, 2001). The initial phase is thought to be due to binding of neurotoxins to the receptor sites (Caratsch *et al.*, 1981; Harris, 1991). As previously mentioned, an N-type receptor in neurons and an M-type receptor in skeletal muscle have been identified and characterised on the basis of binding by PLA_2 toxins such as OS_1 and OS_2 (Lambeau *et al.*, 1989; Lambeau *et al.*, 1991a,b; Lambeau & Lazdunski, 1999). Several other binding proteins for taipoxin (Tzeng *et al.*, 1989), ammodytoxin (Križaj *et al.*, 1994; Križaj *et al.*, 1995), crotoxin (Hseu *et al.*, 1999) and β-bungarotoxin (Breeze & Dolly, 1989) have also been detected and characterised (Križaj & Gubenšek, 2000). This initial inhibitory phase is seen in frog preparations (Alderdice & Volle, 1981; Caratsch *et al.*, 1981) and mouse preparations exposed to low Ca^{2+} (Chang *et al.*, 1977b), but not in rat (Chang *et al.*, 1977a), and perhaps a very subtle or absent effect in chick preparations (Chang & Su, 1982; Mollier *et al.*, 1989; Fletcher & Rosenberg, 1997). It has been shown that during the enhanced transmitter release phase β-bungarotoxin causes a shift of the Ca^{2+} dose response curve to the left (Su & Chang, 1984; Rowan, 2001). However, it is not known whether this is due to a Ca^{2+} influx or an inhibition of Ca^{2+} uptake during acetylcholine release (Rowan, 2001). While the first and second phases are phospholipase-independent in mammalian preparations, the third phase is phospholipase-dependent. Unlike the third phase, the first two phases were present when β-bungarotoxin was treated with the PLA_2 enzyme inhibitor *p*-bromophenacyl bromide

(Abe *et al.*, 1977; Kondo *et al.*, 1978c; Chang & Su, 1982). The triphasic characteristics of presynaptic neurotoxicity are not always present as described above. There seems to be variability in this triphasic response depending on the β-neurotoxin tested and the animal nerve-muscle preparation utilised (Chang & Huang, 1974; Harris, 1991; Rowan, 2001).

The morphological changes induced by presynaptic neurotoxins can be seen from electron microscopy studies. Dramatic reduction in vesicles, presence of clathrin-coated Ω-shaped indentations on the plasma-membrane, damaged mitochondria and swollen axon terminals are prominent features of neuromuscular junctions exposed to β-neurotoxins (Cull-Candy *et al.*, 1976; Gopalakrishnakone & Hawgood, 1984; Dixon & Harris, 1999). To date, the precise mechanism of presynaptic neurotoxicity that accounts for all the pharmacological data and morphological changes has not been described and tested. Recently, a comprehensive model for presynaptic neurotoxicity was proposed by Montecucco and Rossetto (2000). In brief, β-neurotoxins bind to specific receptors on the presynaptic terminal (Lambeau *et al.*, 1989; Lambeau *et al.*, 1991a,b). Montecucco & Rossetto (2000) suggest that the β-neurotoxin-receptor complex then migrate to the presynaptic active zone. Here the toxins enter the lumen of the small synaptic vesicles after these have released their neurotransmitter to the extracellular space. Thereby, β-neurotoxins are internalised into the small synaptic vesicles by endocytosis. The toxins are then thought to hydrolyse the inner membrane of the small synaptic vesicles and so release fatty acids and lysophospholipids. After the protein layer that mediates endocytosis is lost, the ATPase proton pump creates a pH gradient across the membrane of the small synaptic vesicles. This in turn, causes the uptake of neurotransmitter into the synaptic vesicles. Given the acidic environment inside the vesicles the fatty acids are protonated. The protonated fatty acids flip-flop to the outer surface of the vesicles leaving behind the

lysophospholipids inside the vesicles. With fatty acids on the outer surface of the vesicles, these become highly fusogenic (Chernomordik *et al.*, 1995; Chernomordik *et al.*, 1997; Montecucco & Rossetto, 2000). Vesicle fusion then takes place anywhere along the plasma membrane and releases the neurotransmitter. Both fatty acids and lysophospholipids in the fused vesicles prevent further endocytosis and vesicle recycling (Montecucco & Rossetto, 2000). This causes the formation of the Ω-shaped indentations and eventual depletion of vesicles.

As previously mentioned, PLA_2 isomers called acanthoxin A1 and A2 were isolated from *A. antarcticus* venom (van der Weyden *et al.*, 1997). Acanthoxin A1 showed a high degree of amino acid sequence identity with other elapid presynaptic neurotoxins, especially pseudexin A (77%) from the red-bellied black snake (*Pseudechis porphyriacus*) (van der Weyden *et al.*, 1997; Hains *et al.*, 1999; van der Weyden *et al.*, 2001). Based on sequence identity and lethality in mice, acanthoxin A1 was considered to be the first neurotoxic PLA_2 from a death adder venom (van der Weyden *et al.*, 2001). Interestingly, acanthoxin A1 shared highest sequence identity with acanthin II (96%), an antiplatelet component from *A. antarcticus* venom (Chow *et al.*, 1998; van der Weyden *et al.*, 2001). Furthermore, toxicity of acanthoxin A1 was uncharacteristically low for a presynaptic neurotoxin (van der Weyden *et al.*, 1997; Montecucco & Rossetto, 2000). Therefore, the neurotoxic activity of acanthoxin A1 needs to be confirmed in further pharmacological studies.

Myotoxic Phospholipase A_2 Components

Myotoxicity, in the toxinology literature, is broadly defined as structural or functional disruption of muscle tissue as measured by pharmacological, biochemical or pathological

terms in either *in vivo* or *in vitro* conditions (Gopalakrishnakone *et al.*, 1997). To assist structure-function studies and comparisons between myotoxins, Gopalakrishnakone *et al.* (1997) suggest classifying myotoxic PLA_2 components into general myotoxins and local myotoxins. General myotoxins or myoglobinuric myotoxins are those that cause systemic effects away from the site of injection (eg. *P. australis* VIIIA). Local myotoxins or myonecrotic toxins are those that cause muscle damage at the site of application, either in *in vivo* or *in vitro* situations (eg. notexin).

Several pharmacological and biochemical assays have been used to determine the myotoxic activity of isolated components. *In vivo* assays consist of myotoxins being injected via a subcutaneous or an intramuscular route into animals (usually mice) (Harris, 1991). The animals are then sacrificed at a predetermined time point, skeletal muscles removed and examined under the microscope. In the absence of histological examination the detection of myoglobinuria is considered a reliable indicator of myotoxicity (Harris, 1991; Gopalakrishnakone *et al.*, 1997). Some authors have also used the elevation of creatine kinase (CK) as an indicator of myotoxic activity (Sutherland *et al.*, 1981; Mebs & Ownby, 1990; Melo & Ownby, 1996,1999). Others have suggested that CK results should be interpreted with caution since the correlation between the level of CK and the extent of muscle damage is not always supported (Rowland, 1980; Harris, 1991; Fletcher *et al.*, 1997). However, CK measurements are routinely used clinically to diagnose severe muscle diseases. For *in vitro* studies of myotoxicity, the nerve-muscle preparations from mice and especially that of chicks have been utilised (Harvey *et al.*, 1994). Since the integrity of muscle fibres is essential for the directly stimulated twitches, myotoxicity is indicated by the loss of direct twitches. In addition to the inhibition of direct twitches, myotoxins may also cause an increase in baseline tension (Harvey *et al.*, 1994). This contraction is thought to be due to an elevation in Ca^{2+} concentrations within the myoplasm (Fletcher *et al.*,

1997). While the inhibition of direct twitches or a contracture is indicative of myotoxicity, neither provides adequate evidence to confirm this activity (Harris, 1991). Therefore, supplementary histological studies are required to confirm myotoxicity.

Morphological changes are usually evident under light microscopy after about 3 – 6 hours of exposure to myotoxic PLA_2s (Harris, 1991). By 6 hours of exposure to notexin or taipoxin most fibres of the rat soleus muscle were damaged, and by 12 – 24 hours almost every muscle fibre was broken down (Harris *et al.*, 2000). Morphological changes induced by myotoxins typically include vacuolation, delta lesions, interstitial edema, cellular infiltrate, necrotic cells and loss of striation (Harris & Maltin, 1982; Fletcher *et al.*, 1997; Gopalakrishnakone *et al.*, 1997; Ali *et al.*, 2000). At the electron microscopy level, the first signs of myotoxicity are delta lesions, ruptured plasma membrane, clear areas of cytoplasm, swollen mitochondria and dilated sarcoplasmic reticulum (Fletcher *et al.*, 1997; Gopalakrishnakone *et al.*, 1997). Other pathological changes observed are broken sarcomeres and areas of hypercontracted myofilaments. Degeneration of skeletal muscle as a result of exposure to taipoxin lasted for about 48 hours, and regeneration occurred thereafter (Harris & Maltin, 1982). Formation of myotubes occurred by 3 days, immature muscle fibres were seen by 5 days, and complete recovery was noted by 3 weeks (Harris & Maltin, 1982).

Slow-twitch, oxidative and oxidative-glycolytic muscle fibres are thought to be more susceptible to myotoxic PLA_2s than the fast-twitch, glycolytic muscle fibres (Harris, 1991; Ownby *et al.*, 1999). Furthermore, immature muscle fibres are quite resistant to myotoxic PLA_2s in both *in vivo* and *in vitro* conditions (Harris & Johnson, 1978; Harris, 1991). There may also be differences in sensitivity to myotoxins depending on the species tested (Harris, 1985; Mebs & Ownby, 1990). Some studies have found that there is a good correlation between enzymatic activity and toxicity of myotoxic PLA_2 components (Fatehi

et al., 1994; Gopalakrishnakone *et al.*, 1997). Furthermore, others have shown that inhibition of PLA_2 activity with *p*-bromophenacyl bromide causes an inhibition of myotoxic activity (Chen *et al.*, 1994; Gao *et al.*, 2001). However, some studies have found that there is no correlation between the level of PLA_2 activity and myotoxicity (Dhillon *et al.*, 1987; Rosenberg *et al.*, 1989; Rosenberg, 1997b; Ownby *et al.*, 1999). Hence, it is thought that PLA_2 activity plays a less important role in the mechanism of myotoxicity than was earlier thought, at least in some cases (Fletcher & Rosenberg, 1997). This view gained further acceptance with the isolation of Lys-49 PLA_2 myotoxins. Studies showed that Lys-49 PLA_2 myotoxins lacked enzymatic activity on artificial substrates (Soares *et al.*, 2000; Soares *et al.*, 2001). Therefore, it was thought that enzymatic activity was not essential for these toxins to cause myotoxicity. However, recently it was shown that Lys-49 PLA_2 myotoxins are indeed catalytically active on biological substrates (Soares *et al.*, 2002). Therefore, it is still possible that enzymatic activity may play a role in Lys-49 PLA_2 induced myotoxicity. Further research will be required to examine the role of PLA_2 activity in myotoxicity.

In a hypothetical model it was suggested that PLA_2 toxins may target specific proteins rather than the lipid domain to cause myotoxicity (Kini & Evans, 1989). As previously mentioned, an M-type receptor was characterised from rabbit skeletal muscle cells that bound certain PLA_2 components with high affinity (Lambeau *et al.*, 1990; Lambeau & Lazdunski, 1999). In the hypothetical model for PLA_2 induced myotoxicity, proposed by Fletcher *et al.* (1997), the target recognition site would bind with high affinity to proteins such as M-type receptors. The target recognition site would then determine tissue specificity and potency of a myotoxin. The cytolytic site on the PLA_2s disrupts the sarcolemma, and this happens most probably in the presence of enzymatic activity (Fletcher *et al.*, 1997). It is also suggested that fatty acids are produced at the binding sites

by PLA_2 activity. The acylation of fatty acids to membrane proteins would then result in an inhibition of normal protein function and lead to a loss of membrane integrity (Fletcher *et al.*, 1997). Clearly, further studies on myotoxins are required before it can be possible to definitively describe the mechanism of PLA_2 induced myotoxicity.

As previously mentioned (refer Whole Venom Studies), studies have shown that *A. antarcticus* venom has no myotoxic activity in monkeys *in vivo* and fails to produce myoglobinuria in mice (Mebs & Samejima, 1980; Sutherland *et al.*, 1981). However, in *in vitro* studies using the chick biventer cervicis nerve-muscle preparation, *A. praelongus* venom (30 µg/ml) induced a significant direct contracture of muscle (Wickramaratna & Hodgson, 2001), indicating possible myotoxic activity (Harvey *et al.*, 1994). Furthermore, a clinical study reported myotoxic activity following death adder envenomations, in Papua New Guinea, by a species thought to be different to *A. antarcticus* (Lalloo *et al.*, 1996). In this study, two thirds of envenomed patients had significantly elevated creatine kinase levels and one patient developed renal failure following delayed presentation. Therefore, it is possible that some of the death adder venoms may be myotoxic. Further studies based on histological examination are required to confirm any such activity.

Other Constituents of Snake Venoms

Elapid venom cardiotoxins have a similar structure to postsynaptic neurotoxins with the four disulfide bonds in almost identical positions (Dufton & Hider, 1983,1991). However, in terms of pharmacology and amino acid sequence, they are quite different from neurotoxins. Cardiotoxins cause systolic heart arrest and severe tissue necrosis in *in vivo*, and are cytotoxic to cardiomyocytes and erythrocytes *in vitro* (Tzeng & Chen, 1988; Huang *et al.*, 2003). Although no cardiotoxins have been isolated from death adder venoms a

clinical study found that 2 out of 6 patients envenomed by death adders in Papua New Guinea had electrocardiographic abnormalities (Lalloo *et al.*, 1997).

Snake venoms exert many effects on the blood. Elapid venoms are known to have components with procoagulant and anticoagulant activity (Tu, 1996; Fry, 1999). The ratio of procoagulant and anticoagulant components in the venom determines whether the whole venom would display procoagulant or anticoagulant activity, at a particular concentration (Tu, 1996). To date, no components with procoagulant or anticoagulant activity have been isolated from death adder snake venoms. However, several components with antiplatelet activity have been isolated from *A. antarcticus* (acanthin I and II) and *A. praelongus* (Praelongin 2bIII, 2cII and 2cIV) venoms (Chow *et al.*, 1998; Sim, 1998). These components are potent inhibitors of collagen-induced platelet aggregation. Elapid venoms are also known to contain haemolytic factors that act either directly or indirectly. Direct haemolytic factors are highly basic polypeptides, while indirect factors possess PLA_2 activity (Tu, 1996). Other venom components such as cardiotoxins and myotoxins may also induce haemolysis (Fletcher & Jiang, 1993; Tu, 1996).

In addition to the toxins discussed above, venoms contain many other components such as enzymes (eg. hyaluronidase) and nerve growth factor. Hyaluronidase is thought to facilitate toxin diffusion into the tissues, while nerve growth factor potentiates the toxicity of other components (Hider *et al.*, 1991; Tu, 1996).

Antivenom Treatment

Two recent reviews have covered antivenoms broadly (Hodgson & Wickramaratna, 2002; Fry *et al.*, 2003; refer appendix). Here, antivenom studies relevant to death adders will be covered.

Antivenom remains the principal therapy for death adder envenomation (White, 1998a). In Australia, death adder antivenom is prepared by CSL Ltd. from the plasma of horses immunised with common death adder (*A. antarcticus*) venom. Initially, antivenom production ran into problems as high levels of antibodies were not produced by horses immunised with death adder venom. However, with improved immunisation schedules, death adder antivenom first became available for treatment in 1958 (Sutherland & Tibballs, 2001). An ampoule of death adder antivenom consists of 6000 units in an approximate volume of 22 ml. Similarly, CSL polyvalent antivenom contains 6000 units of death adder antivenom. In Australia, the current cost of an ampoule of death adder antivenom is about $869 and that of CSL polyvalent antivenom is about $1440. Campbell (1966) reported that, given in large amounts (i.e. 3 to 6 ampoules of CSL polyvalent antivenom and 3000 to 12000 units of monovalent death adder antivenom), antivenom was very effective in reversing the effects of death adder envenomation in Papua New Guinea. After antivenom treatment the reversal of paralysis was reported to occur within hours (Campbell, 1966). In another study, 13 death adder envenomed patients in Papua New Guinea were treated with antivenom, and all survived (Lalloo *et al.*, 1996). Two patients received more than one ampoule of antivenom. After antivenom treatment, one seriously envenomed patient with respiratory arrest recovered almost completely within 18 hours. Jelinek and Wambeek (1992) reported the first *A. antarcticus* envenomation in Perth, Western Australia. In this case, the patient recovered completely after receiving one ampoule of death adder antivenom. In contrast, another recent envenomation in Australia required three ampoules of death adder antivenom plus anticholinesterase therapy to resolve severe neurotoxicity (Little & Pereira, 2000).

In a recent study, the efficacy of CSL death adder antivenom was examined *in vitro* against the neurotoxicity of *A. antarcticus* (Queensland geographic variant), *A. praelongus*

and *A. pyrrhus* venoms. This study found that CSL death adder antivenom was effective against all three death adder venoms (Wickramaratna & Hodgson, 2001). However, antivenom was significantly less effective against *A. antarcticus* venom compared to the other two venoms. In light of this finding, the efficacy of CSL death adder antivenom should be examined against venoms from other *Acanthophis* species.

Anticholinesterase Therapy

A placebo-controlled, double-blind crossover trial of edrophonium treatment against the neurotoxicity of cobra (*Naja naja philippinensis*) venom suggested that anticholinesterases could be beneficial in the management of snake envenomation (Watt *et al.*, 1986). Following this, neostigmine methylsulphate was administered to a death adder envenomed patient in Papua New Guinea (Currie *et al.*, 1988). Although the patient showed marked improvement of ptosis and chest wall movement, antivenom was later administered. Again in Papua New Guinea another patient who received simultaneous antivenom and neostigmine recovered rapidly (Lalloo *et al.*, 1996). As mentioned above, Little & Pereira (2000) administered neostigmine after three ampoules of death adder antivenom because the hospital had exhausted the supply of antivenom. Once again the anticholinesterase treatment was thought to have a beneficial effect in the management of death adder envenomation. In an *in vivo* rat study neostigmine helped to prolong the survival at only the lower concentrations of *A. antarcticus* venom (Flachsenberger & Mirtschin, 1994). Concomitant anticholinesterase treatment has been suggested as a means to reduce the costs involved with antivenom therapy in Papua New Guinea (Currie *et al.*, 1988; Flachsenberger & Mirtschin, 1994). However, others have raised concerns given that the effects of anticholinesterases are thought to be transient (Sutherland & Tibballs, 2001).

To date, no *in vitro* studies have examined the effectiveness of anticholinesterases against the neurotoxicity of death adder venoms.

Focus of this Study

Venoms from four geographic variants of common death adder (*A. antarcticus*) were obtained. These are *A. antarcticus* (Gosford; New South Wales), *A. antarcticus* (Surfers Paradise; Gold Coast, Queensland), *A. antarcticus* (Middleback, Whyalla, South Australia) and *A. antarcticus* (Darling Ranges, Western Australia). In addition, venoms from *A. praelongus* (Cairns, Queensland), *A. pyrrhus* (Alice Springs, Northern Territory), *A. rugosus* (Merauke, Irian Jaya, Indonesia), *A. wellsi* (Newman, Pilbarra, Western Australia), *A. hawkei* (Goydnor's Lagoon, Barkley Tableland, Northern Territory) and *A.* sp. Seram (Seram, Indonesia) were obtained. To date, several neurotoxic components have been isolated from *A. antarcticus* venom. However, a detailed analysis of the neurotoxicity of *Acanthophis* venoms has not been undertaken. In fact, no pharmacological studies have been undertaken on whole venoms from *A. hawkei*, *A. rugosus*, *A.* sp. Seram and *A. wellsi*. Neither has any study compared venoms of the four geographic variants of *A. antarcticus*. Furthermore, the efficacy of CSL death adder antivenom against venoms of *A. hawkei*, *A. rugosus*, *A.* sp. Seram and *A. wellsi* is unknown.

The aim of this thesis is to study both the whole venoms and their isolated components for pharmacological activity with a particular emphasis on neurotoxicity and myotoxicity. Studies will also determine the efficacy of CSL death adder antivenom against the *in vitro* neurotoxicity of death adder venoms. Furthermore, isolated neurotoxins will be characterised at a number of different nicotinic acetylcholine receptor subtypes. It is

anticipated that this study will contribute to better treatment of envenomation, and isolation of components may lead to the identification of valuable research tools.

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CHAPTER 2

Neurotoxicity of Death Adder Venoms

Declaration for Thesis Chapter 2

This chapter is made up of the following publication:

Species and regional variations in the effectiveness of antivenom against the in vitro neurotoxicity of death adder (*Acanthophis*) venoms

Published in Toxicology and Applied Pharmacology (2001), vol. 175, pp. 140 – 148.

I/we declare that over 80 % of this work has been done by the candidate. Except the preparation of Figure 1 and Table 1 this manuscript has been written solely by the candidate taking into consideration the advice and recommendations of co-authors. Figure 1 and Table 1 were prepared by Dr. Bryan Fry. LC-MS analysis was also done by Dr. Bryan Fry under the overall supervision of Dr. Alun Jones and Prof. Paul Alewood at the University of Queensland, Australia. Some of the venoms were supplied by Dr. Bryan Fry. All other experiments were performed by the candidate. First authorship of Dr. Bryan Fry reflects honoring of a prior agreement before the commencement of the study. Another paper (Fry *et al.* (2002). *Rapid Commun. Mass Spectrom.* **16**, 600 – 608) found in the appendix of this thesis represents the original work of Dr. Bryan Fry that was meant to be submitted in place of this publication.

The original data are stored at the Department of Pharmacology, Monash University, Clayton Campus, Australia and will be held for at least seven years from the date of publication.

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Species and Regional Variations in the Effectiveness of Antivenom against the *in Vitro* Neurotoxicity of Death Adder (*Acanthophis*) Venoms

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Although viperlike in appearance and habit, death adders belong to the Elapidae family of snakes. Systemic envenomation represents a serious medical problem with antivenom, which is raised against *Acanthophis antarcticus* venom, representing the primary treatment. This study focused on the major *Acanthophis* variants from Australia and islands in the Indo-Pacific region. Venoms were profiled using liquid chromatography–mass spectrometry, and analyzed for *in vitro* neurotoxicity (0.3–10 μg/ml), as well as the effectiveness of antivenom (1–5 units/ml; 10 min prior to the addition of 10 μg/ml venom). The following death adder venoms were examined: *A. antarcticus* (from separate populations in New South Wales, Queensland, South Australia, and Western Australia), *A. hawkei*, *A. praelongus*, *A. pyrrhus*, *A. rugosus*, *A. wellsi*, and venom from an unnamed species from the Indonesian island of Seram. All venoms abolished indirect twitches of the chick isolated biventer cervicis nerve-muscle preparation in a dose-dependent manner. In addition, all venoms blocked responses to exogenous acetylcholine (1 mM) and carbachol (20 μM), but not KCl (40 mM), suggesting postsynaptic neurotoxicity. Death adder antivenom (1 unit/ml) prevented the neurotoxic effects of *A. pyrrhus*, *A. praelongus*, and *A. hawkei* venoms, although it was markedly less effective against venoms from *A. antarcticus* (NSW, SA, WA), *A. rugosus*, *A. wellsi*, and *A.* sp. Seram. However, at 5 units/ml, antivenom was effective against all venoms tested. Death adder venoms, including those from *A. antarcticus* geographic variants, differed not only in their venom composition but also in their neurotoxic activity and susceptibility to antivenom. For the first time toxicological aspects of *A. hawkei*, *A. wellsi*, *A. rugosus*, and *A.* sp. Seram venoms were studied.

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Death adders (genus *Acanthophis*) are unique among Australian snakes in both morphology and venom composition (Campbell, 1966; Fry, 1999). Although classified into the Elapidae family of snakes they are viperlike in appearance and habit (Campbell, 1966). They are characterized by a somewhat flattened, almost triangular head and a short, stout body terminating to a thin ratlike tail (Cogger, 1996). This makes them among the most specialized of all elapids and closely convergent in many respects with members of the family Viperidae.

Death adders 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 there have been up to 12 species and 3 subspecies of death adders described thus far (Hoser, 1998), considerable debate remains about species identification (Wuster *et al.*, 1999). Of these, only the venoms of the common (*A. antarcticus*), northern (*A. praelongus*), and desert (*A. pyrrhus*) death adders have been studied.

In terms of biochemical and pharmacological properties, species variations between the venoms of *A. antarcticus*, *A. praelongus*, and *A. pyrrhus* have been reported. Many differences between venom profiles were found when each venom was applied to a cation-exchange Mono-S column (van der Weyden *et al.*, 2000). Another study showed significant differences in *in vitro* neurotoxicity between these three venoms (Wickramaratna and Hodgson, 2001).

In a comparative study of 25 snake venoms, Broad *et al.* (1979) ranked *A. antarcticus* venom as the ninth most lethal venom with a murine LD50 of 0.338 mg/kg (sc, in 0.1% bovine serum albumin in saline). Kellaway *et al.* (1932) found the neurotoxic action of *A. antarcticus* venom to be attributable to a peripheral curare-like neuromuscular block. More recently, five postsynaptic neurotoxins have been isolated and sequenced from *A. antarcticus* venom (Sheumack *et al.*, 1979,

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1990; Kim and Tamiya, 1981a,b; Tyler *et al.*, 1997). In the chick isolated stimulated biventer cervicis nerve-muscle preparation, *A. antarcticus* (Qld), *A. praelongus*, and *A. pyrrhus* venoms (3–10 μg/ml) caused time-dependent inhibition of twitches and blocked contractile responses to exogenous acetylcholine and carbachol, suggesting the presence of postsynaptic neurotoxins (Wickramaratna and Hodgson, 2001). In the same study the three venoms (3–10 μg/ml) were ranked according to their *in vitro* neurotoxicity as follows: *A. antarcticus* (Qld) ≥ *A. pyrrhus* > *A. praelongus*.

Death adder envenomations are a rare occurrence in Australia, although these are still a significant health problem in Papua New Guinea (Currie *et al.*, 1991; Sutherland, 1992; Lalloo *et al.*, 1995). Clinical symptoms of envenomation by *Acanthophis* spp. include those relating to the paralysis of bulbar and ocular muscles, enlargement of regional lymph nodes, and death occurs through inhibition of respiration resulting from paralysis of the voluntary muscles (Campbell, 1966; Lalloo *et al.*, 1996). CSL death adder antivenom, which has been raised against *A. antarcticus* venom, remains the principal therapy for death adder envenomation (White, 1998). This antivenom was found to be very effective in reversing the effects of death adder envenomation in Papua New Guinea (Campbell, 1966; Lalloo *et al.*, 1996). A recent *in vitro* study showed that CSL death adder antivenom, while very effective against *A. praelongus* and *A. pyrrhus* venoms, was significantly less effective against the neurotoxicity of *A. antarcticus* (Qld) venom (Wickramaratna and Hodgson, 2001). Although death adder antivenom was raised against *A. antarcticus* venom it is possible that it may not have been raised against a pool of *A. antarcticus* venoms representative of all geographic variations. Thus, the antivenom may lack the ability to neutralize some neurotoxic components of venoms from subpopulations of *A. antarcticus* species (Schenberg, 1963; Wickramaratna and Hodgson, 2001). This explanation, however, remains to be investigated. With many new species of death adders described, from a clinical perspective, it is useful to know the effectiveness of CSL death adder antivenom against their venoms (Currie, 2000). However, no such study has been published.

To date, no pharmacological studies have been undertaken on whole venoms from the Barkly tableland (*A. hawkei*), black head (*A. wellsi*), or Irian Jayan (*A. rugosus*) death adders, nor from death adders from the Indonesian island of Seram (*A.* sp. Seram), considered by some herpetologists to be another possible species (Mark O'Shea, personal communication, 2000). Therefore, this study examined the *in vitro* neurotoxicity of these venoms and compared these to the previously studied *A. antarcticus*, *A. praelongus*, and *A. pyrrhus* venoms. In light of our recent findings, the efficacy of CSL death adder antivenom against the *in vitro* neurotoxicity of these unstudied venoms was also examined. In addition, death adder venoms were profiled using on-line liquid chromatography–mass spectrometry (LC-MS) to determine the level of variations in venom composition resulting from geographic location or species differences.

MATERIALS AND METHODS

Venom Collection

A. antarcticus venoms were obtained from populations in New South Wales (NSW), Queensland (Qld), South Australia (SA), and Western Australia (WA). *A. praelongus* venom was milked from populations in Cairns, Queensland; *A. pyrrhus* venom from Alice Springs, Northern Territory; *A. wellsi* venom from the Pilbarra region of Western Australia; *A. hawkei* venom from the Barkly tableland region of Northern Territory; *A. rugosus* venom from Irian Jaya (West Papua), and *A.* sp. Seram from the island of Seram, Indonesia. Venoms were either purchased from Venom Supplies Pty. Ltd., South Australia, or milked by the first author. For each geographic variant or species venoms were collected and pooled to minimize the effects of individual variations (Chippaux *et al.*, 1991).

Venom Preparation and Storage

Freeze-dried venoms and stock solutions of venoms, prepared in 0.1% bovine serum albumin in 0.9% saline, were stored at −20°C until required.

Liquid Chromatography–Mass Spectrometry (LC-MS)

Venoms were dissolved in 0.1% trifluoroacetic acid (TFA) to a concentration of 1 mg/ml. On-line LC-MS of venoms was performed on a Vydac C18 analytical column (2.1 × 250 mm, 5μ particle size, 300 Å) with solvent A (0.05% TFA) and solvent B (90% acetonitrile in 0.045% TFA) at a flow rate of 130 μl/min. The solvent delivery and gradient formation of a 1% gradient from 0 to 60% acetonitrile/0.05% TFA over 60 min was achieved using an Applied Biosystems 140 B solvent delivery system. Electrospray mass spectra were acquired on a PE-SCIEX triple quadrupole mass spectrometer equipped with an ionspray atmospheric pressure ionization source. Samples (10 μl) were injected manually into the LC-MS system and analysed in positive ion mode. Full scan data were acquired at an orifice potential of 80 V over the mass range 400–2100 Da with a step size of 0.2 amu. Data processing was performed with the aid of the software package Biomultiview (PE-SCIEX, Canada).

Chick Isolated Biventer Cervicis Nerve-Muscle Preparation

Male White leg horn chicks aged between 4 and 8 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 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; and glucose, 11.1. The Krebs solution was bubbled with carbogen (95% O_2 and 5% CO_2) and maintained at 34°C. Twitches were evoked by stimulating the motor nerve every 10 s with pulses of 0.2 ms duration at a supramaximal voltage (Harvey *et al.*, 1994) using a Grass S88 stimulator. After a 30-min equilibration period, *d*-tubocurarine (10 μM) was added. Subsequent abolition of twitches confirmed selective stimulation of nerves. Twitches were then reestablished 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 (Harvey *et al.*, 1994). Electrical stimulation was then recommenced and the preparations allowed to equilibrate for a further 30-min period before commencement of the experiment. Venoms were left in contact with the preparations until complete twitch blockade occurred, or for a 4-h period. Contractile responses to ACh, CCh, and KCl were then obtained as previously described. Where indicated, death adder antivenom (1–5 units/ml) was added 10 min prior (Barfaraz and Harvey, 1994; Crachi *et al.*, 1999; Wickramaratna and Hodgson, 2001) to the addition of venoms (10 μg/ml).

Drugs

The following drugs were used: acetylcholine chloride (Sigma, St. Louis, MO); bovine serum albumin (BSA; Sigma); carbamylcholine chloride (carbachol; Sigma); *d*-tubocurarine chloride (Sigma). Except where indicated, stock solutions were made up in distilled water. Death adder antivenom, which is raised against *A. antarcticus* venom in horses, was obtained from CSL Ltd. (Australia).

Analysis of Results and Statistics

For isolated tissue experiments, responses were measured via a Grass force displacement transducer (FT03) and recorded on a MacLab System. To compare the neurotoxicity of venoms, the time taken to cause 90% inhibition of nerve-mediated twitches (i.e., t_{90} values) was determined. t_{90} values were calculated for each experiment by determining the elapsed time after venom addition at 10% of the initial twitch height, and then the means and standard error of the means were calculated. A two-way ANOVA was performed for comparison of t_{90} values between venoms and concentrations. Where indicated, curves were compared by a two-way repeated measures ANOVA. Contractile responses to ACh, CCh, and KCl were expressed as a percentage of the respective initial response. These were analyzed using either Student's paired *t*-tests or, where stated, compared against the control response via a one-way ANOVA. All ANOVAs were followed by a Bonferroni post hoc test. Statistical significance was indicated when $p < 0.05$.

RESULTS

Liquid Chromatography–Mass Spectrometry Analysis of Venoms

Venoms were profiled using LC-MS to determine differences in venom composition. All venoms had essentially the same generalized elution profile: an early eluting component (~24% acetonitrile), a few percentage pause without any appreciable components eluting, and then the vast majority of components eluting between about 30 to 40% acetonitrile (Fig. 1). The first eluting component in all venoms had a mass similar to that of the short-chain neurotoxin Aa-c, previously isolated from *A. antarcticus* venom. A greater number of components were shared by *A. antarcticus* geographic variants (NSW, Qld, SA, WA) than between species (Table 1).

Neurotoxicity Studies

All *Acanthophis* venoms (10 μg/ml) caused time-dependent inhibition of nerve-mediated twitches, whereas vehicle (i.e., BSA) had no inhibitory effect on twitch height ($n = 4–8$; Figs. 2a and 2b). There was no significant difference in neurotoxicity between the *A. antarcticus* venoms at this concentration (two-way repeated measures ANOVA, $p < 0.05$). However, *A. hawkei, A. praelongus,* and *A. pyrrhus* venoms were significantly less neurotoxic than *A.* sp. Seram venom (10 μg/ml; two-way repeated measures ANOVA, $p < 0.05$). All venoms at 10 μg/ml totally abolished contractile responses to exogenous ACh (1 mM) and CCh (20 μM), but not KCl (40 mM), whereas the vehicle had no significant inhibitory effect on the contractile responses to exogenous agonists ($n = 4–5$; Student's paired *t*-test, $p < 0.05$, data not shown).

All *Acanthophis* venoms (0.3–10 μg/ml; $n = 4–15$) caused a concentration-dependent inhibition of nerve-mediated twitches (two-way ANOVA, $p < 0.05$; Figs. 3a and 3b). Because experiments were terminated after 4 h, t_{90} values for venoms (0.3 μg/ml) from *A. antarcticus* (SA), *A. praelongus, A. rugosus, A. wellsi,* and *A. hawkei* were not determined. Although not shown in Fig. 3b, the t_{90} values for *A. pyrrhus* and *A.* sp. Seram venoms (0.3 μg/ml; $n = 8$) were 180 ± 18 and 212 ± 32 min, respectively. *A. antarcticus* (SA) venom was significantly less neurotoxic than *A. antarcticus* (Qld) and *A. antarcticus* (NSW) venoms (two-way ANOVA, $p < 0.05$; Fig. 3a). *A. hawkei* venom was significantly less neurotoxic than *A.* sp. Seram venom (two-way ANOVA, $p < 0.05$; Fig. 3b). At lower concentrations (0.3–1.0 μg/ml) there was a greater degree of spread of neurotoxicity than at higher concentrations (3–10 μg/ml). In the case of *A. wellsi* and *A. pyrrhus* venoms, the rank order of venom neurotoxicity, based on t_{90} values, altered with the change in concentration (Fig. 3b).

Efficacy of Death Adder Antivenom

Prior incubation (10 min) of death adder antivenom (1 unit/ml) significantly delayed twitch inhibition produced by all *Acanthophis* venoms (10 μg/ml; $n = 4–8$; two-way repeated measures ANOVA, $p < 0.05$; Figs. 4a and 5a). Among the *A. antarcticus* geographic variants, antivenom markedly attenuated the neurotoxicity of *A. antarcticus* (Qld) venom, while having a significantly lesser effect on *A. antarcticus* (NSW) and *A. antarcticus* (WA) venoms ($n = 5–7$; two-way repeated measures ANOVA, $p < 0.05$; Fig. 4a). In addition, in the presence of antivenom (1 unit/ml) all four *A. antarcticus* venoms (10 μg/ml) continued to significantly inhibit contractile responses to exogenous ACh (1 mM) and CCh (20 μM) compared to the antivenom control (i.e., antivenom only; $n = 5–7$; one-way ANOVA, $p < 0.05$; Fig. 4b).

Prior incubation of antivenom (1 unit/ml) prevented twitch inhibition by *A. hawkei, A. praelongus,* and *A. pyrrhus* venoms (10 μg/ml). However, in the presence of antivenom, *A. wellsi, A. rugosus,* and *A.* sp. Seram venoms (10 μg/ml) continued to significantly inhibit the twitch response compared to the antivenom control ($n = 4–8$; two-way repeated measures ANOVA, $p < 0.05$; Fig. 5a). Furthermore, in the presence of antivenom (1 unit/ml), *A. wellsi, A. rugosus,* and *A.* sp. Seram venoms continued to significantly inhibit contractile responses to exogenous ACh and CCh compared to the antivenom control ($n = 4–8$; one-way ANOVA, $p < 0.05$; Fig. 5b). However, antivenom prevented *A. hawkei, A. praelongus,* and *A. pyrrhus* venoms from inhibiting contractile responses to exogenous ACh and CCh ($n = 4–8$; Fig. 5b).

Prior incubation of antivenom (5 units/ml) prevented twitch inhibition by *A. antarcticus* (NSW, Qld, SA, WA), *A. rugosus, A.* sp. Seram, and *A. wellsi* venoms (10 μg/ml; $n = 3–4$; data not shown). Similarly, antivenom (5 units/ml) prevented these venoms from inhibiting contractile responses to exogenous ACh and CCh ($n = 3–4$; data not shown).

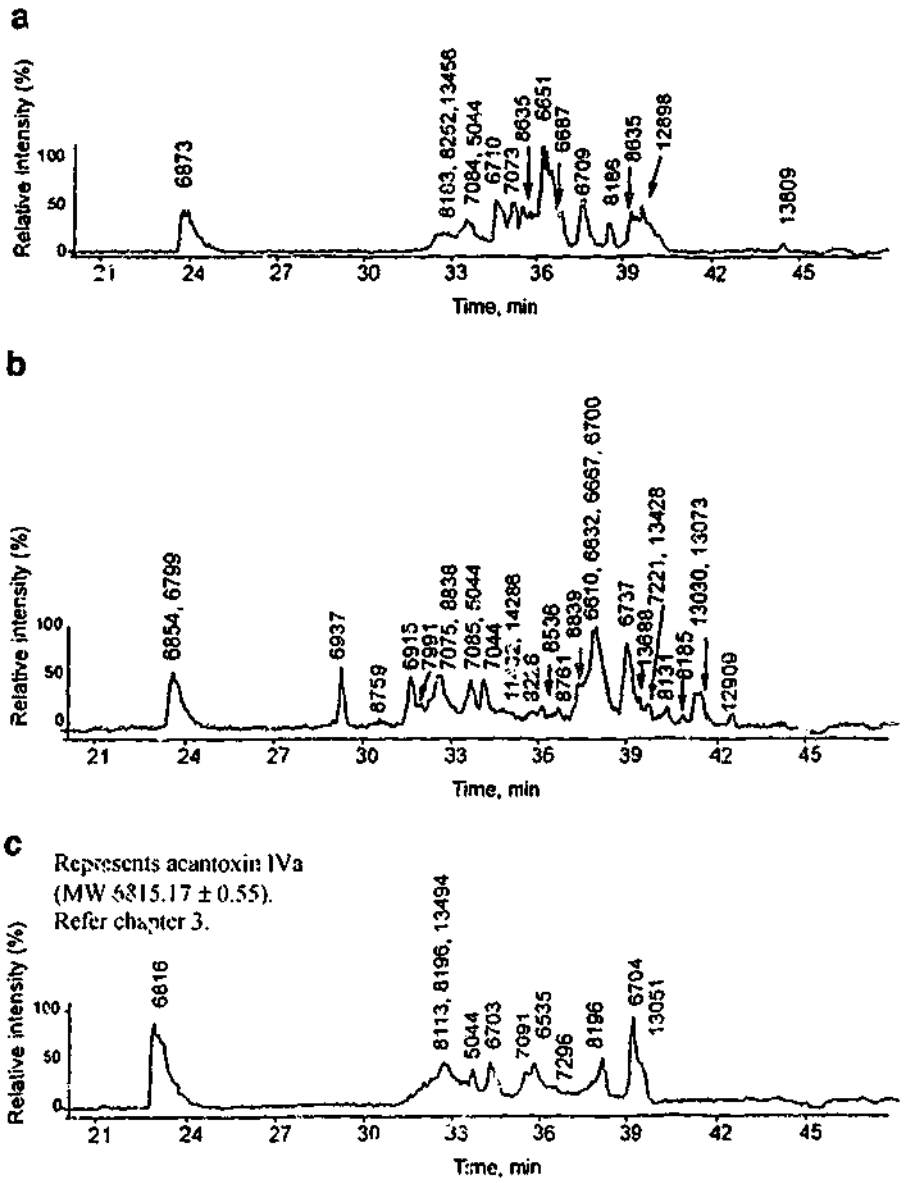

FIG. 1. Representative LC-MS profiles: comparison of the two main Australian *Acanthophis* species (a) *A. antarcticus* (NSW population) and (b) *A. praelongus* with (c) the venom of the species from the Indonesian island of Seram. LC-MS profiles for other venoms not shown.

DISCUSSION

A. antarcticus crude venom was previously examined for lethality, neurotoxicity, myotoxicity, and its effects on blood coagulation, both experimentally and clinically (Fairley, 1929; Kellaway, 1929a,b; Campbell, 1966; Broad *et al.*, 1979; Sutherland, 1983). Recently, *A. praelongus* and *A. pyrrhus* venoms were studied for *in vitro* neurotoxicity, myotoxicity, and phospholipase A_2 activity (van der Weyden *et al.*, 2000; Wickramaratna and Hodgson, 2001). To date, no pharmacological studies have been carried out on whole venoms from *A. hawkei, A. wellsi, A. rugosus,* and *A.* sp. Seram. Therefore, this study examined the *in vitro* neurotoxicity of these crude venoms and compared these to the previously studied *A. antarcticus, A. praelongus,* and *A. pyrrhus* venoms. In addition, the efficacy of CSL death adder antivenom against the *in vitro* neurotoxicity of these venoms was examined.

Venoms were profiled using on-line liquid chromatography–mass spectrometry to determine basic biochemical differences. As previously detailed, all venoms had essentially the same generalized elution profile. Given that these venoms are from snakes belonging to the same genus this is not surprising. However, close examination and comparison of each profile showed many differences in peak distribution and complexity between venoms from different species of death adder. Such species variations in chromatographic profiles were previously observed for *A. antarcticus, A. praelongus,* and *A. pyrrhus* venoms (van der Weyden *et al.*, 2000). In the present study, LC-MS profiles of venoms from *A. antarcticus* geographic

TABLE 1
Components Shared by Two or More *Acanthophis* Venoms

Venom* (*m/z*)									
ant									
NSW	Qld	SA	WA	Ser	haw	pra	pyr	rug	wel
6873	6873	6873	6873						
						6914	6913		
8186	8184	8183	8183						
8252	8252	8252	8252						
5044	5044	5044	5044	5044	5044			5044	
6710	6710	6710	6711		6709				
7073	7070		7070				7075		
6651	6652	6652	6654		6653			6657	
6687	6686		6688						
	6676	6676	6679		6675		6676		6678
					13427	13428			
12898			12898						
13809	13810								

Note. Molecular weights of components are presented by order of elution.
* ant = *A. antarcticus;* Ser = *A.* sp. Seram; haw = *A. hawkei;* pra = *A. praelongus;* pyr = *A. pyrrhus;* rug = *A. rugosus;* wel = *A. wellsi.*

variants showed a lesser degree of variability. Furthermore, a greater number of components were shared by *A. antarcticus* geographic variants than between species. Previous reports suggest that variations in venom composition as a result of geographic location or difference in species are not unique to death adders (Jimenez-Porras, 1964; Williams *et al.*, 1988; Yang *et al.*, 1991; Assakura *et al.*, 1992; Daltry *et al.*, 1996). The large distances separating the *A. antarcticus* geographic variants may account for the variations in their venom composition. Daltry *et al.* (1996) showed that variations in venom composition as a result of geographic location reflect natural selection for feeding on local prey. LC-MS venom profiles may be used to suggest taxonomic relationships among death adder species, as was previously suggested for some spider venoms (Escoubas *et al.*, 1997).

Although it is beyond the scope of this study to fully characterize species variations in venom composition, preliminary observations can be made. The venom profile of *A. antarcticus* varies little over its vast range, with the majority of the venom components being conserved in this species but with minimal conservation among other species. Certain venoms, such as that of *A. praelongus,* appear to be much more complex than those of other species, such as *A.* sp. Seram. This may be the case but may also indicate that *A. praelongus,* in fact, is a species complex. Given that venoms used in the study were the result of pooling of venoms of *A. praelongus* across its range this has implications upon homology of samples. The apparent diversity in venom composition will be the focus of a follow-up study

The first eluting components have masses corresponding to isoforms of the short-chain neurotoxin Aa-c (Kim and Tamiya, 1981b). *A. praelongus* is notable in being the only species with an intermediate eluting component. The characteristic masses for each species of this first peak allows for preliminary *m/z* fingerprinting and these components have been isolated for characterization. Molecular weights corresponding with PLA_2 toxins are also present in all the venoms, in greater quantities and molecular weight diversities than were expected. These components have also been isolated and are presently being characterized. A component of mass 5044 is present in the vast majority of venoms and does not correspond in molecular weight with other isolated components from elapids and thus may represent a new class of venom molecule.

Clinically, the most important symptoms of death adder envenomations are those relating to neurotoxicity, such as ptosis, generalized paralysis, and respiratory failure (Campbell, 1966; Lalloo *et al.*, 1996). However, nothing is known about the neurotoxicity of *A. hawkei, A. wellsi, A. rugosus,* and *A.* sp. Seram venoms. Furthermore, we compared the venoms from *A. antarcticus* geographic variants to determine whether variations in venom composition, as a result of geographic location, are reflective of neurotoxic activity. Therefore, the neurotoxicity of death adder venoms was investigated using the chick biventer cervicis nerve-muscle preparation. In this preparation, all venoms caused time-dependent inhibition of indirect twitches. Furthermore, all venoms abolished contractile responses to acetylcholine and carbachol but not KCl, thus indicating the presence of postsynaptically acting neurotoxins in these death adder venoms. This is in agreement with previous studies showing that the neurotoxicity of *A. antarcticus* venom is mainly attributed to the presence of postsynaptic neurotoxins (Sheumack *et al.*, 1979, 1990; Kim and Tamiya,

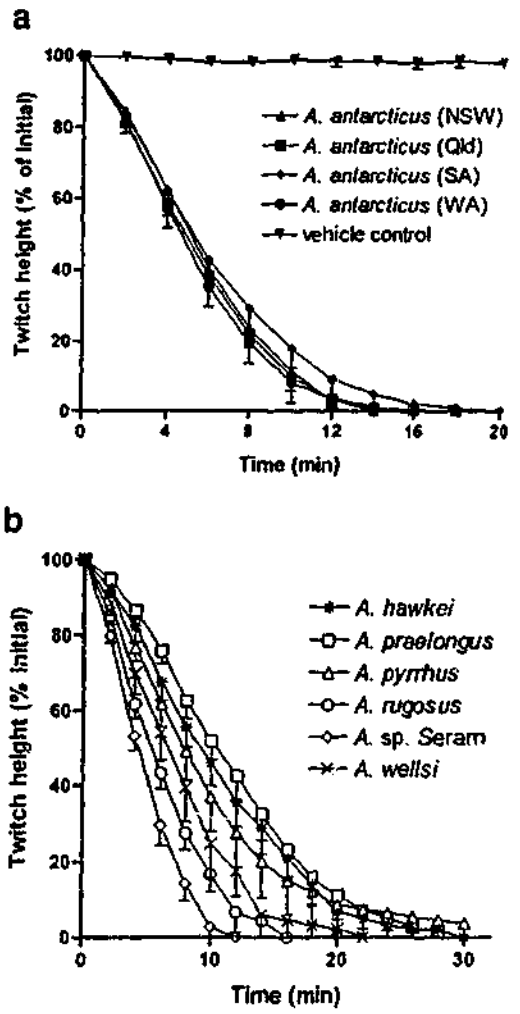

FIG. 2. (a) Effect of venoms (10 μg/ml; n = 4–5) from *A. antarcticus* geographic variants or vehicle (n = 8) on nerve-mediated twitches in the chick isolated biventer cervicis nerve-muscle preparation. (b) Effect of venoms (10 μg/ml; n = 4–5) from other *Acanthophis* spp. on nerve-mediated twitches in the chick isolated biventer cervicis nerve-muscle preparation.

1981a,b; Tyler *et al.*, 1997; Wickramaratna and Hodgson, 2001).

Because of the complex regulatory requirements involved in gaining approval for murine LD50 studies in many countries, including Australia, they have been largely superseded by *in vitro* studies. One method of measuring the neurotoxicity of venoms is by determining t_{90} values in isolated skeletal muscle preparations (Harvey *et al.*, 1994). Comparison of t_{90} values, at 10 μg/ml, indicated the following rank order of neurotoxicity: *A.* sp. Seram ≥ *A. antarcticus* (Qld) ≥ *A. antarcticus* (WA) ≥ *A. antarcticus* (NSW) ≥ *A. rugosus* ≥ *A. antarcticus* (SA) ≥ *A. wellsi* ≥ *A. hawkei* ≥ *A. pyrrhus* ≥ *A praelongus*. However, for some venoms the rank order of neurotoxicity altered with a change in venom concentration. This was especially the case with regard to the potency of *A. wellsi* and *A. pyrrhus* venoms. This possibly results from the fact that death adder venoms contain a number of neurotoxins with various quantities of each (Sheumack *et al.*, 1979, 1990; Kim and Tamiya, 1981a,b; Tyler *et al.*, 1997). Thus, the neurotoxicity of the whole venom is dependent not only on the toxicity of each neurotoxin but also on the quantity of each neurotoxin within the venom. Therefore, particularly at lower concentrations of whole venom, the quantity of each neurotoxin within the venom becomes important. Although there were no significant differences in neurotoxicity between venoms from *A. antarcticus* geographic variants at a concentration of 10 μg/ml, there were significant differences when other concentrations were taken into account. *A. antarcticus* (SA) venom was significantly less neurotoxic than *A. antarcticus* (Qld) and *A. antarcticus* (NSW) venoms. Therefore, venoms from *A. antarcticus* geographic variants differ not only in their venom composition but also in their neurotoxic activity.

Whether the rank order of neurotoxicity is representative of the rank order of venom LD50 values for this genus remains to be elucidated. However, it is tempting to speculate that this could be the case because lethality of death adder venoms is

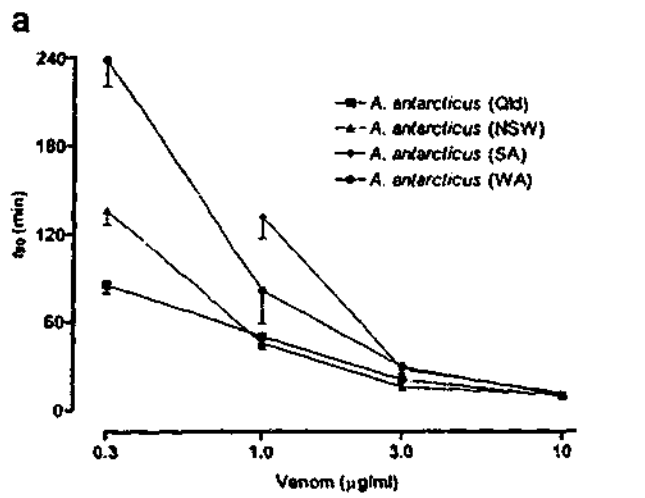

FIG. 3. (a) Graph showing t_{90} values (min) against venom concentrations (n = 4–15) for *A. antarcticus* geographic variants. (b) Graph showing t_{90} values (min) against venom concentrations (n = 4–15) for other *Acanthophis* species.

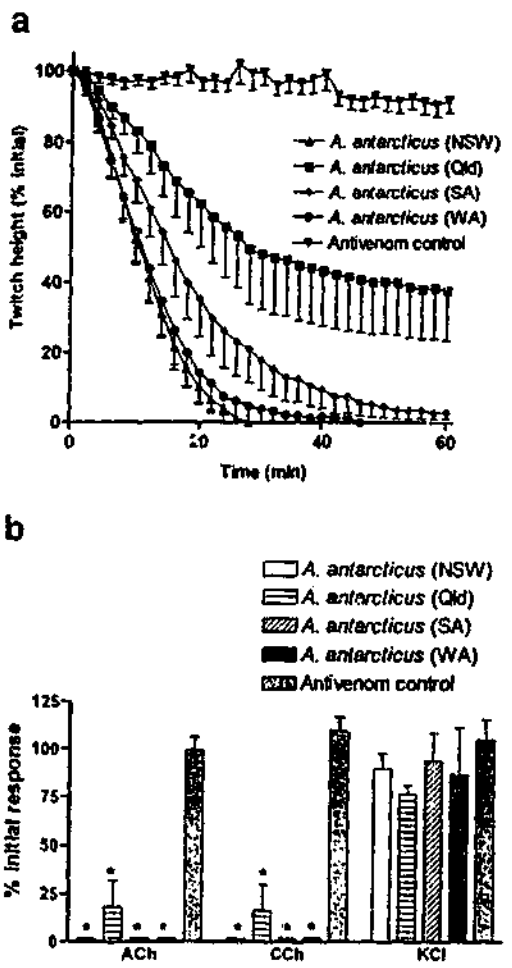

FIG. 4. (a) Effect of venoms (10 μg/ml; n = 5–7) from *A. antarcticus* geographic variants, in the presence of antivenom (1 unit/ml), or antivenom control (n = 8) on nerve-mediated twitches in the chick isolated biventer cervicis nerve-muscle preparation. (b) Effect of venoms (10 μg/ml; n = 5–7) from *A. antarcticus* geographic variants, in the presence of antivenom (1 unit/ml), or antivenom control (n = 8) on contractile responses to exogenous ACh, CCh, and KCl in the chick isolated biventer cervicis nerve-muscle preparation. *$p < 0.05$, significantly different from antivenom control response, one-way ANOVA.

largely the result of respiratory failure (Kellaway, 1929b, 1932; Campbell, 1966). Although it should be noted that murine LD50 determinations are based on "quantity" (i.e., what concentration of venom kills 50% of mice usually over a 24- to 48-h period), whereas t_{90} values are based on how "quick" a venom acts. Therefore, it is possible to have an extremely "lethal" venom (based on LD50 values), which takes a long time to produce its effects. Therefore, knowledge of both parameters is desirable. In the present study, it was not possible to determine the presence of presynaptic neurotoxins in death adder crude venoms. Presynaptic neurotoxins have a slow onset of action, with a latency period of up to 60 min resulting from internalization and disruption of the presynaptic processes (Chang and Su, 1982; Chang, 1985). Therefore, it is possible that the action of any presynaptic neurotoxins was masked by the faster acting postsynaptic neurotoxins in the death adder crude venoms (Lee, 1979). However, fractionation of these venoms will allow the identification of any presynaptic neurotoxins.

In Australia, CSL death adder antivenom is indicated for use in envenomation by any death adder species (AMH, 1998). A previous *in vitro* study by us showed that CSL death adder antivenom (1 unit/ml), although very effective against *A. praelongus* and *A. pyrrhus* venoms, was significantly less effective against the neurotoxicity of *A. antarcticus* (Qld) venom (Wickramaratna and Hodgson, 2001). However, no studies have examined the efficacy of antivenom against venoms from *A. hawkei, A. wellsi, A. rugosus,* and *A.* sp. Seram. Furthermore, it was of interest to determine the efficacy of antivenom against venoms from other *A. antarcticus* geographic variants. Therefore, the efficacy of CSL death adder antivenom was determined according to the procedure described by Barfaraz and Harvey (1994). Prior incubation of antivenom (1 unit/ml) significantly attenuated twitch inhibition produced by all venoms. Antivenom (1 unit/ml) totally pre-

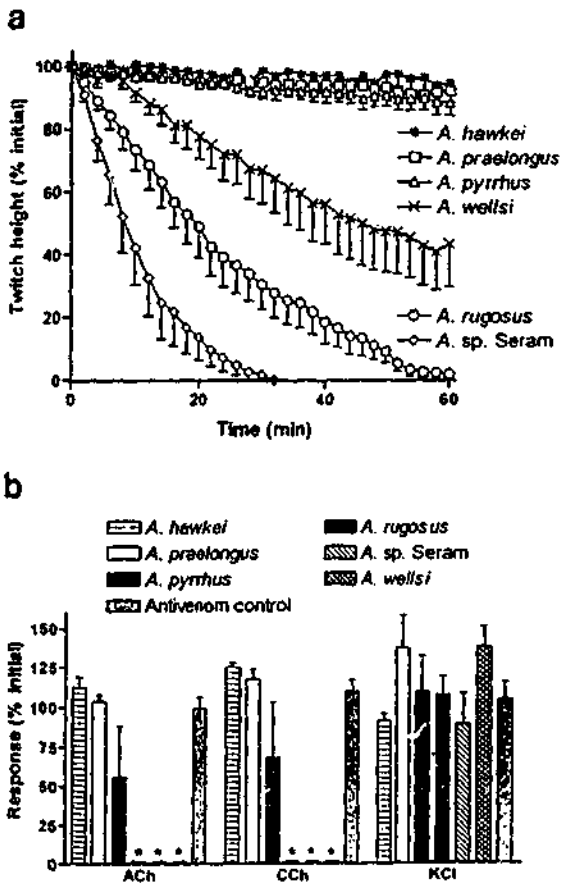

FIG. 5. (a) Effect of venoms (10 μg/ml; n = 4–8) from other *Acanthophis* spp., in the presence of antivenom (1 unit/ml) on nerve-mediated twitches in the chick isolated biventer cervicis nerve-muscle preparation. (b) Effect of venoms (10 μg/ml; n = 4–8) from other *Acanthophis* spp., in the presence of antivenom (1 unit/ml), or antivenom control (n = 8) on contractile responses to exogenous ACh, CCh, and KCl in the chick isolated biventer cervicis nerve-muscle preparation. *$p < 0.05$, significantly different from antivenom control response, one-way ANOVA.

vented twitch inhibition by *A. hawkei, A. praelongus,* and *A. pyrrhus* venoms. In addition, antivenom prevented *A. hawkei, A. praelongus,* and *A. pyrrhus* venoms from inhibiting contractile responses to exogenous ACh and CCh. Thus, antivenom raised against *A. antarcticus* venom is very effective against the neurotoxicity of *A. hawkei, A. praelongus,* and *A. pyrrhus* venoms. However, antivenom (1 unit/ml) was markedly less effective against venoms from *A. antarcticus* (Qld), *A. antarcticus* (SA), *A. rugosus,* and *A. wellsi.* Interestingly, antivenom (1 unit/ml) only delayed the neurotoxicity of venoms from *A.* sp. Seram, *A. antarcticus* (NSW), and *A. antarcticus* (WA). Furthermore, in the presence of antivenom (1 unit/ml), *A. antarcticus* (Qld, SA, NSW, WA), *A. rugosus, A. wellsi,* and *A.* sp. Seram venoms continued to significantly inhibit contractile responses to exogenous ACh and CCh.

Given that *A. hawkei, A. praelongus,* and *A. pyrrhus* venoms (10 μg/ml) were the least neurotoxic, it makes sense that these venoms were the most affected by the antivenom. However, it is surprising that *A. antarcticus* (Qld) venom was the most affected by antivenom compared to the other *A. antarcticus* geographic variants. In this case, *A. antarcticus* (Qld) venom was the most neurotoxic, whereas *A. antarcticus* (SA) venom was the least neurotoxic. Thus, it may have been expected that antivenom would have a greater effect on *A. antarcticus* (SA) venom than on *A. antarcticus* (Qld) venom. Furthermore, it is surprising that antivenom raised against *A. antarcticus* venom was markedly less effective against the neurotoxicity of all venoms from *A. antarcticus* geographical variants compared to venoms from other death adder species. It is possible that death adder antivenom may neutralize some neurotoxins within the venoms better than others. Thus, the effectiveness of antivenom may be dependent on its ability to neutralize the different neurotoxins within the whole venoms.

To further study the efficacy of CSL death adder antivenom the concentration of antivenom was increased. Prior incubation of antivenom (5 units/ml) prevented twitch inhibition by *A. antarcticus* (NSW, Qld, SA, WA), *A. rugosus, A.* sp. Seram, and *A. wellsi* venoms. Similarly, antivenom (5 units/ml) prevented these venoms from inhibiting contractile responses to exogenous ACh and CCh. Thus, at higher concentrations antivenom is capable of completely neutralizing all death adder venoms. Usually, in clinical situations antivenom is administered until symptoms of neurotoxicity (e.g., ptosis) are reversed (AMH, 1998; Currie, 2000). Clinical studies have shown that CSL death adder antivenom is very effective in reversing the effects of death adder envenomation in Papua New Guinea (Campbell, 1966; Lalloo *et al.*, 1996). This is not surprising if, as suggested by O'Shea (1996) and Lalloo *et al.* (1996), *A. praelongus* or a variant of this species is responsible for envenomations in some parts of Papua New Guinea. A detailed taxonomic study of the Indonesian/New Guinean death adders may be required before the taxonomy of this widespread and complicated genus can be fully understood.

In conclusion, all death adder venoms are predominately postsynaptically neurotoxic. Some venoms were significantly more neurotoxic than other death adder venoms. CSL death adder antivenom (1 units/ml) was found to be very effective against the neurotoxicity of *A. hawkei, A. praelongus,* and *A. pyrrhus* venoms, while markedly less effective against *A. antarcticus* (NSW, SA, WA), *A. rugosus, A. wellsi,* and *A.* sp. Seram venoms. However, a higher concentration of antivenom was effective against all death adder venoms. Death adder venoms, including those from *A. antarcticus* geographic variants, differed not only in their venom composition but also in their neurotoxic activity. Although this study was based on an *in vitro* preparation, it is anticipated that these findings will have a clinical significance in the event of death adder envenomation.

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Anticholinesterase Studies

Additional experiments undertaken but not included in the manuscript.

MATERIALS AND METHODS (Continued)

Chick Isolated Biventer Cervicis Nerve-Muscle Preparation (*continued*)

In other 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 venom (3 μg/ml) or *d*-tubocurarine (dTC; 8 μM).

Drugs (*continued*)

Neostigmine methyl sulfate was obtained from Sigma Chemical Co., St. Louis, MO, USA.

Analysis of Results and Statistics (*continued*)

To determine the effect of anticholinesterase on the neurotoxicity of venoms, the t_{90} values of venoms alone were compare with t_{90} values of venoms in the presence of neostigmine (10 μM) using a two-way ANOVA statistical test. All statistical tests were carried out using the SigmaStat (ver. 1.0; Jandel Corporation, CA, USA) software package.

RESULTS (Continued)

Anticholinesterase Studies

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 ± 2.6% of the initial height and was maintained at this level for the next 30 min (n = 4; Fig. 6a). In contrast, when neostigmine (10 μM) was added at t_{50}, i.e. after the addition of venom (3 μg/ml), no sustained recovery of the twitch height was observed for any of the venoms (n = 4). The transient recovery of twitch height with neostigmine was quickly overcome by all of the venoms (n = 4; Fig. 6b, c and d; data not shown for others). The t_{90} values of venoms in the absence and presence of neostigmine (10 μM) are shown in Table 2. Neostigmine (10 μM) added at t_{50} did not significantly affect the t_{90} value of any of the venoms (n = 4 – 15; two-way ANOVA, $P < 0.05$). Higher concentrations of neostigmine (eg. 30 – 100 μM) were found to be not appropriate for this study as they produced a contractile response *per se* which caused a reduction in twitch height.

TABLE 2

Neurotoxicity (t_{90} values) of *Acanthophis* venoms with and without neostigmine

Death Adder Venom	t_{90} Value at 3μg/ml (min)	t_{90} Value at 3μg/ml + Neostigmine (10 μM) (min)
A. antarcticus (NSW)	15.8 ± 1.3	17.9 ± 0.5
A. antarcticus (Qld)	20.7 ± 1.8[†]	21.4 ± 0.7
A. antarcticus (SA)	28.2 ± 2.5	24.1 ± 1.1
A. antarcticus (WA)	29.8 ± 5.0	23.9 ± 2.9
A. hawkei	40.9 ± 5.5	35.1 ± 3.4
A. praelongus	32.6 ± 3.0[†]	35.3 ± 3.2
A. pyrrhus	28.5 ± 1.8[†]	27.2 ± 4.7
A. rugosus	30.6 ± 3.5	28.1 ± 4.1
A. wellsi	23.7 ± 5.4	23.3 ± 2.4
A. sp. Seram	21.1 ± 1.0	17.1 ± 1.6

Data shown are mean ± SEM (n = 4 – 15).

[†] Data from Wickramaratna and Hodgson, 2001.

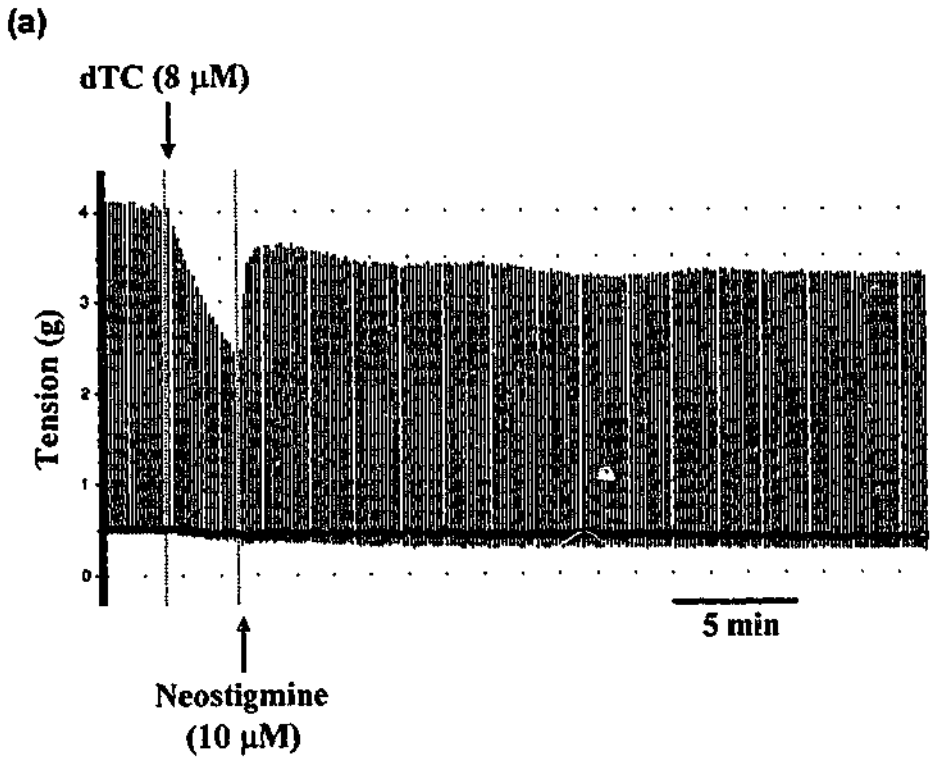

Fig. 6 A typical trace of the effect of (a) dTC (8 μM) or (b) *A. antarcticus* (Qld; 3 μg/ml) venom, with neostigmine (10 μM) added at t_{50}, on nerve-mediated twitches in the chick biventer cervicis nerve-muscle preparation.

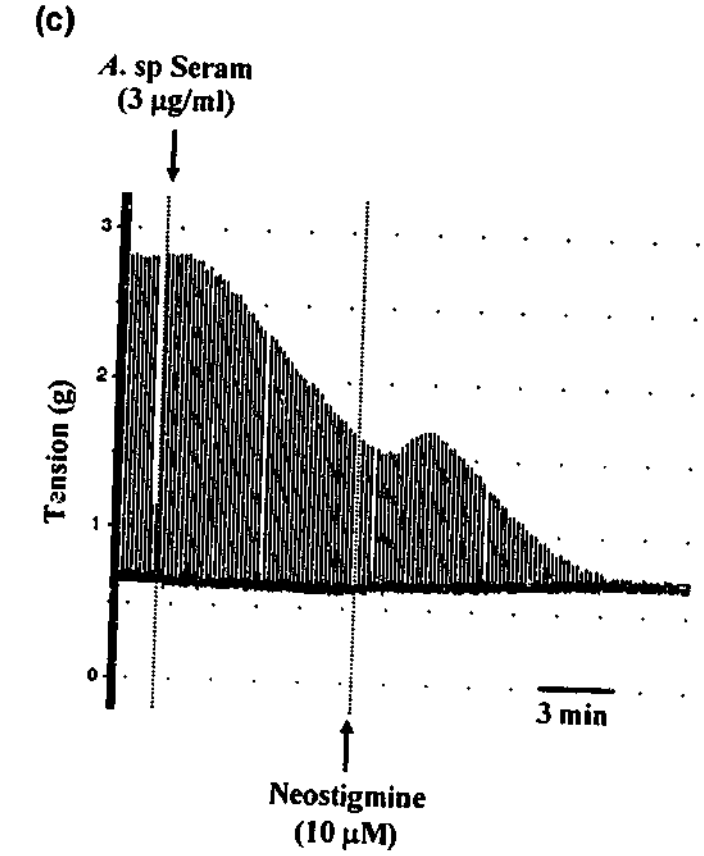

Fig. 6 A typical trace of the effect of (c) *A*. sp. Seram (3 µg/ml) venom or (d) *A. rugosus* (3 µg/ml) venom, with neostigmine (10 µM) added at t_{50}, on nerve-mediated twitches in the chick biventer cervicis nerve-muscle preparation.

DISCUSSION (Continued)

Given that the most important clinical symptoms of death adder envenomations are due to postsynaptic neurotoxicity, anticholinesterase therapy has been suggested as a supplement to death adder antivenom (Currie *et al.*, 1988). Indeed, several clinicians have used anticholinesterases (neostigmine and edrophonium) successfully to reduce the amount of antivenom administered (Currie *et al.*, 1988; Hudson, 1988; Lalloo *et al.*, 1996; Little & Pereira, 2000). Following anticholinesterase treatment a marked recovery of neurotoxicity in death adder envenomed patients has been reported. However, Sutherland and Tibballs (2001) cautioned that anticholinesterase treatment was no substitute for antivenom therapy as the effects are transient. They also suggested that a neurophysiological study should be undertaken to confirm the effectiveness of anticholinesterases against death adder envenomation. Therefore, in the present study the effects of anticholinesterase on the *in vitro* neurotoxicity of death adder venoms on the chick isolated stimulated biventer cervicis nerve-muscle preparation were examined. When the anticholinesterase neostigmine was added at the t_{50} time point a transient recovery of the venom-induced neuromuscular blockade was observed. This is in contrast to the effect on d-tubocurarine induced blockade, where a sustained recovery of the neuromuscular blockade was observed following neostigmine. Furthermore, neostigmine had no significant effect on the overall neurotoxicity of any death adder venom as determined by the t_{90} values. This confirms the suggestion by Sutherland and Tibballs (2001) that the reversal of the death adder venom induced neuromuscular blockade with neostigmine is transient in nature.

Perhaps, a more convincing reversal with neostigmine would have been obtained had a much lower concentration of these venoms been examined. For example, in *in vivo* rat studies neostigmine prolonged the survival at only the lower concentrations of *A. antarcticus* venom (Flachsenberger & Mirtschin, 1994). However, given the pseudo-

irreversible nature of the antagonism caused by neurotoxins present in death adder venoms (refer chapter 3), it is no surprise that the neurotoxicity is not easily reversed. This is in contrast to the effects of neostigmine on the competitive antagonism caused by d-tubocurarine. Perhaps, further studies could be carried out examining the combination of anticholinesterase and antivenom.

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CHAPTER 3

Isolation and Pharmacological Characterisation of Neurotoxins from the Venoms of *Acanthophis* sp. Seram and *Acanthophis rugosus* Death Adders

Summary

1 The present study describes the isolation and characterisation of acantoxin IVa from *A.* sp. Seram venom and acantoxin Va from *A. rugosus* venom. The efficacy of antivenom against the *in vitro* neurotoxicity of acantoxin IVa and acantoxin Va was also determined.

2 Acantoxin IVa (MW 6815; 0.1 – 1.0 μM) and acantoxin Va (MW 7991; 0.1 – 1.0 μM) caused concentration-dependent inhibition of indirect twitches (0.1Hz, 0.2ms, supramaximal V) and blocked contractile responses to exogenous nicotinic agonists in the chick biventer cervicis nerve-muscle (n = 5 – 8), confirming that these toxins are postsynaptic neurotoxins.

3 Prior incubation (10 min) of death adder antivenom (1 unit/ml; n = 7) had no significant effect on the twitch inhibition caused by acantoxin IVa (0.3 μM). However, a higher concentration of antivenom (5 units/ml; n = 4) prevented the twitch inhibition by acantoxin IVa (0.3 μM). In contrast to acantoxin IVa, antivenom (1 unit/ml; n = 4) prevented the twitch inhibition by acantoxin Va (0.3 μM).

4 Acantoxin IVa (1 – 10 nM; n = 4 – 6) and acantoxin Va (30 – 70 nM; n = 4 - 5) caused pseudo-irreversible antagonism at skeletal muscle nicotinic acetylcholine receptors (nAChR) with a depression in maximum response of the cumulative concentration-response curve to carbachol. With an estimated pA_2 of 8.36 ± 0.17, acantoxin IVa was about 2 fold less potent than the long-chain neurotoxin, α-bungarotoxin. Acantoxin Va (pA_2 = 7.72 ± 0.12) was about 10 fold less potent than α-bungarotoxin.

5 With a pK_i value of 4.48, acantoxin IVa (n = 4) was approximately 25,000 fold less potent than α-bungarotoxin at α7-type neuronal nAChR. Acantoxin Va (pK_i = 7.02 ± 0.15; n = 4) was approximately 350 fold more potent than acantoxin IVa. In contrast to α-

bungarotoxin and acantoxin Va, acantoxin IVa completely inhibited specific [^{3}H]-methyllycaconitine ([^{3}H]-MLA) binding in rat hippocampus homogenate (n = 4).

6 Acantoxin IVa (1 nM – 0.1 mM; n = 4) and acantoxin Va (1 nM – 0.01 mM; n = 4) had no activity at α4β2 subtype neuronal nAChR or cytisine-resistant [^{3}H]-epibatidine binding sites. Acantoxin IVa (1 μM; n = 4) was also devoid of activity at ganglionic nAChR. Since only a small amount of acantoxin Va was isolated it was not possible to examine its activity at ganglionic nAChR.

7 In conclusion, the first neurotoxins from *A*. sp. Seram and *A*. *rugosus* death adder venoms were isolated and characterised. In addition, while long-chain neurotoxin resistant [^{3}H]-MLA binding in hippocampus homogenate requires further investigation, we have shown that a short-chain neurotoxin is capable of fully inhibiting specific [^{3}H]-MLA binding.

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

Introduction

Using the chick isolated biventer cervicis nerve-muscle (CBCNM) preparation we have previously shown that *A.* sp. Seram and Irian Jayan death adder (*A. rugosus*) venoms cause concentration-dependent inhibition of indirect twitches and block contractile responses to exogenous nicotinic agonists (Fry *et al.*, 2001). This suggests the presence of postsynaptic neurotoxins in these venoms. The rank order of neurotoxicity showed that *A.* sp. Seram venom was one of the most neurotoxic of the ten death adder venoms tested (Fry *et al.*, 2001; Hodgson & Wickramaratna, 2002). We have also shown that CSL death adder antivenom, raised against *A. antarcticus* venom, was markedly less effective against the neurotoxic effects of both *A.* sp. Seram and *A. rugosus* venoms compared to *A. hawkei*, *A. praelongus* and *A. pyrrhus* venoms (Fry *et al.*, 2001). We postulated that the antivenom may lack the ability to neutralise some neurotoxic components of *A.* sp. Seram and *A. rugosus* venoms (Fry *et al.*, 2001).

Snake α-neurotoxins, also called curaremimetic or postsynaptic neurotoxins, are generally classified as short-chain or long-chain neurotoxins based on their amino acid sequence (Dufton & Harvey, 1989; Endo & Tamiya, 1991). Short-chain neurotoxins consist of 60 to 62 amino acid residues and 4 disulfide bridges (Endo & Tamiya, 1991). Long-chain neurotoxins have 66 to 74 amino acid residues and usually 5 disulfide bridges (Endo & Tamiya, 1991). The major functional difference between the two types of α-neurotoxins was thought to be in the kinetics of association and dissociation with the skeletal muscle nicotinic receptor (Chicheportiche *et al.*, 1975). However, it has now been shown that long-chain neurotoxins containing a fifth disulfide bridge bind to neuronal α7-type nicotinic acetylcholine receptors with higher affinity than short-chain neurotoxins (Servent *et al.*, 1997). Therefore, it should be possible to classify snake postsynaptic

neurotoxins on the basis of pharmacological activity on nicotinic acetylcholine receptors (nAChR).

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 (Arias, 2000). 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 (Paterson & Nordberg, 2000). To date, eight neuronal nAChR α (α2-α7, α9, α10) subunits and three β (β2-β4) subunits have been identified in the mammal, and an additional α subunit, α8, has been identified in the chick optic lobe (Schoepfer *et al.*, 1990; Lindstrom *et al.*, 1996; Lips *et al.*, 2002). 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 (Whiteaker *et al.*, 2000; Dwoskin & Crooks, 2001). 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 (Couturier *et al.*, 1990; Seguela *et al.*, 1993; Elgoyhen *et al.*, 1994; Servent *et al.*, 1997; Utkin *et al.*, 2001). On the other hand, κ-neurotoxins such as neuronal bungarotoxin are selective for α3β2 neuronal nAChR (Harvey & Luetje, 1996; Luetje *et al.*, 1998). Due to its availability and historic significance in this field, the nicotinic receptor pharmacology of α-bungarotoxin has been studied extensively. However, most other snake α-neurotoxins are poorly studied in this respect.

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 (Sheumack *et al.*, 1979; Kim & Tamiya, 1981a,b; Sheumack *et al.*,

1990; Tyler *et al.*, 1997). 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 neurotoxins from the venoms of *A. rugosus* and *A.* sp. Seram death adders and to examine their pharmacological activity on nAChR. In light of our previous findings, the efficacy of CSL death adder antivenom against the *in vitro* neurotoxicity of these toxins was also examined.

Methods

Venom preparation and storage

A. sp. Seram and *A. rugosus* venoms were purchased from Venom Supplies Pty. Ltd., South Australia. Freeze dried venoms and stock solutions of venoms prepared in 0.1% bovine serum albumin (BSA) in 0.9% saline were stored at -20° C until required.

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 (250×20 mm, 10 µm, 300 Å) and Phenomenex Jupiter semi-preparative (250×10 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 to 60% solvent B over sixty min (1% gradient) and then 60% to 80% solvent B in five min (4% gradient). The eluant was monitored at 214 nm 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 (150×2 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 to 20% solvent B over five min (4% gradient), 20% to 60%

solvent B in forty min (1% gradient) and then 60% to 80% solvent B over five min (4% gradient). The eluant was monitored at 214 nm.

Molecular mass determination by electrospray mass spectrometry

The sample was dissolved in 50% acetonitrile and analysed 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 analysed in positive ion mode. Data processing was performed with the aid of the software package Biomultiview (PE-Sciex, Thronton, Canada).

Amino acid sequence determination

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

Chick isolated biventer cervicis nerve-muscle preparation

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 and 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 (Harvey *et al.*, 1994) 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. 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 (Harvey *et al.*, 1994). Electrical stimulation was then recommenced and the preparations were allowed to equilibrate for a further 30 min period before commencement of the experiment. Toxins (0.1 – 1.0 μM) were left in contact with the preparations until complete twitch blockade occurred, or for a 1 h period. Contractile responses to ACh, CCh and KCl were then obtained as previously described. In additional experiments, the reversibility of the toxins (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). Where indicated, CSL death adder antivenom (1 – 5 units/ml) was added 10 min prior (Barfaraz & Harvey, 1994; Crachi *et al.*, 1999a; Fry *et al.*, 2001; Wickramaratna & Hodgson, 2001) to the addition of toxin (0.3 μM).

To further study the activity of the toxins 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), acantoxin IVa (1

– 10 nM) or acantoxin Va (30 – 70 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.

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 acantoxin IVa at ganglionic nAChR, a discrete 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, mecamylamine (0.3 – 10 μM) or toxin (1 μM) 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.

Membrane preparation for radio-ligand binding assays

Female Sprague-Dawley rats weighing 180 – 220 g were gassed with 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 homogenised in 50 volumes of ice-cold

homogenising 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). Homogenised membranes were centrifuged (20000 g, 15 min, 4 °C) and pellets were resuspended in fresh homogenising buffer (Whiteaker *et al.*, 1999). 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.

[^{3}H]-Methyllycaconitine binding assays

Competition binding studies with [^{3}H]-methyllycaconitine ([^{3}H]-MLA) were done according to the method described by Whiteaker *et al.* (1999). 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 ± 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, PerkinElmer 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).

[^{3}H]-Epibatidine binding assays

Binding studies with [^{3}H]-epibatidine were done by modifying a method previously described by Marks *et al.* (1998). 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 ug of protein), binding buffer and 0.4 nM [^{3}H]-epibatidine (Marks *et al.*, 1999).

Chemicals and drugs

The following drugs and chemicals were used: acetonitrile (Fisher Scientific, UK); α-bungarotoxin, acetylcholine chloride, bovine serum albumin (BSA), 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). Death adder antivenom, which is raised against *A. antarcticus* venom in horses, was obtained from CSL Ltd (Melbourne, Australia). [^{3}H]-MLA and [^{3}H]-epibatidine were diluted in binding buffer. Except where indicated, stock solutions were made up in distilled water.

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 corresponding initial response. These were compared against the vehicle control response via a one-way ANOVA. Concentration-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

analysed 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 (ver. 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 (Schild, 1957), Lew and Angus method (Lew & Angus, 1995) or modified Lew and Angus method (Christopoulos *et al.*, 1999; Christopoulos *et al.*, 2001). 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 (Lew & Angus, 1995; Christopoulos *et al.*, 1999):

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

or

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

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 equation 1 the pK_b is given directly as a fitted parameter. However, in equation 2 when s is not significantly different from unity then it is constrained to be as such and hence the pK value is equivalent to pK_b (Christopoulos *et al.*, 1999; Christopoulos *et al.*, 2001). 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 equation 1 with equation 2 using an *F* test (Lew & Angus, 1995).

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 (Christopoulos *et al.*, 1999; Christopoulos *et al.*, 2001). Here the dependent variable (i.e. pEC_{50}) in the equations was replaced with $pEC_{25\%}$ (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.* (1999). 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 analysed 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 (Cheng & Prusoff, 1973):

$$K_i = IC_{50} / 1 + ([D] / K_D)$$

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

Results

Isolation and purification of acantoxin IVa and acantoxin Va

Acantoxin IVa and acantoxin Va were isolated from *A.* sp. Seram and *A. rugosus* venoms, respectively, 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 (Figs. 3.1a & b). Acantoxin IVa eluted as a clean peak separating away from minor contaminants at about 28% solvent B (i.e. at approximately 13 min).

The initial fractionation of *A. rugosus* venom using a preparative column produced eleven major peaks. The third peak was subjected to further purification by RP-HPLC. In order to determine homogeneity and location of acantoxin Va in relation to other peaks of the whole venom both *A. rugosus* venom and acantoxin Va were run on the same conditions using an analytical column (Figs. 3.2a & b). Acantoxin Va eluted as a clean peak separating away from minor contaminants at about 35% solvent B (i.e. at approximately 20 min).

Purity and molecular mass determination

Homogeneity and molecular mass of acantoxin IVa and acantoxin Va were determined by electrospray mass spectrometry (Figs. 3.3a & b). 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 daltons. Similarly, the molecular mass of acantoxin Va

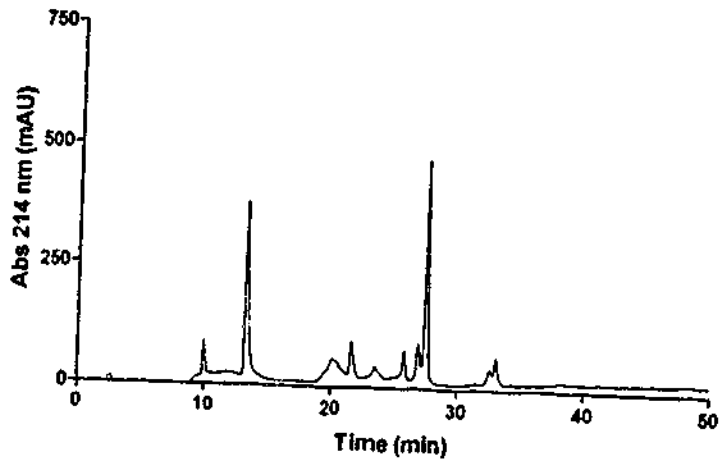
was determined to be 7991.00 ± 1.45 daltons. The estimated molecular masses of previously isolated death adder neurotoxins are indicated in Table 3.1 for comparison.

N-terminal amino acid sequence

The N-terminal amino acid sequence of acantoxin IVa and acantoxin Va was determined (Table 3.1). 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 of acantoxin IVa 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*) venom and taipan toxin 1 (75%) from coastal taipan (*Oxyuranus s. scutellatus*) venom. Acantoxin IVa shared much lower identity with other previously isolated death adder long-chain neurotoxins such as toxin Aa b and acanthophin d.

In contrast to acantoxin IVa, molecular weight gains after reduction/alkylation indicated that acantoxin Va contains ten cysteines. Within the first 20 amino acid residues, the location of half-cystines of acantoxin Va was typical of long-chain neurotoxins (Table 3.1). Acantoxin Va shared highest identity with the long-chain neurotoxins toxin Aa b (80%) and Aa e (75%) isolated from common death adder (*A. antarcticus*) venom.

(a)



(b)

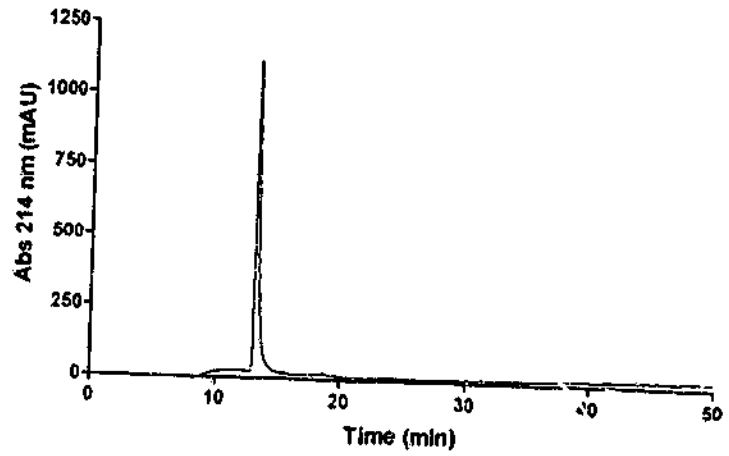
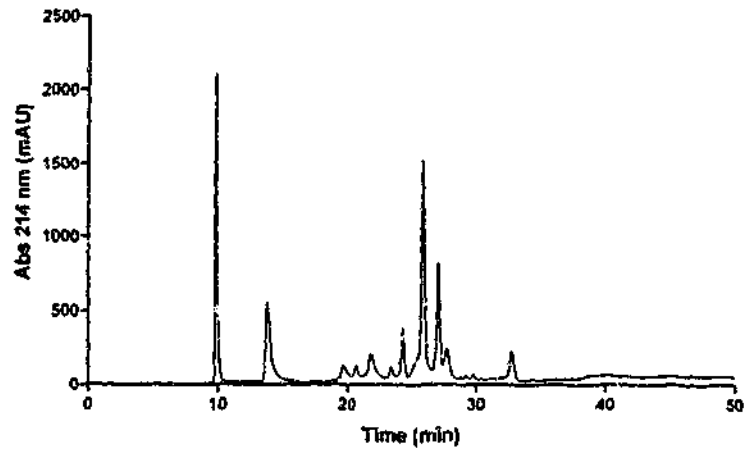


Fig. 3.1 RP-HPLC chromatograph of (a) *A.* sp. Seram venom or (b) acantoxin IVa run on a Jupiter analytical C18 column, equilibrated with solvent A (0.1% TFA) and eluted with the following gradient conditions of solvent B (90% acetonitrile in 0.09% TFA) and solvent A: 0 to 20% solvent B over five min, 20% to 60% solvent B in forty min and then 60% to 80% solvent B over five min.

(a)



(b)

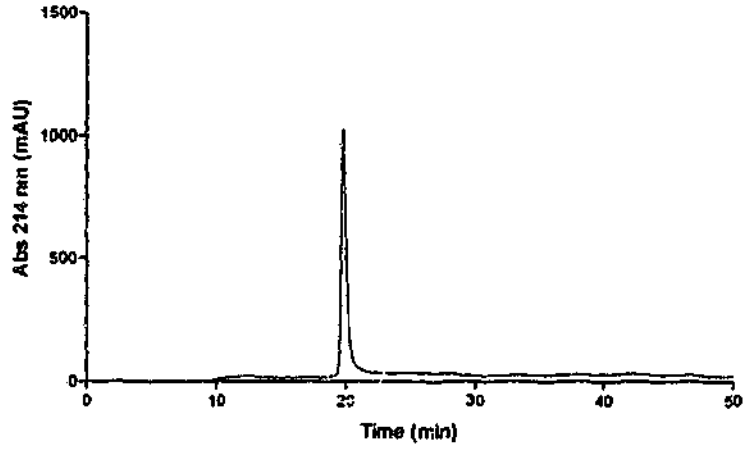
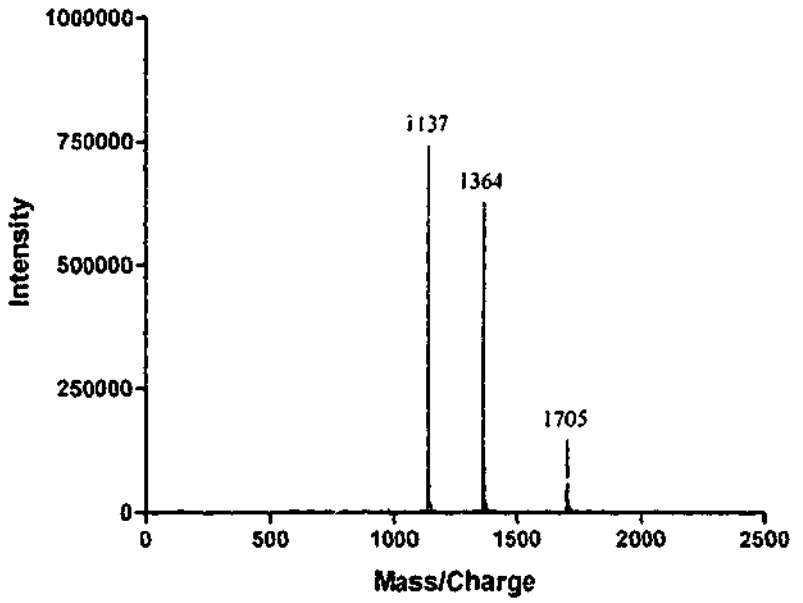


Fig. 3.2 RP-HPLC chromatograph of (a) *A. rugosus* venom or (b) acantoxin Va run on a Jupiter analytical C18 column, equilibrated with solvent A (0.1% TFA) and eluted with the following gradient conditions of solvent B (90% acetonitrile in 0.09% TFA) and solvent A: 0 to 20% solvent B over five min, 20% to 60% solvent B in forty min and then 60% to 80% solvent B over five min.

(a)



(b)

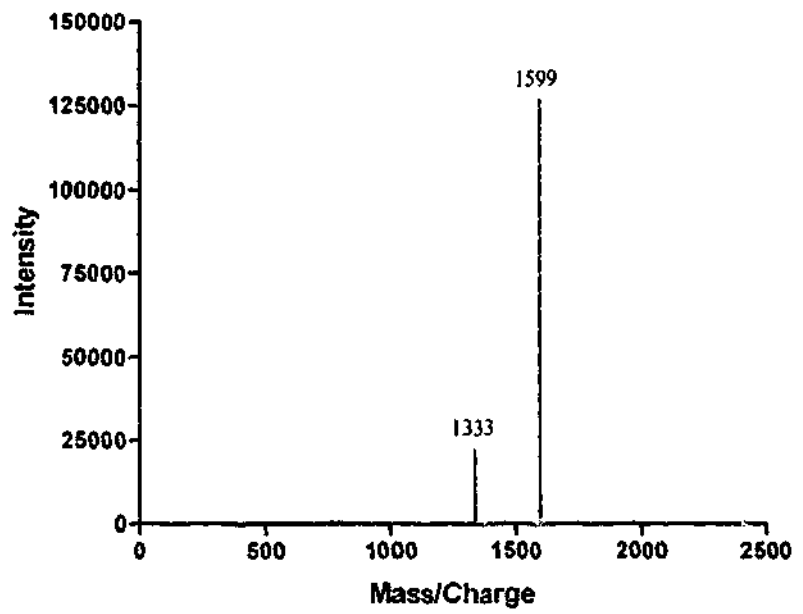


Fig. 3.3 Electrospray mass spectrometry of (a) acantoxin IVa showing a series of multiple-charged ions which could be reconstructed to a single molecular mass of 6815.17 ± 0.55 daltons, and (b) acantoxin Va showing a series of multiple-charged ions which could be reconstructed to a single molecular mass of 7992.00 ± 1.45 daltons.

Table 3.1 Partial N-terminal sequence of neurotoxic components isolated from death adder and some other elapid snake venoms

Species	Neurotoxin	Estimated molecular mass	N-terminal sequence
A. sp. Seram	**acantoxin IVa**	**6815**	**MQCCNQQSSQ PKTTTTCPGG**
A. antarcticus	toxin Aa c[a]	6898	MQCCNQQSSQ PKTTTTCPGG
Pseudechis australis	toxin Pa a[b]	6758	MTCCNQQSSQ PKTTTICAGG
Oxyuranus s. scutellatus	taipan toxin I[c]	6726	MTCYNQQSSE AKTTTTCSGG
A. antarcticus	acanthophin a[d]	7155	Not determined
A. rugosus	**acantoxin Va**	**7992**	**VICYLGYNYA -QPCPPGENVC**
A. antarcticus	toxin Aa b[e]	8135	VICYRGYNNP -QTCPPGENVC
A. antarcticus	acanthophin d[f]	8387	VICYRKYTNN VKTCPDGENVC
A. antarcticus	Aa e[g]	8752	VICYVGYNNP -QTCPPGGNVC

[a] Kim & Tamiya (1981b); [b] Takasaki & Tamiya (1985); [c] Zamudio *et al.* (1996); [d] Sheumack *et al.* (1979); [e] Kim & Tamiya (1981a); [f] Sheumack *et al.* (1990); [g] Tyler *et al.* (1997)

Chick isolated biventer cervicis nerve-muscle preparation

Neurotoxicity studies

While the vehicle (i.e. BSA) had no inhibitory effect on twitch height, acantoxin IVa (0.1 – 1 μM; Fig. 3.4a) and acantoxin Va (0.1 – 1 μM; Fig. 3.5a) caused time-dependent inhibition of indirect twitches (n = 5 – 8). 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). The t_{90} values for 0.1, 0.3 and 1.0 μM acantoxin Va were 73.9 ± 8.9, 24.3 ± 2.5 and 12.1 ± 0.8 min, respectively (n = 5). Acantoxin IVa caused concentration-dependent inhibition of indirect twitches (n = 5; one-way ANOVA of t_{90} values, $P < 0.05$; Fig. 3.4a). Similarly, acantoxin Va caused concentration-dependent inhibition of indirect twitches (n = 5; one-way ANOVA of t_{90} values, $P < 0.05$; Fig. 3.5a).

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. 3.4b; data not shown for 0.1 and 1 μM acantoxin IVa). Similarly, acantoxin Va (0.1 – 1 μM) significantly inhibited contractile responses to exogenous ACh and CCh, but not KCl, compared to vehicle (n = 5 – 8; one-way ANOVA, $P < 0.05$; Fig. 3.5b; data not shown for 0.1 and 1 μM acantoxin Va).

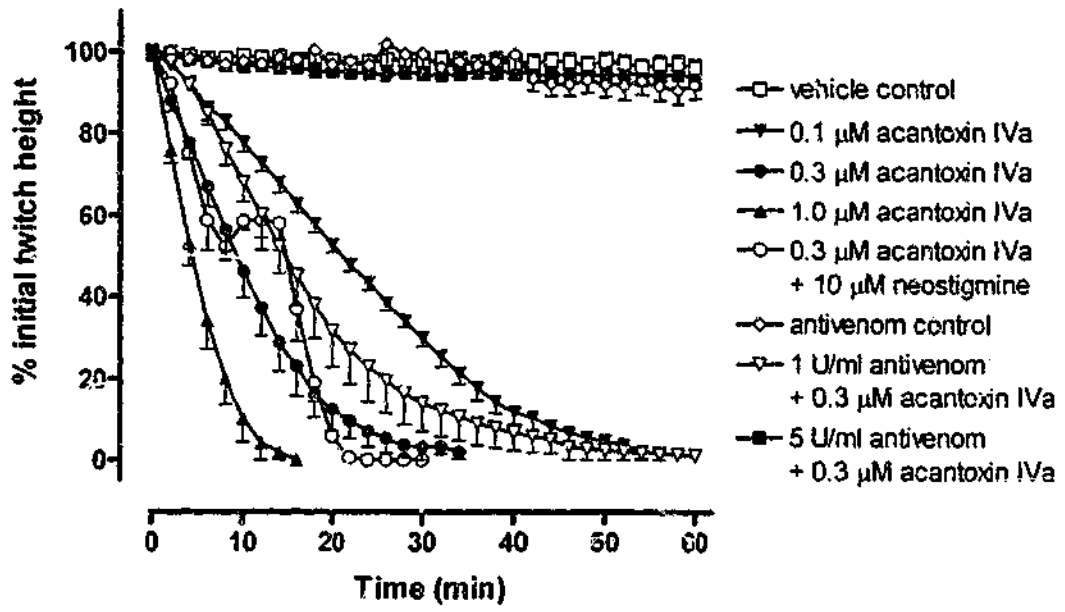
Toxin reversal and antivenom studies

Washing of the preparation every 10 min for 3 h, after acantoxin IVa (0.3 μM) or acantoxin Va (0.3 μM) had caused complete twitch blockade, resulted in 0% and 4.7 ± 2.7% recovery of indirect twitches, respectively (n = 4; data not shown). When neostigmine (10 μM) was added at t_{50}, i.e. after the addition of acantoxin IVa (0.3 μM) or acantoxin Va (0.3 μM), no sustained recovery of the twitch height was observed in either

case (n = 4; Figs. 3.4a & 3.5a). 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). Similarly, with a t_{90} value of 23.9 ± 2.3 min for acantoxin Va (0.3 μM) plus neostigmine (10 μM) there was no significant difference between the t_{90} values when compared with acantoxin Va (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 ± 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 abolished by acantoxin IVa (0.3 μM) plus neostigmine (10 μM; n = 4; Fig. 3.4b) and acantoxin Va (0.3 μM) plus neostigmine (10 μM; n = 4; Fig. 3.5b).

Prior incubation (10 min) of death adder antivenom (1 unit/ml) only slightly delayed the twitch inhibition caused by acantoxin IVa (0.3 μM; n = 7; Fig. 3.4a). However, with a t_{90} value of 31.3 ± 5.1 min, antivenom (1 unit/ml) plus acantoxin IVa (0.3 μM) was not significantly different from acantoxin IVa (0.3 μM) alone (n = 5 – 7; Student's unpaired t-test). Furthermore, acantoxin IVa (0.3 μM) in the presence of antivenom (1 unit/ml) still abolished contractile responses to exogenous nicotinic agonists (n = 7; Fig. 3.4b). However, a higher concentration of antivenom (5 units/ml) prevented the twitch inhibition by acantoxin IVa (0.3 μM; n = 4; Fig. 3.4a). Antivenom (5 units/ml) also prevented the inhibition of contractile responses to exogenous nicotinic agonists caused by acantoxin IVa (0.3 μM; n = 4; Fig. 3.4b).

(a)



(b)

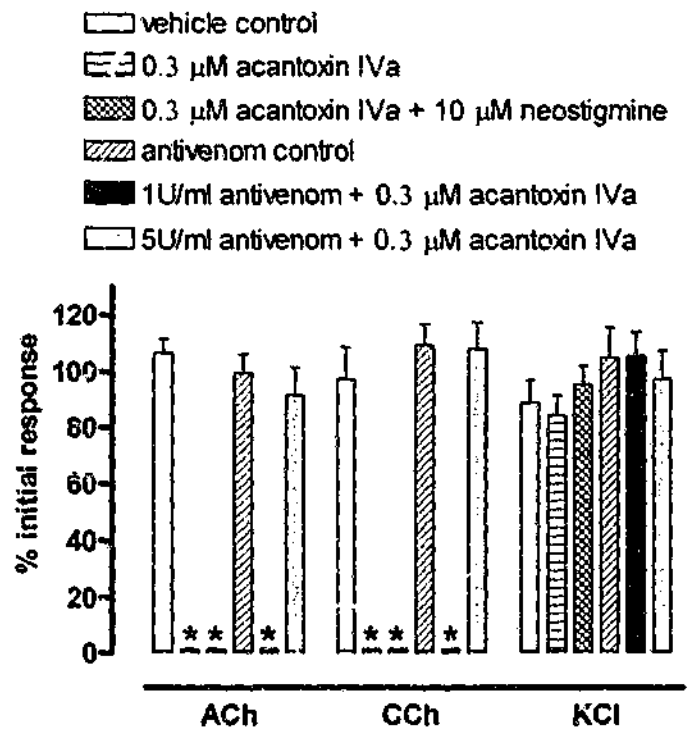
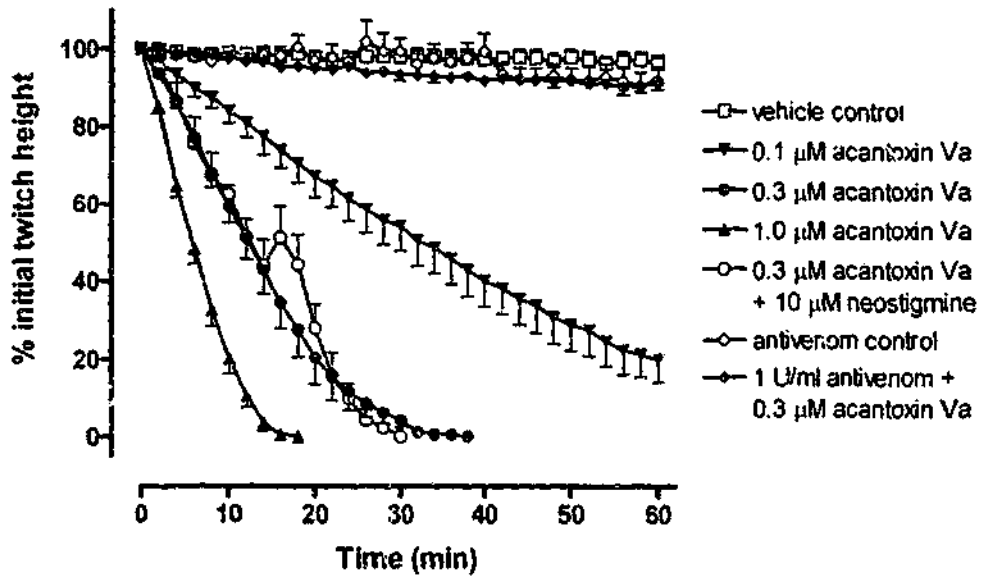


Fig. 3.4 The effect of acantoxin IVa (0.1 – 1 μM; n = 5), acantoxin IVa (0.3 μM; n = 4) with neostigmine (10 μM) at t_{50}, acantoxin IVa (0.3 μM; n = 4 – 7) in the presence of antivenom (1 – 5 units/ml), or vehicle (n = 8) on (a) indirect twitches or (b) contractile responses to exogenous ACh, CCh and KCl in the CBCNM preparation (data not shown for 0.1 & 1 μM acantoxin IVa). *$P < 0.05$, significantly different from contractile responses in the presence of vehicle, one-way ANOVA.

(a)



(b)

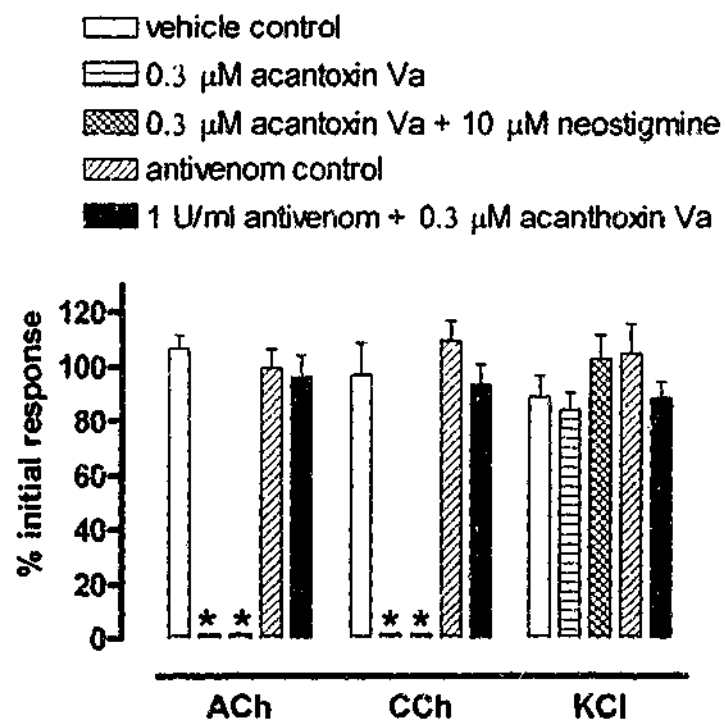


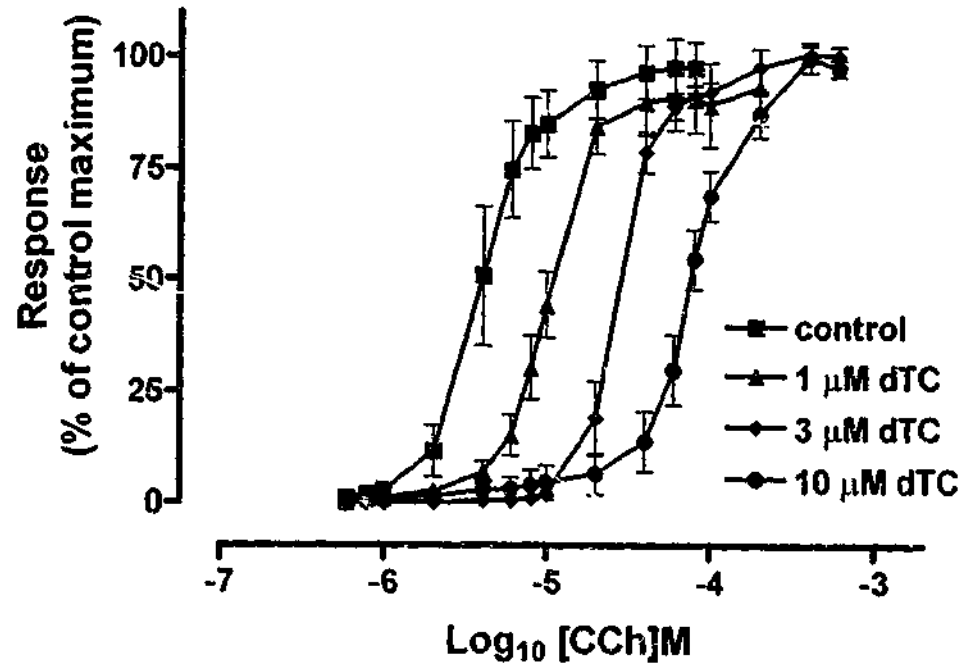
Fig. 3.5 The effect of acantoxin Va (0.1 – 1 μM; n = 5), acantoxin Va (0.3 μM; n = 4) with neostigmine (10 μM) at t_{50}, acantoxin Va (0.3 μM; n = 4) in the presence of antivenom (1 unit/ml), or vehicle (n = 8) on (a) indirect twitches or (b) contractile responses to exogenous ACh, CCh and KCl in the CBCNM preparation (data not shown for 0.1 & 1 μM acantoxin Va). *$P < 0.05$, significantly different from contractile responses in the presence of vehicle, one-way ANOVA.

In contrast to acantoxin IVa, prior incubation of death adder antivenom (1 unit/ml) prevented the twitch inhibition by acantoxin Va (0.3 μM; n = 4; Fig. 3.5a). Antivenom (1 unit/ml) also prevented the inhibition of contractile responses to exogenous nicotinic agonists caused by acantoxin Va (0.3 μM; n = 4; Fig. 3.5b).

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 displayed no depression of the maximum response (n = 5 - 6; Fig. 3.6a). 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 (NSDU) since equation 1 was deemed to be a better fit by using an *F* test as stated in the "Methods" section. 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; n = 4 – 6; Fig. 3.6b), acantoxin Va (30 – 70 nM; n = 4 – 5; Fig. 3.7a) and α-bungarotoxin (2 – 7 nM; n = 4 – 5; Fig. 3.7b) caused a concentration-dependent depression of the maximum CCh response (one-way ANOVA, $P < 0.05$). Therefore, the modified Lew and Angus method was used to determine the potency of these toxins at skeletal muscle nAChR (Table 3.2). In all three cases the slope factor was found to be NSDU since equation 1 was deemed to be a better fit by using an *F* test as stated in the "Methods" section.

(a)



(b)

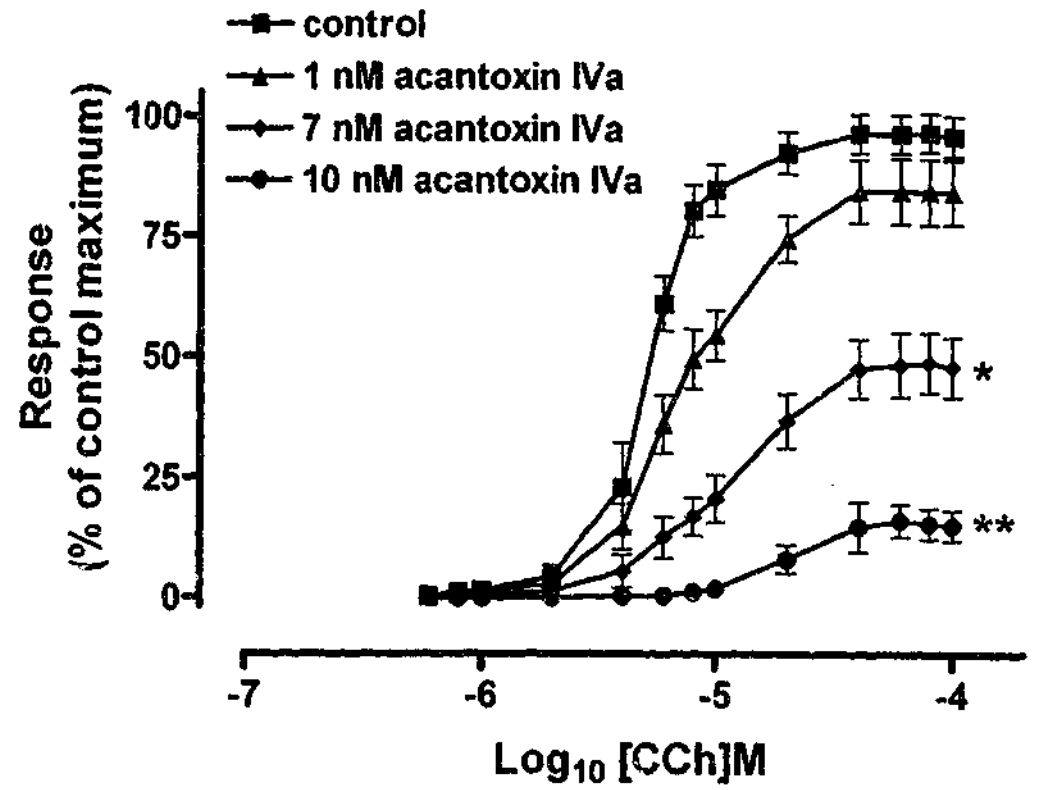
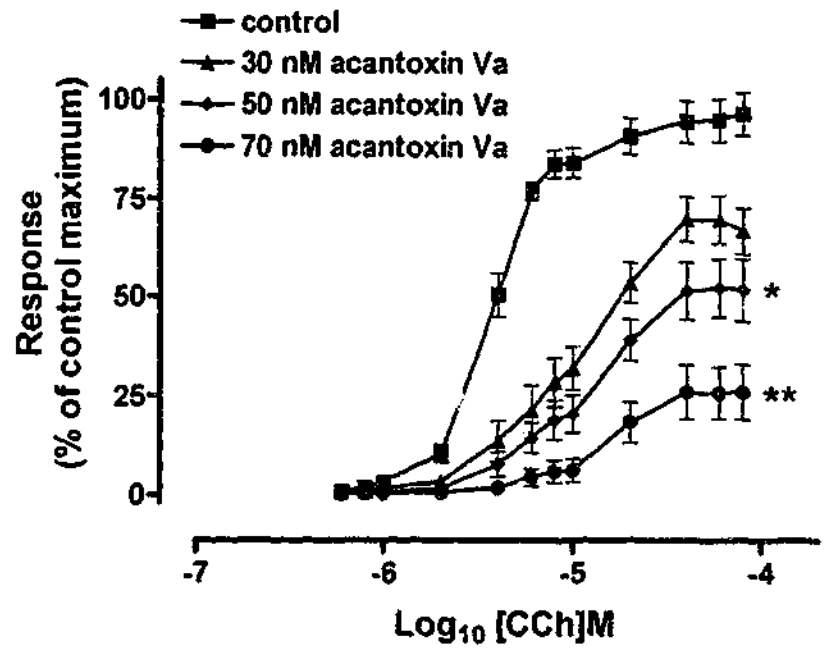


Fig. 3.6 The effect of (a) dTC (1 – 10 μM; n = 5 – 6) or (b) acantoxin IVa (1 – 10 nM; n = 4 – 6) on responses to cumulative additions of carbachol in the CBCNM preparation. *$P < 0.05$, significantly different from 1 nM acantoxin IVa, one-way ANOVA. **$P < 0.05$, significantly different from 7 nM acantoxin IVa, one-way ANOVA.

(a)



(b)

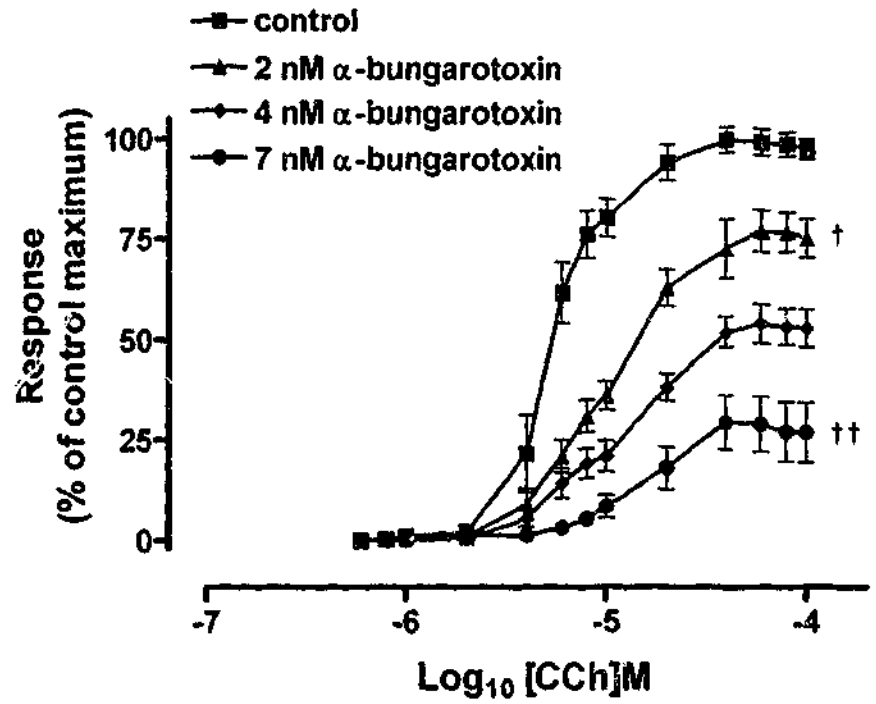


Fig. 3.7 The effect of (a) acantoxin Va (30 – 70 nM; n = 4 – 5) or (b) α-bungarotoxin (2 – 7 nM; n = 4 – 5) on responses to cumulative additions of carbachol in the CBCNM preparation. *$P < 0.05$, significantly different from control, one-way ANOVA. **$P < 0.05$, significantly different from 30 nM acantoxin Va, one-way ANOVA. †$P < 0.05$, significantly different from control, one-way ANOVA. ††$P < 0.05$, significantly different from 2 nM α-bungarotoxin, one-way ANOVA.

Table 3.2 Potency of toxins at skeletal muscle and α7-type nicotinic acetylcholine receptors

Toxin	Skeletal muscle nACR		α7-type nAChR
	pA_2[a]	Slope[b]	pK_i
α-bungarotoxin	8.71 ± 0.06	NSDU	8.89 ± 0.13
acantoxin IVa	8.36 ± 0.17	NSDU	4.48 ± 0.13
acantoxin Va	7.72 ± 0.12	NSDU	7.02 ± 0.15

[a] pA_2 value estimated by the modified Lew and Angus method

[b] NSDU - not significantly different from unity

Ganglionic nicotinic acetylcholine receptors

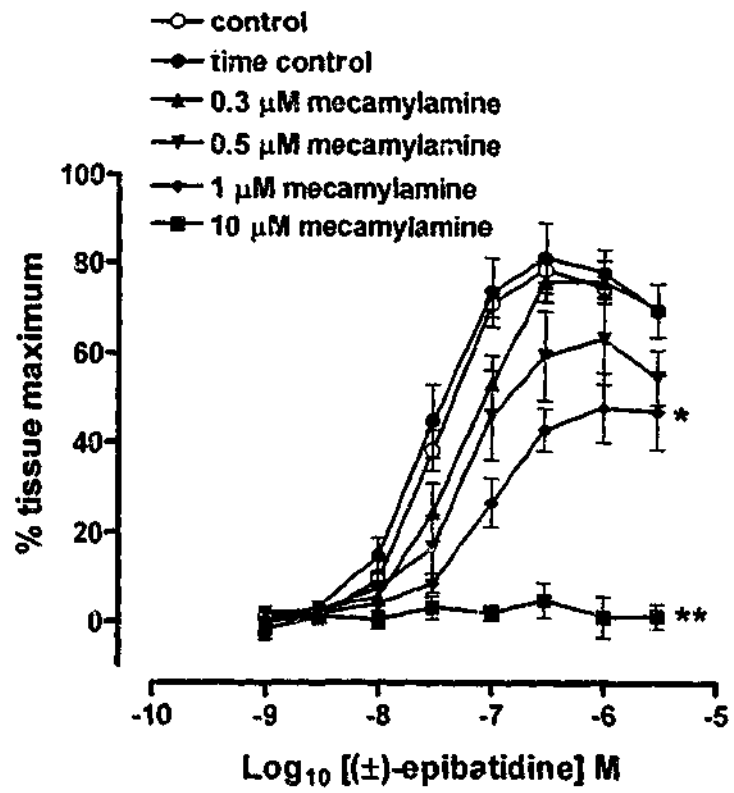
Discrete concentration-response curves to (±)-epibatidine were repeatable after an incubation period of 1 h with no desensitisation being observed (n = 8; Fig. 3.8a). Mecamylamine (0.3 – 10 μM) caused a concentration-dependent depression of the maximum response (n = 4 – 8; one-way ANOVA, $P < 0.05$; Fig. 3.8a). 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. 3.8b).

NB: Since only a small quantity of acantoxin Va was isolated it was not possible to examine its activity at ganglionic nAChR.

α7-type neuronal nicotinic acetylcholine receptors

In hippocampus homogenate, unlabelled MLA, α-bungarotoxin and acantoxin Va were potent inhibitors of specific [^{3}H]-MLA binding (n = 4; Fig. 3.9). A one-site binding model was deemed to be a better fit by using an *F* test as stated in the "Methods" section. While α-bungarotoxin (pK_i = 8.89 ± 0.13) seemed to be slightly more potent than unlabelled MLA (pK_i = 8.22 ± 0.12) there was no significant difference in potency (n = 4; one-way ANOVA, $P < 0.05$). Acantoxin IVa (n = 4; Fig. 3.9; Table 3.2) was ≈25,000 fold less potent than α-bungarotoxin at α7-type neuronal nAChR. In contrast, acantoxin Va (n = 4; Fig. 3.9; Table 3.2) was ≈75 fold less potent than α-bungarotoxin, but ≈350 fold more potent than acantoxin IVa. While both unlabelled MLA and acantoxin IVa completely inhibited specific binding, this was not the case with α-bungarotoxin or acantoxin Va (Fig. 3.9). Approximately 8% and 16% of specific binding was resistant to high concentrations of α-bungarotoxin and acantoxin Va, respectively. Non-specific binding was close to 30%

(a)



(b)

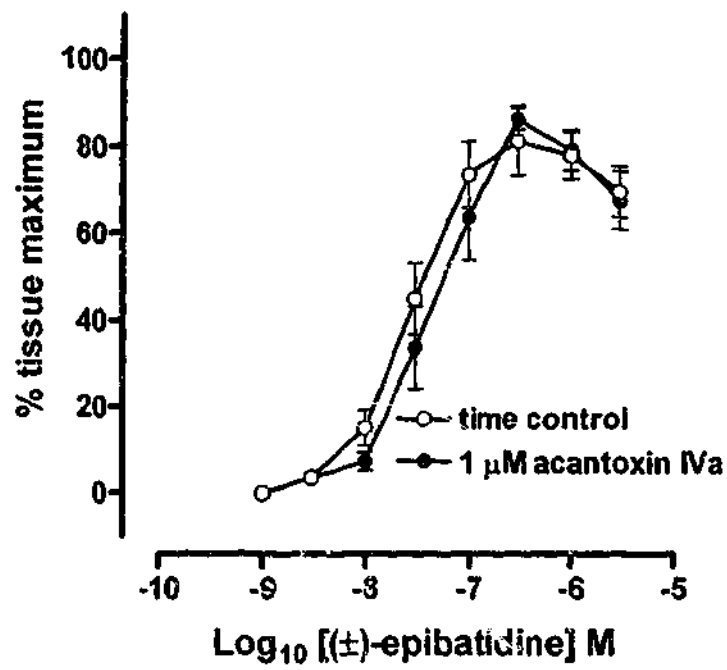


Fig. 3.8 The effect of (a) mecamylamine (0.3 – 10 μM; n = 4 – 8) or (b) acantoxin IVa (1 μM; n = 4) on responses to discrete additions of (±)-epibatidine in the guinea-pig ileum. *$P < 0.05$, significantly different from 0.3 μM mecamylamine, one-way ANOVA. **$P <$ 0.05, significantly different from 1 μM mecamylamine, one-way ANOVA.

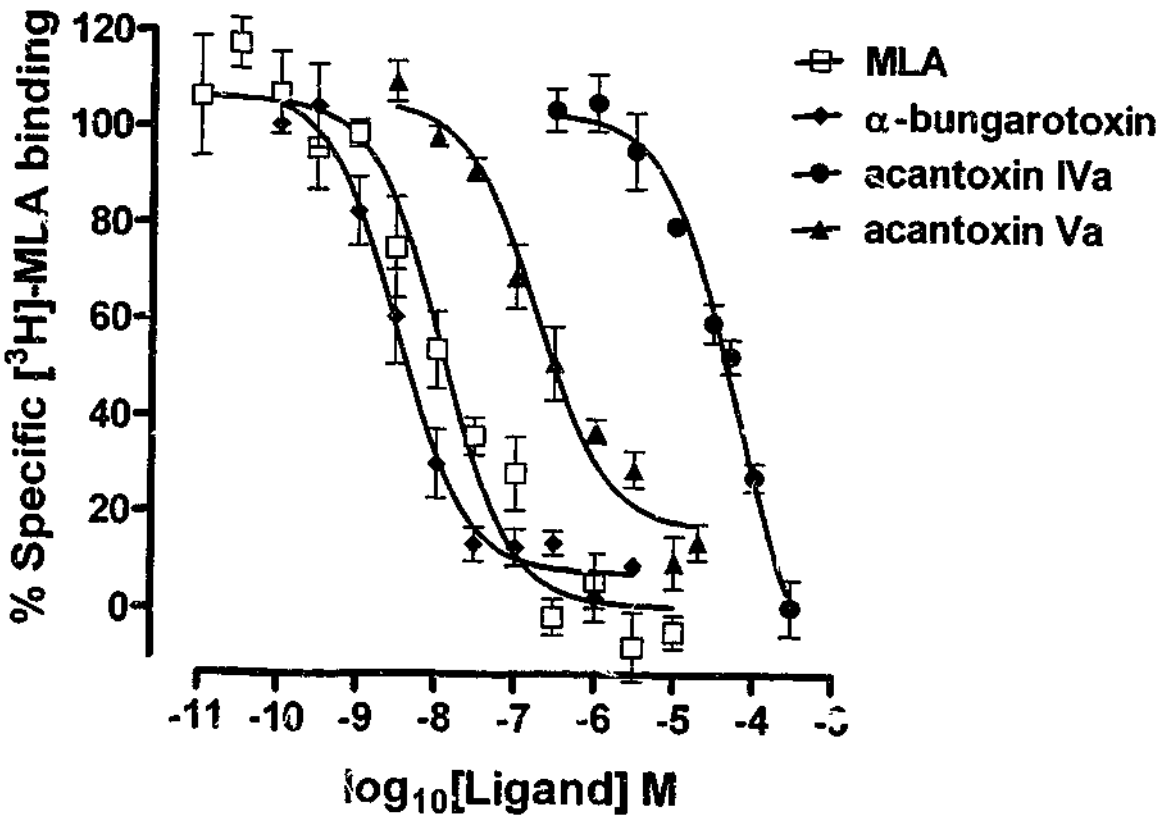


Fig. 3.9 Inhibition of specific [^{3}H]-MLA binding by unlabelled MLA (n = 4), α-bungarotoxin (n = 4), acantoxin IVa (n = 4) and acantoxin Va (n = 4) in rat hippocampus homogenate. Data were fitted to a one-site binding model.

of total binding. This non-specific binding was comparable to levels reported in previous studies (Davies *et al.*, 1999; Whiteaker *et al.*, 1999).

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 the "Methods" section (n = 4; Fig. 3.10). 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. 3.11). A two-site binding model was deemed to be a better fit by using an *F* test as stated in the "Methods" section. (-)-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. 3.11). Similarly, Acantoxin Va caused no inhibition of specific binding up to a concentration of 0.01 mM (n = 4; Fig. 3.11). Non-specific binding was less than 6% of total binding.

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. 3.12). This binding followed a one-site binding model with an IC_{50} of 0.96 ± 0.14 μM (n = 4). In contrast, acantoxin IVa or acantoxin Va caused no inhibition of binding up to a concentration of 0.1 mM and 0.01 mM, respectively (n = 4; Fig. 3.12). Non-specific binding was about 18% of total binding.

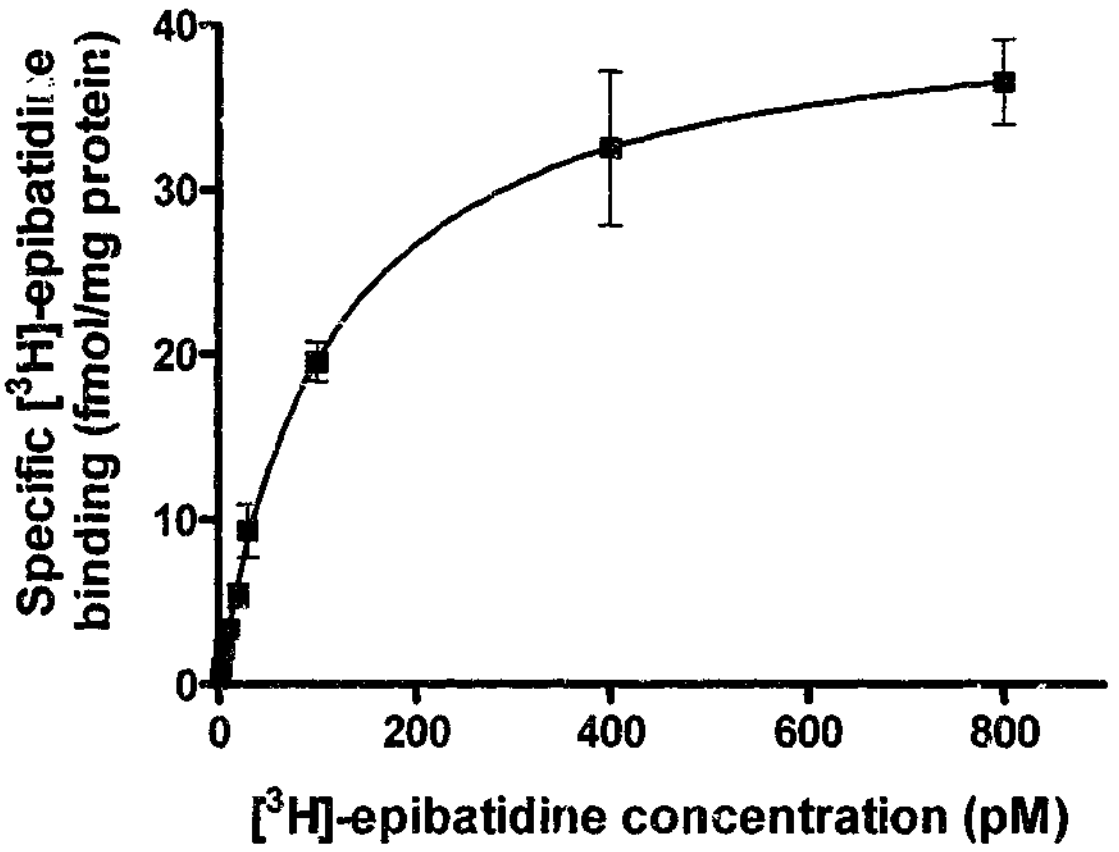


Fig. 3.10 Saturation binding of [^{3}H]-epibatidine to rat midbrain homogenate (n = 4). Data were fitted to a one-site binding model.

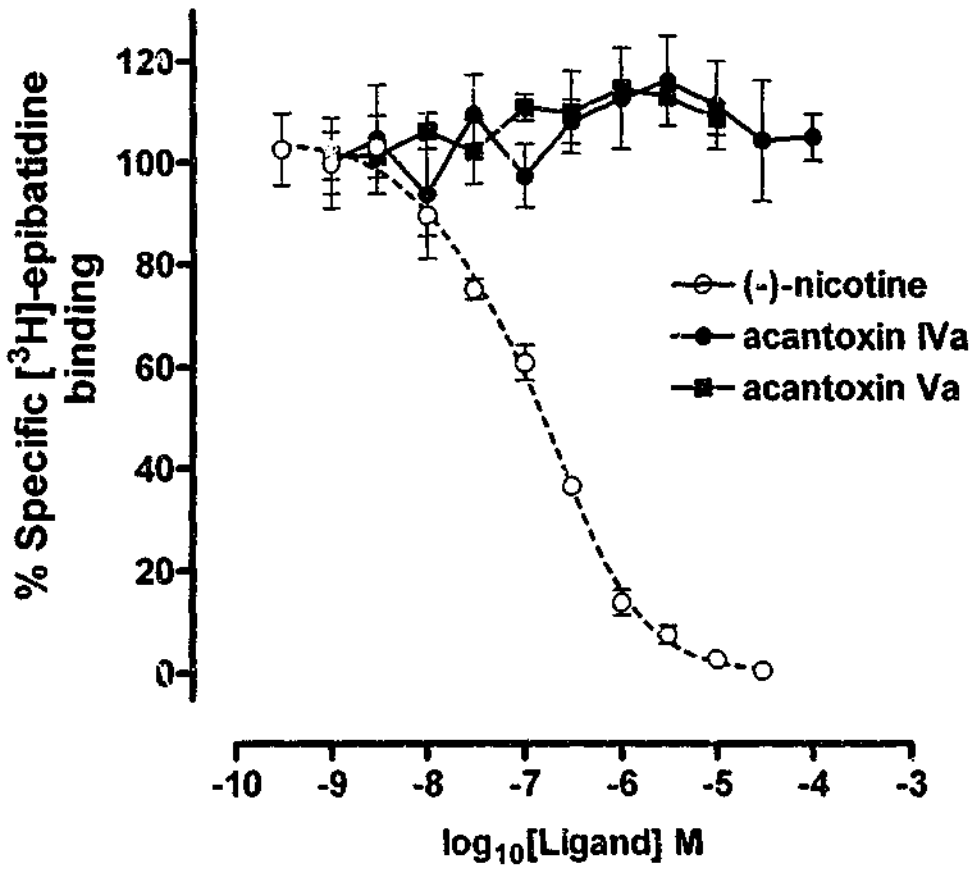


Fig. 3.11 Inhibition of specific [^{3}H]-epibatidine binding by (-)-nicotine (n = 4), acantoxin IVa (n = 4) and acantoxin Va (n = 4) in rat midbrain homogenate. Dashed line represents data fitted to a two-site binding model.

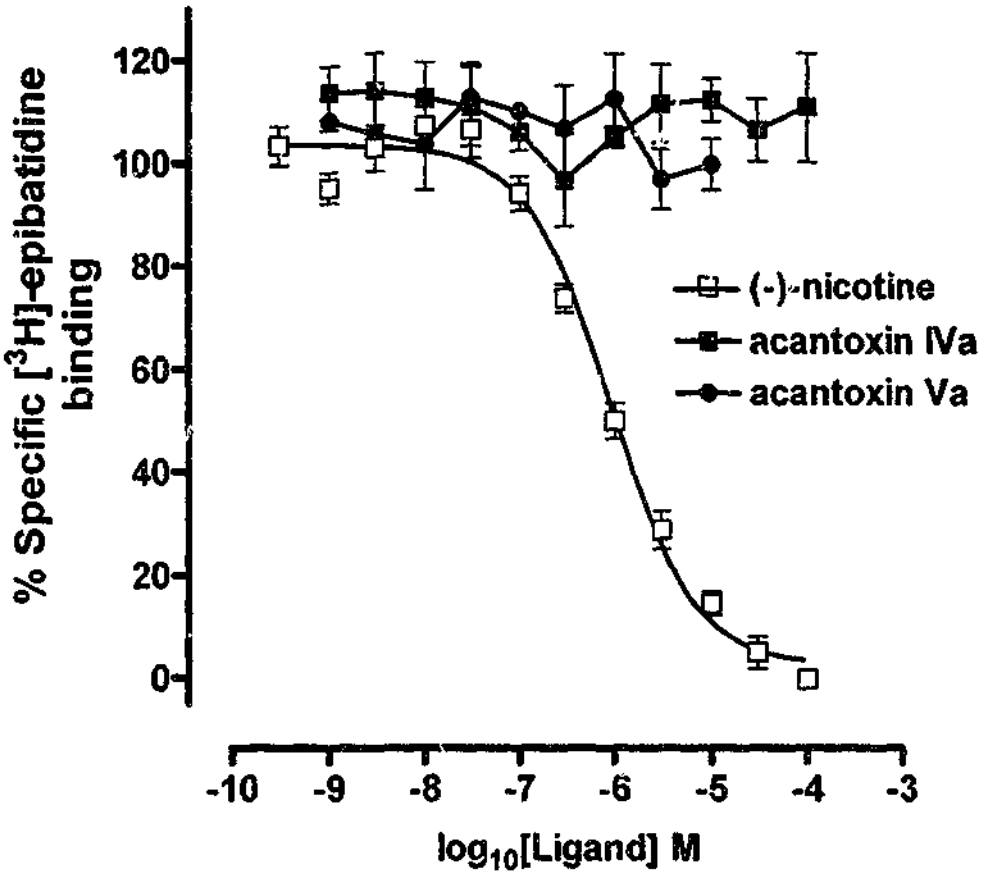


Fig. 3.12 Inhibition of specific [^{3}H]-epibatidine binding by (-)-nicotine (n = 4), acantoxin IVa (n = 4) and acantoxin Va (n = 4) in rat midbrain homogenate with the presence of cytisine (100 nM). Unbroken line represents data fitted to a one-site binding model.

Discussion

We have previously shown, based on *in vitro* studies, that death adder venoms are among the most neurotoxic Australasian venoms (Crachi *et al.*, 1999b; Fry *et al.*, 2001; Hodgson & Wickramaratna, 2002). With five postsynaptic neurotoxins isolated from *A. antarcticus* venom, death adder venoms are thought to be rich in these components. 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 and characterisation of the first neurotoxins from *A.* sp. Seram and *A. rugosus* death adder venoms. The efficacy of CSL death adder antivenom against the *in vitro* neurotoxicity of acantoxin IVa and acantoxin Va was also determined.

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. In contrast, acantoxin Va elutes as the third peak and is a minor component of *A. rugosus* venom. Using electrospray mass spectrometry, the molecular mass of acantoxin IVa was determined to be 6815 daltons. It is well documented that elapid snake venom postsynaptic neurotoxins usually have a molecular mass in the range of 6 – 9 kDa (Zamudio *et al.*, 1996; Tyler *et al.*, 1997; Fry, 1999). In fact, a molecular mass of 6815 daltons is in the range expected for short-chain neurotoxins as opposed to long chain neurotoxins (Fry, 1999). 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 daltons, respectively, calculated from their amino acid composition (Sheumack *et al.*, 1979; Kim & Tamiya, 1981a). With a

molecular mass of 7991 daltons, acantoxin Va is smaller than toxin Aa b, acanthophin d and Aa e, however, it is in the range expected for long-chain neurotoxins (Kim & Tamiya, 1981b; Sheumack *et al.*, 1990; Tyler *et al.*, 1997; Fry, 1999). Comparison of the N-terminal sequences showed that acantoxin IVa shares very high identity with other short-chain neurotoxins from elapid snake venoms, in particular toxin Aa c. In contrast, acantoxin Va shares high identity with previously isolated long-chain neurotoxins from *A. antarcticus* venom.

Due to the sequence homology and molecular mass resemblance of acantoxin IVa and acantoxin Va to other elapid venom postsynaptic neurotoxins, these components were examined for *in vitro* neurotoxicity using the indirectly stimulated CBCNM preparation. Both acantoxin IVa and acantoxin Va caused concentration-dependent inhibition of indirect twitches and blocked contractile responses to exogenous nicotinic agonists. Thus, confirming that these components are indeed postsynaptic neurotoxins. However, unlike d-tubocurarine or the snake venom neurotoxin candoxin (Nirthanan *et al.*, 2002a), neuromuscular blockade by acantoxin IVa or acantoxin Va was not readily reversible by washing. To further study the reversibility of these toxins, the anticholinesterase neostigmine was added at the t_{50} time point (i.e. after the addition of either acantoxin IVa or acantoxin Va had reduced the twitch height by 50%). Although there was a transient recovery of the neuromuscular blockade with neostigmine this was quickly overcome by the toxins. Similar observations were made when neostigmine (10 μM) was added at t_{50} after the addition of *A.* sp. Seram venom or *A. rugosus* venom (3 μg/ml; refer chapter 2). However, several clinicians have used anticholinesterases successfully to reduce the amount of antivenom administered following envenomation by death adders in Australia and Papua New Guinea (Currie *et al.*, 1988; Lalloo *et al.*, 1996; Little & Pereira, 2000).

Perhaps, a more convincing reversal with neostigmine would have been obtained had a much lower concentration of these toxins been examined.

Since our previous study showed that CSL death adder antivenom was markedly less effective against *A.* sp. Seram and *A. rugosus* venoms compared to other death adder venoms (Fry *et al.*, 2001), the efficacy of antivenom against the *in vitro* neurotoxicity of acantoxin IVa and acantoxin Va was examined. Prior incubation of antivenom (1 unit/ml) had no significant effect on the twitch inhibition caused by acantoxin IVa. However, under the same conditions antivenom prevented the twitch inhibition produced by acantoxin Va. This suggests that acantoxin IVa is one of the neurotoxic components present in *A.* sp. Seram venom that is not readily neutralised by death adder antivenom. The same cannot be said of acantoxin Va. The higher concentration of antivenom (i.e. 5 units/ml) was successful in preventing the twitch inhibition caused by acantoxin IVa. Therefore, as with the *A.* sp. Seram whole venom (Fry *et al.*, 2001; refer chapter 2), a much larger dose of antivenom is required to prevent the neurotoxicity of acantoxin IVa.

In order to determine the potency of acantoxin IVa and acantoxin Va at skeletal muscle nAChR, the CBCNM unstimulated preparation was utilised. 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, acantoxin Va or α-bungarotoxin. These snake neurotoxins produced depression of the maximum CCh response. It is well known that α-bungarotoxin and most elapid postsynaptic neurotoxins dissociate very slowly from the skeletal muscle nAChR once they are bound (Chicheportiche *et al.*, 1975; Blanchard *et al.*, 1979; Chiappinelli, 1991). Given the pseudo-irreversible antagonism caused by these toxins, the observed depression of the

cumulative concentration-response curve to CCh is to be expected (Lew *et al.*, 2000). Pseudo-irreversible antagonism is a distinct pharmacological property of acantoxin IVa and acantoxin Va and is not caused by every elapid venom neurotoxin (Nirthanan *et al.*, 2002a). However, given that these snake neurotoxins caused depression of the maximum CCh response, the classical Schild plot analysis cannot be utilised 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 (Christopoulos *et al.*, 1999; Christopoulos *et al.*, 2001). In the current study, the pA_2 value of dTC determined via the modified Lew and Angus method was in 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 (Lew & Angus, 1995). With an estimated pA_2 value of 8.36, acantoxin IVa is at least 100 fold more potent than d-tubocurarine and only about 2 fold less potent than α-bungarotoxin. With an estimated pA_2 value of 7.72, acantoxin Va is about 10 fold less potent than α-bungarotoxin but only 4 fold less potent than acantoxin IVa. 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 (Lew & Angus, 1995; Christopoulos *et al.*, 1999).

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 (Hayashi *et al.*, 1977). 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 (Nirthanan *et al.*, 2002b). Given the requirement for discrete additions of (±)-epibatidine (i.e. due to receptor desensitisation), large amounts of toxin are consumed during this assay. Hence, the activity of acantoxin Va was not examined at this receptor.

Hippocampus homogenate was utilised to study the activity of acantoxin IVa and acantoxin Va at α7-type neuronal nAChR as it has been shown to be rich in this nicotinic receptor (Davies *et al.*, 1999; Whiteaker *et al.*, 1999). 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 fold less potent than the long-chain neurotoxin, α-bungarotoxin, at this receptor. While acantoxin Va was only 10 fold less potent than α-bungarotoxin at skeletal muscle nAChR, it was about 75 fold less potent than α-bungarotoxin at α7-type neuronal nAChR. Therefore, it would be of interest to elucidate the full primary sequence and the NMR structure of acantoxin Va to allow for detailed structure-function studies. Previously, it has been shown in both chick optic lobe homogenate and chimeric α7 receptors that long-chain neurotoxins are 300 – 20,000 fold more potent at α7-type neuronal nAChR than short-chain neurotoxins (Zamudio *et al.*, 1996; Servent *et al.*, 1997). Thus, in line with the molecular mass and N-terminal sequence, acantoxin IVa behaves like a short-chain neurotoxin pharmacologically. Also in line with the molecular mass and N-terminal sequence, acantoxin Va behaves like a long-chain neurotoxin.

Interestingly, while both acantoxin IVa and unlabelled MLA completely inhibited specific [^{3}H]-MLA binding, this was not the case with α-bungarotoxin or acantoxin Va. 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 (Davies *et al.*, 1999; Whiteaker *et al.*, 1999).

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 (Davies *et al.*, 1999; Whiteaker *et al.*, 1999). As suggested by Davies *et al.* (1999) and Whiteaker *et al.* (1999), 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 for the first time that a short-chain neurotoxin (only about 1 kD smaller than α-bungarotoxin) is capable of fully inhibiting specific [^{3}H]-MLA binding in hippocampus homogenate.

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 (Dajas-Bailador *et al.*, 1998). Hence, acantoxin IVa and acantoxin Va were examined for activity at other neuronal nAChR. Both acantoxin IVa and acantoxin Va 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 (Whiteaker *et al.*, 2000).

In conclusion, the first neurotoxins from *A.* sp. Seram and *A. rugosus* death adder venoms were isolated. Antivenom studies showed that acantoxin IVa is a potent neurotoxic component present in *A.* sp. Seram venom that is not readily neutralised by death adder antivenom. In contrast, acantoxin Va was readily neutralised by antivenom. Although acantoxin IVa has a similar potency to α-bungarotoxin for skeletal muscle nAChR it is about 25,000 fold less potent at α7-type nAChR. While acantoxin Va was several fold less

potent than acantoxin IVa at skeletal muscle nAChR, it was about 350 foid more potent than acantoxin IVa at α7-type neuronal nAChR. Although 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 studies are required to determine whether short-chain neurotoxins preferentially bind to another neuronal nAChR compared to long-chain neurotoxins.

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CHAPTER 4

Isolation and Pharmacological Characterization of a Phospholipase A_2 Myotoxin from the Venom of the Irian Jayan Death Adder (*Acanthophis rugosus*)

Declaration for Thesis Chapter 4

This chapter is made up of the following publication:

Isolation and pharmacological characterization of a phospholipase A_2 myotoxin from the venom of the Irian Jayan death adder (*Acanthophis rugosus*)

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I/we declare that over 80 % of this work has been done by the candidate. This manuscript has been written solely by the candidate taking into consideration the advice and recommendations of co-authors. Dr. Bryan Fry was responsible for obtaining the N-terminal amino acid sequence of acanmyotoxin-1. Dr. Bryan Fry also supervised the candidate during the isolation and purification of acanmyotoxin-1 while the candidate was in Prof. Manjunatha Kini's laboratory, Department of Biological Sciences, Faculty of Science, National University of Singapore, Singapore. Dr Marie-Isabel Aguilar (co-supervisor) supervised the candidate during the HPLC analysis of acanmyotoxin-1 in the Department of Biochemistry and Molecular Biology, Monash University. Prof. Manjunatha Kini provided valuable advice on the 4-BPB modification of acanmyotoxin-1. All other experiments were performed by the candidate.

The original data are stored at the Department of Pharmacology, Monash University, Clayton Campus, Australia and will be held for at least seven years from the date of publication.

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Isolation and pharmacological characterization of a phospholipase A_2 myotoxin from the venom of the Irian Jayan death adder (*Acanthophis rugosus*)

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1 It has long been thought that death adder venoms are devoid of myotoxic activity based on studies done on *Acanthophis antarcticus* (Common death adder) venom. However, a recent clinical study reported rhabdomyolysis in patients following death adder envenomations, in Papua New Guinea, by a species thought to be different to *A. antarcticus*. Consequently, the present study examined *A. rugosus* (Irian Jayan death adder) venom for myotoxicity, and isolated the first myotoxin (acanmyotoxin-1) from a death adder venom.

2 *A. rugosus* (10–50 $\mu g\ ml^{-1}$) and acanmyotoxin-1 (MW 13811; 0.1–1 μM) were screened for myotoxicity using the chick directly (0.1 Hz, 2 ms, supramaximal V) stimulated biventer cervicis nerve-muscle (CBCNM) preparation. A significant contracture of skeletal muscle and/or inhibition of direct twitches were considered signs of myotoxicity. This was confirmed by histological examination.

3 High phospholipase A_2 (PLA_2) activity was detected in both *A. rugosus* venom ($140.2 \pm 10.4\ \mu mol\ min^{-1}\ mg^{-1}$; $n=6$) and acanmyotoxin-1 ($153.4 \pm 11\ \mu mol\ min^{-1}\ mg^{-1}$; $n=6$). Both *A. rugosus* venom (10–50 $\mu g\ ml^{-1}$) and acanmyotoxin-1 (0.1–1 μM) caused dose-dependent inhibition of direct twitches and increase in baseline tension ($n=4-6$). In addition, dose-dependent morphological changes in skeletal muscle were observed.

4 Prior incubation (10 min) of CSL death adder antivenom (5 units ml^{-1}; $n=4$) or inactivation of PLA_2 activity with 4-bromophenacyl bromide (1.8 mM; $n=4$) prevented the myotoxicity caused by acanmyotoxin-1 (1 μM).

5 Acanmyotoxin-1 (0.1 μM; $n=4$) displayed no significant neurotoxicity when it was examined using the indirectly (0.1 Hz, 0.2 ms, supramaximal V) stimulated CBCNM preparation.

6 In conclusion, clinicians may need to be mindful of possible myotoxicity following death adder envenomation in Irian Jaya.

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Keywords: Death adder; *Acanthophis*; antarcticus; rugosus; myotoxic; Phospholipase A_2; acanmyotoxin-1; rhabdomyolysis; antivenom; venom

Abbreviations: 4-BPB, 4-bromophenacyl bromide; CBCNM, chick biventer cervicis nerve-muscle; CCh, carbachol; PLA_2, Phospholipase A_2; TFA, trifluoroacetic acid

Introduction

Death adders (genus *Acanthophis*) are unique among Australian snakes in both morphology and behaviour. Although classified into the Elapidae family of snakes they are viper-like in appearance and habit (Campbell, 1966). They are characterized by a somewhat flattened, almost triangular head and a short, stout body terminating to a thin rat-like tail (Cogger, 2000). This makes them among the most specialized of all elapids and closely convergent in many respects with members of the family Viperidae.

Death adders 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 there have been up to 12 species and three subspecies of death adders described thus far (Hoser, 1998), considerable debate remains about species identification (Wuster *et al.*, 1999). Of these, only the venoms of the common (*A. antarcticus*) and northern (*A. praelongus*) death adders have been studied in some detail. However, recently the venoms of the major species and regional variants have been investigated by liquid chromatography/mass spectrometry (Fry *et al.*, 2002). This study revealed a great diversity in the venoms.

Previously, using the chick isolated stimulated biventer cervicis nerve-muscle (CBCNM) preparation, we have shown that Irian Jayan death adder (*A. rugosus*) venom (1–

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10 μg ml^{-1}) caused time-dependent inhibition of indirect twitches and blocked contractile responses to exogenous acetylcholine and carbachol (Fry *et al.*, 2001). Thus, suggesting the presence of postsynaptic neurotoxins. In addition, the efficacy of CSL death adder antivenom against the *in vitro* neurotoxicity of *A. rugosus* venom was studied. It was found that CSL death adder antivenom (1 unit ml^{-1}), raised against *A. antarcticus* venom, was markedly less effective against *A. rugosus* venom compared to *A. hawkei*, *A. praelongus* and *A. pyrrhus* venoms (Fry *et al.*, 2001). However, a higher concentration of antivenom (5 units ml^{-1}) completely neutralized the *in vitro* neurotoxicity of *A. rugosus* venom (Fry *et al.*, 2001). To date, no components have been studied from *A. rugosus* venom.

In contrast to *A. rugosus* venom, *A. antarcticus* venom has previously been examined for lethality, neurotoxicity, myotoxicity and its effects on blood coagulation, both experimentally and clinically (Kellaway, 1929a, b; Campbell, 1966; Broad *et al.*, 1979; Mebs & Samejima, 1980, Sutherland *et al.*, 1981). In addition, five postsynaptic neurotoxins have been isolated and sequenced from *A. antarcticus* venom (Sheumack *et al.*, 1979; Kim & Tamiya, 1981a, b; Sheumack *et al.*, 1990; Tyler *et al.*, 1997). In terms of phospholipase A_2 (PLA_2) components, acanthin I and II, both potent inhibitors of platelet aggregation have been isolated from *A. antarcticus* venom (Chow *et al.*, 1998). In addition, acanthoxin A1 and A2, two PLA_2 isoforms with weak neurotoxic activity, have been isolated from *A. antarcticus* venom (van der Weyden *et al.*, 1997; 2001). Three PLA_2 isoenzymes, praelongins 2bIII, 2cII and 2clV, with antiplatelet activity have also been isolated from *A. praelongus* venom (Sim, 1998). However, no myotoxic components have been isolated from any death adder venom to date.

It has long been thought that death adder venoms are devoid of myotoxic activity based on studies done on *A. antarcticus* venom. This venom had no myotoxic activity in Rhesus monkeys (*Macaca fascicularis*) (Sutherland *et al.*, 1981). Mebs & Samejima (1980) fractionated *A. antarcticus* venom by size exclusion chromatography. None of the isolated fractions were capable of causing myoglobinuria in mice after subcutaneous injection. Furthermore, *A. antarcticus* venom (30 μg ml^{-1}) had no myotoxic activity *in vitro* in the directly stimulated CBCNM preparation (Wickramaratna & Hodgson, 2001). However, a recent clinical study reported myotoxic activity *in vivo* following death adder envenomations, in Papua New Guinea, by a species thought to be different to *A. antarcticus* (Lalloo *et al.*, 1996). In this study there was one patient who developed renal failure following delayed presentation after a suspected death adder bite. There were significantly elevated creatine kinase levels (median of 411 IU l^{-1}, range of 164–4220 IU l^{-1}) in two thirds of envenomed patients (Lalloo *et al.*, 1996). However, these levels may not be clinically important in terms of causing renal failure (GK Isbister, personal communication 2002). Renal failure and elevated creatine kinase levels suggest rhabdomyolysis and the presence of myotoxic activity in the venom (Sutherland *et al.*, 1981).

The first aim of this study was to examine the venom from death adders (*A. rugosus*) found in Irian Jaya (West Papua) to determine any possible myotoxic activity. Secondly, to isolate and pharmacologically characterize myotoxins from this venom. Thirdly, to determine the effectiveness of CSL death adder antivenom, which has been raised against *A. antarcticus* venom, in neutralizing myotoxic activity.

Methods

Venom preparation and storage

A. rugosus 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 in 0.9% saline (BSA) were stored at −20°C until required.

Fractionation of venom

Freeze dried venom was dissolved in distilled water and filtered through a 0.45 μm Millipore (Bedford, MA, U.S.A.) filter. Reverse phase high performance liquid chromatography (RP-HPLC) separations were performed on the BIOCAD Perfusion Chromatography Workstation (Applied Biosystems, CA, U.S.A.) using Phenomenex Jupiter preparative (250 × 21.2 mm, 10 μ, 300 Å) and semi-preparative (250 × 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^{-1}: 0 to 60% over 60 min (1% gradient) and then 60 to 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, U.S.A.) using a Phenomenex Jupiter analytical (150 × 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^{-1} 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^{-1}: 0 to 20% over 5 min (4% gradient), 20 to 60% in 40 min (1% gradient) and then 60 to 80% over 5 min (4% gradient). The eluant was monitored at 214 nm.

Molecular mass determination by electrospray mass spectrometry

The sample was dissolved in 50% acetonitrile and analysed 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^{-1} while compressed air was the nebulizer gas. The sample (10 μl) was injected manually into the LC-MS system and analysed in positive ion mode. Data processing was performed with the aid of the software package Biomultiview (PE-Sciex, Thronton, Canada).

Amino acid sequence determination

Pure peptide (400 μg) was dissolved in 400 μl of 6 M guanidinium hydrochloride and then 8 μl of 2-mercaptoethanol was added. The sample was then vortexed and briefly centrifuged. Subsequently, 80 μl of 4-vinylpyridine was then 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 494 pulsed-liquid-phased sequencer (Applied Biosystems, CA, U.S.A.).

Determination of phospholipase A_2 activity

The PLA_2 activity of whole venom and isolated component was determined using a secretory PLA_2 colourimetric assay kit (Cayman Chemical, U.S.A.). The assay uses the 1,2-dithio analogue of diheptanoyl phosphatidylcholine as a substrate. Free thiols generated upon hydrolysis of the thio ester bond at the *sn*-2 position by PLA_2 are detected using DTNB (5,5'-dithiobis(2-nitrobenzoic acid)). Colour changes were monitored by the CERES900C microplate reader (Bio-Tek Instruments, U.S.A.) at 405 nm, sampling every min for a 5 min period. PLA_2 activity was expressed as micromoles of phosphatidylcholine hydrolysed per min per milligram of enzyme.

Inactivation of PLA_2 activity with 4-bromophenacyl bromide

PLA_2 activity of acanmyotoxin-1 was inhibited by alkylation with 4-bromophenacyl bromide (4-BPB). Acanmyotoxin-1 (0.1 mM) was made up in sodium cacodylate-HCl buffer (50 μl, 0.1 M, pH 6.0), and 4-BPB made up in acetone was added to give a final concentration of 1.8 mM (Abe *et al.*, 1977; Bell *et al.*, 1998; Crachi *et al.*, 1999a). Each vial containing the above solution was then incubated at 30°C for 16 h. As a positive control, acanmyotoxin-1 (0.1 mM) made up in sodium cacodylate-HCl buffer was incubated with acetone. As a negative control, sodium cacodylate-HCl buffer was incubated with 1.8 mM 4-BPB in acetone.

Chick isolated biventer cervicis nerve-muscle preparation

Male White leg horn chicks aged between 5 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 and 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 (Harvey *et al.*, 1994) using a Grass S88 stimulator. After a 30 min equilibration period, d-tubocurarine (10 μM) was added. Subsequent abolition of twitches confirmed selective stimulation of nerves. Twitches were then 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 (Harvey *et al.*, 1994). Electrical stimulation was then recommenced and the preparations were allowed to equilibrate for a further 30 min period before commencement of the experiment. Venom or toxin was left in contact with the preparations until complete twitch blockade occurred, or for a 3 h period. Contractile responses to ACh, CCh and KCl were then obtained as previously described.

In experiments determining myotoxicity, direct twitches were evoked by stimulating the muscle directly every 10 s with pulses of 2 ms duration at a supramaximal voltage (Harvey *et al.*, 1994). To achieve selective stimulation of muscle, d-tubocurarine (10 μM) was added and left in the organ bath for the duration of the experiment. *A. rugosus* venom (10–50 μg ml^{-1}), acanmyotoxin-1 (0.1–1 μM), 4-BPB modified acanmyotoxin-1 (1 μM) or relevant controls were left in contact with the preparations for a 3 h period. Where indicated, CSL death adder antivenom (5 units ml^{-1}) was added 10 min prior (Barfaraz & Harvey, 1994; Crachi *et al.*, 1999b; Fry *et al.*, 2001; Wickramaratna & Hodgson, 2001) to the addition of acanmyotoxin-1 (1 μM). A significant contracture of skeletal muscle (i.e. a rise in baseline) and/or inhibition of direct twitches were considered signs of myotoxicity (Harvey *et al.*, 1994).

Morphological studies

After the conclusion of the functional myotoxic experiments the tissues were quickly placed in Tissue Tek and frozen with liquid nitrogen. The tissues were stored at −80°C until required. Using a Leica CM1800 cryostat, tissues were cut into transverse sections (14 μm) and placed onto gelatin-coated slides. Tissue sections were post fixed for 15 min in a solution containing 4% paraformaldehyde in phosphate buffered saline (PBS; (mol l^{-1}) NaCl, 0.137; KH_2PO_4, 0.002; and Na_2HPO_4, 0.008). Tissue sections were routinely stained with haematoxylin and eosin and examined under a light microscope (Olympus BH-2, Olympus Optical Co., Japan). Areas exhibiting typical pathological changes were photographed using an Olympus C-35AD (Olympus Optical Co., Japan) camera and Kodak film (Ektachrome P1600).

Chemicals and drugs

The following drugs and chemicals were used: acetonitrile (Fisher Scientific, U.K.); acetylcholine chloride, 4-bromophenacyl bromide (4-BPB), bovine serum albumin (BSA), cacodylic acid (sodium cacodylate), carbamylcholine chloride (carbachol), d-tubocurarine chloride; eosin, Mayer's Haemalum (Sigma Chemical Co., St. Louis, MO, U.S.A.); trifluoroacetic acid, 4-vinylpyridine (Fluka Chemika-Biochemika, Buchs, Switzerland). Sequencing grade chemicals were obtained from Applied Biosystems (Singapore). Except where indicated, stock solutions were made up in distilled water. 4-BPB was made up in acetone. Death adder antivenom, which is raised against *A. antarcticus* venom in horses, was obtained from CSL Ltd (Melbourne, Australia). All reagents were of analytical grade.

Analysis of results and statistics

For isolated tissue experiments, responses were measured *via* a Grass force displacement transducer (FT03) and recorded on a MacLab System. For both neurotoxicity and myotoxicity studies, twitch height was expressed as a percentage of the pre-treated twitch height. Statistical difference was determined by a one-way analysis of variance (ANOVA) on the twitch height at the 180 min time point. Likewise, a one-way ANOVA was performed on the contractile response induced by the venom and acanmyotoxin-1 at the 180 min

time point. Contractile responses to ACh, CCh and KCl were expressed as a percentage of the respective initial response. These were analysed using either Student's paired *t*-tests or, where stated, compared against the control response *via* a one-way ANOVA. All ANOVAs were followed by a Bonferroni *post hoc* test. Statistical significance was indicated when $P<0.05$.

Results

Isolation and purification of acanmyotoxin-1

Acanmyotoxin-1 was isolated from *A. rugosus* venom by successive RP-HPLC separations. The initial fractionation of *A. rugosus* venom using a Phenomenex Jupiter preparative column produced eleven major peaks. The eleventh peak was subjected to further purification by RP-HPLC. In order to determine homogeneity and location of acanmyotoxin-1 in relation to other peaks of the whole venom both *A. rugosus* venom and acanmyotoxin-1 were run on the same conditions using a Phenomenex Jupiter analytical column (Figure 1a,b). Acanmyotoxin-1 eluted as a clean peak separating away from minor contaminants at about 47.5% acetonitrile or at 32.5 min.

Purity and molecular mass determination

Homogeneity and molecular mass of acanmyotoxin-1 were determined by electrospray mass spectrometry (Figure 2). The mass spectra of purified acanmyotoxin-1 displayed several charged states and these could be reconstructed into a single molecular mass of 13811.38 ± 0.81 daltons.

N-terminal amino acid sequence

The N-terminal amino acid sequence of acanmyotoxin-1 was determined (Table 1). The location of half-cystines was typical of elapid PLA_2 enzymes. The N-terminal sequence of acanmyotoxin-1 was compared with other protein sequences at the National Center for Biotechnology Information (NCBI) database using the BLAST service. Acanmyotoxin-1 shared highest identity with taipoxin α chain (75%) from the coastal taipan (*Oxyuranus s. scutellatus*) and Pa-1G (65%) from the Australian king brown snake (*Pseudechis australis*). Acanmyotoxin-1 shared lower identity with other previously isolated death adder PLA_2 components such as acanthin II (55%) and acanthin I (51%).

Phospholipase A_2 activity

High PLA_2 activity was detected in both *A. rugosus* venom and the isolated component acanmyotoxin-1. *A. rugosus* venom had a specific activity of 140.2 ± 10.4 μmol min^{-1} mg^{-1} ($n=6$) while acanmyotoxin-1 had a specific activity of 153.4 ± 11 μmol min^{-1} mg^{-1} ($n=6$). The positive control, bee venom PLA_2 had a specific activity of 287.5 ± 17.5 μmol min^{-1} mg^{-1} ($n=4$). 4-BPB modified acanmyotoxin-1 had no PLA_2 activity ($n=6$).

Chick isolated biventer cervicis nerve-muscle preparation

Neurotoxic studies The positive control and presynaptic neurotoxin, paradoxin (0.07 μM) caused time-dependent

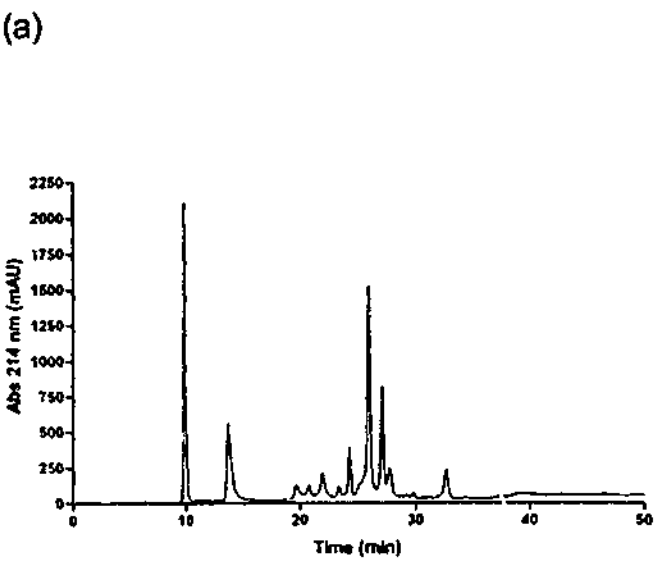


Figure 1 RP-HPLC chromatograph of (a) *A. rugosus* venom or (b) acanmyotoxin-1 run on a Jupiter analytical C18 column, equilibrated with solvent A (0.1% TFA) and eluted with the following gradient conditions of solvent B (90% acetonitrile in 0.09% TFA) and solvent A: 0 to 20% over 5 min, 20 to 60% in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

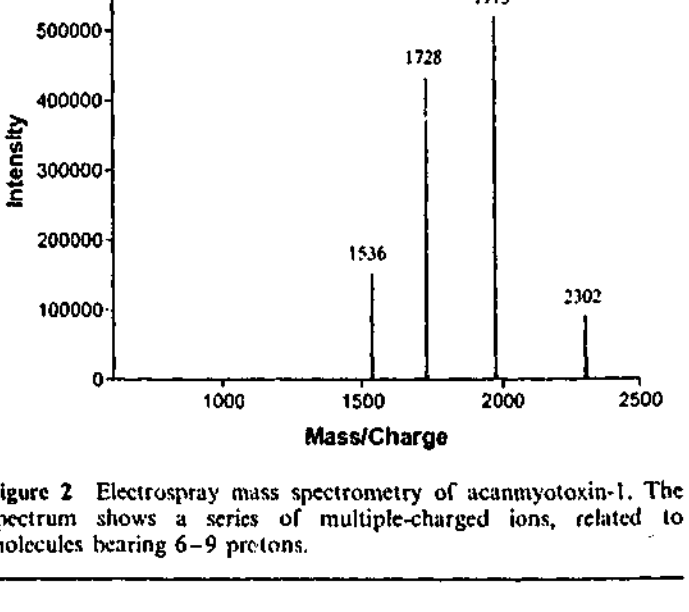


Figure 2 Electrospray mass spectrometry of acanmyotoxin-1. The spectrum shows a series of multiple-charged ions, related to molecules bearing 6–9 protons.

Table 1 N-terminal sequence of PLA_2 components isolated from death adder and some other elapid snake venoms

Common name	*Species*	*PLA_2 component*	*N-terminal sequence*
Irian Jayan death adder	*A. rugosus*	acanmyotoxin-1	NLLQIGIMKR CANKRRRPVF HYRDYGCYC
Common death adder	*A. antarcticus*	acanthin I*	DLFQFGGMIG CANKGARSWL SYVNYGCYC
Common death adder	*A. antarcticus*	acanthin II*	NLYQFGGMIQ CANKGARSWL SYVNYGCYC
Common death adder	*A. antarcticus*	acanthoxin A1**	NLYQFGGMIQ CANKGARSWL SYVNYGCYC
Common death adder	*A. antarcticus*	acanthoxin A2**	DLFQFGGMIG CANKGARSWL SYVNYGCYC
Northern death adder	*A. praelongus*	acanthoxin B†	DLFQFGFMIQ CANKGSRPVF
Desert death adder	*A. pyrrhus*	acanthoxin C†	NLFQFGGMIG CANKGTRSWL SYVNYGCYC
Coastal taipan	*Oxyuranus s. scutellatus*	taipoxin α chain††	NLLQFGFMIR CANRRSRPVW HYMDYGCYC
Australian king brown snake	*Pseudechis australis*	Pa-1G‡	NLIQFGNMIQ CANKGSRPTR HYMDYGCYC

*Chow *et al.* (1998); **van der Weyden *et al.* (1997); †van der Weyden *et al.* (2000); ††Lind & Eaker (1982); ‡Takasaki *et al.* (1990). †No functional studies were done on these toxins.

inhibition of indirect twitches, whereas acanmyotoxin-1 (0.1 μM) had no significant inhibitory effect on twitch height compared to the vehicle, 0.1% bovine serum albumin in 0.9% saline ($n=4-8$; one-way ANOVA, $P<0.05$; Figure 3a). Paradoxin (0.07 μM), acanmyotoxin-1 (0 μM) and vehicle had no significant effect on the contractile responses to exogenous ACh (1 mM), CCh (20 μM) and KCl (40 mM) ($n=4-8$; Student's paired *t*-test, $P<0.05$; Figure 3b). However, acanmyotoxin-1 (0.1 μM) caused a significant increase in baseline tension compared to the vehicle ($n=4-8$; one-way ANOVA, $P<0.05$; data not shown).

Myotoxic studies *A. rugosus* venom (30–50 μg ml^{-1}) caused a significant inhibition of direct twitches compared to the vehicle ($n=4-8$; one-way ANOVA, $P<0.05$; Figure 4a). This effect was dose-dependent with *A. rugosus* venom (50 μg ml^{-1}) causing a significant inhibition of direct twitches compared to *A. rugosus* venom at 10 μg ml^{-1} ($n=4-5$; one-way ANOVA, $P<0.05$; Figure 4a). In addition, *A. rugosus* venom (10–50 μg ml^{-1}) induced a significant increase in baseline tension compared to the vehicle control ($n=4-8$; one-way ANOVA, $P<0.05$; Figure 4b). Again this effect was dose-dependent with *A. rugosus* venom (50 μg ml^{-1}) causing a significantly greater contraction compared to *A. rugosus* venom at 10 μg ml^{-1} ($n=4-5$; one-way ANOVA, $P<0.05$; Figure 4b).

Acanmyotoxin-1 (1 μM) caused a significant inhibition of direct twitches compared to the vehicle, whereas acanmyotoxin-1 (0.1 μM) had no significant effect on the twitch height ($n=4-6$; one-way ANOVA, $P<0.05$; Figure 5a). In addition, acanmyotoxin-1 (0.1–1 μM) induced a significant increase in baseline tension compared to the vehicle ($n=4-6$; one-way ANOVA, $P<0.05$; Figure 5b). This response was dose-dependent with acanmyotoxin-1 (1 μM) causing a significantly greater contraction than acanmyotoxin-1 at 0.1 μM ($n=4$; one-way ANOVA, $P<0.05$; Figure 5b).

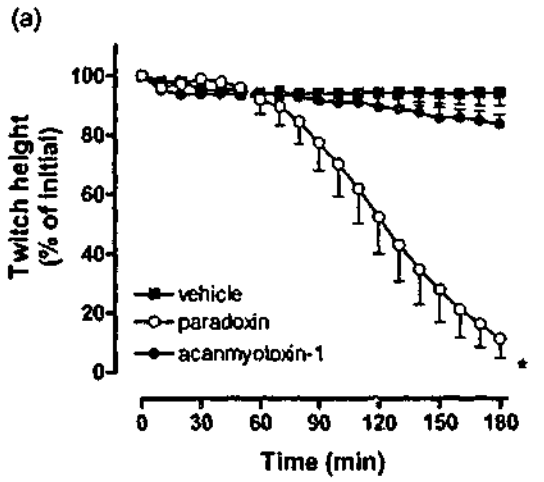
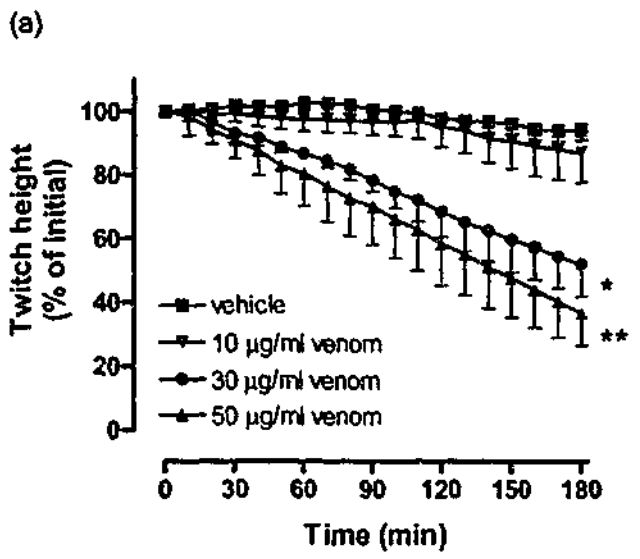


Figure 3 The effect of acanmyotoxin-1 (0.1 μM; $n=4$), paradoxin (positive control; 0.07 μM; $n=4$) or vehicle ($n=8$) on (a) indirect twitches or (b) contractile responses to exogenous ACh, CCh and KCl in the CBCNM preparation. *$P<0.05$, significantly different from vehicle, one-way ANOVA.

Antivenom studies Prior incubation (10 min) of CSL death adder antivenom (5 units ml^{-1}) prevented the inhibition of direct twitches and the increase in baseline tension caused by acanmyotoxin-1 (1 μM; $n=4$; Figures 6a,b).

4-BPB modified acanmyotoxin-1 studies Acanmyotoxin-1 (1 μM) plus vehicle (acetone) significantly inhibited direct twitches compared to 4-BPB plus vehicle (sodium cacodylate; $n=4-6$; one-way ANOVA, $P<0.05$; Figure 6a). However, acanmyotoxin-1 (1 μM) plus 4-BPB had no significant inhibitory effect on direct twitches compared to 4-BPB plus vehicle ($n=4-6$; one-way ANOVA, $P<0.05$; Figure 6a). In addition, acanmyotoxin-1 (1 μM) plus vehicle induced a significant increase in baseline tension compared to 4-BPB plus vehicle, while acanmyotoxin-1 (1 μM) plus 4-BPB had no significant effect on the baseline tension ($n=4-6$; one-way ANOVA, $P<0.05$; Figure 6b).



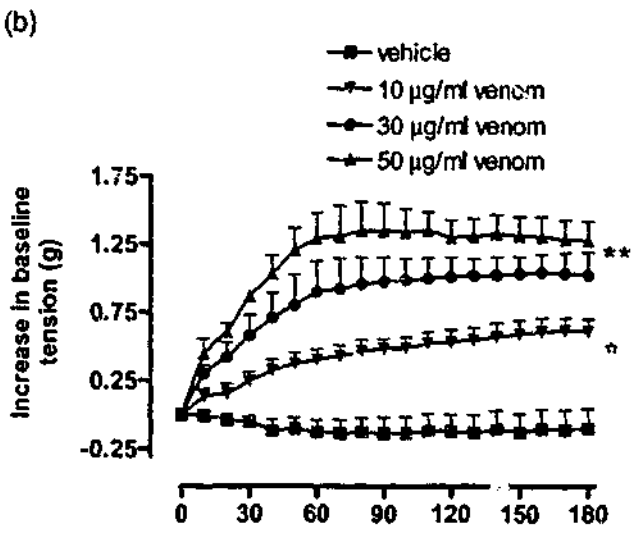
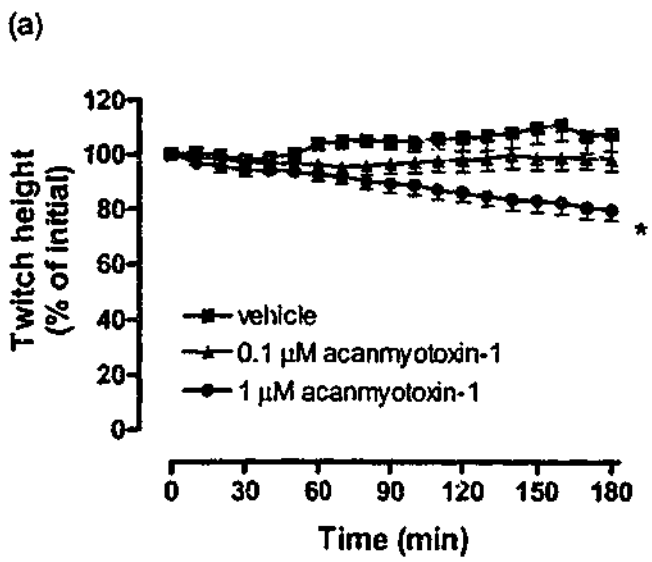


Figure 4 The effect of *A. rugosus* venom (10–50 µg ml^{-1}; $n=4$) or vehicle ($n=8$) on (a) direct twitches or (b) baseline tension of the CBCNM preparation. *$P<0.05$, significantly different from vehicle, one-way ANOVA. **$P<0.05$, significantly different from *A. rugosus* venom (10 µg ml^{-1}), one-way ANOVA.



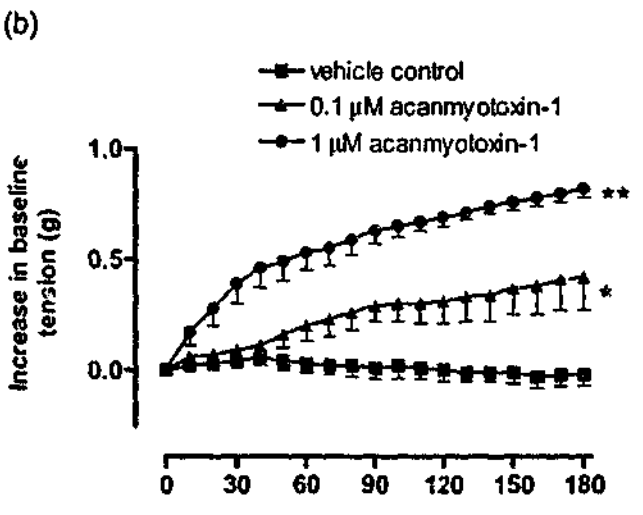


Figure 5 The effect of acanmyotoxin-1 (0.1–1 µM; $n=4$) or vehicle ($n=6$) on (a) direct twitches or (b) baseline tension of the CBCNM preparation. *$P<0.05$, significantly different from vehicle, one-way ANOVA.

Morphological studies

Light microscopy studies of tissues exposed to *A. rugosus* venom (10–50 µg ml^{-1}) and acanmyotoxin-1 (0.1–1 µM) showed dose-dependent morphological changes in skeletal muscle compared to the vehicle control tissues (Figures 7a,b,c,d; data not shown for 10 and 50 µg ml^{-1} *A. rugosus* venom). These changes included muscle fibre damage and vacuolation of the muscle cells. Prior incubation of CSL death adder antivenom (5 units ml^{-1}) prevented morphological changes from occurring due to acanmyotoxin-1 (1 µM; Figure 7e). No detectable morphological changes were seen in tissues equilibrated with antivenom alone (data not shown). While acanmyotoxin-1 (1 µM) plus vehicle (i.e. acetone) induced morphological changes similar to acanmyotoxin-1 (1 µM) alone, no detectable morphological changes were seen in acanmyotoxin-1 (1 µM) plus 4-BPB or vehicle plus 4-BPB treated tissues (data not shown).

Discussion

Until recently, research on death adders has been largely focused on the venom from the Australian *A. antarcticus* (common death adder). Both *in vivo* and *in vitro* studies have shown that this venom has no significant myotoxic activity (Mebs & Samejima, 1980; Sutherland *et al.*, 1981; Wickramaratna & Hodgson, 2001). Therefore, it has been thought that death adder venoms are devoid of myotoxic activity. However, a recent clinical study reported evidence of rhabdomyolysis in patients following death adder envenomations, in Papua New Guinea, by a species not closely aligned with *A. antarcticus* (Lalloo *et al.*, 1996). Consequently, the present study examined *A. rugosus* venom for myotoxic activity, and isolated the first myotoxin from a death adder venom.

Acanmyotoxin-1 was isolated as a single peak from *A. rugosus* venom by successive RP-HPLC separations. As seen from the RP-HPLC chromatogram of the *A. rugosus* venom,

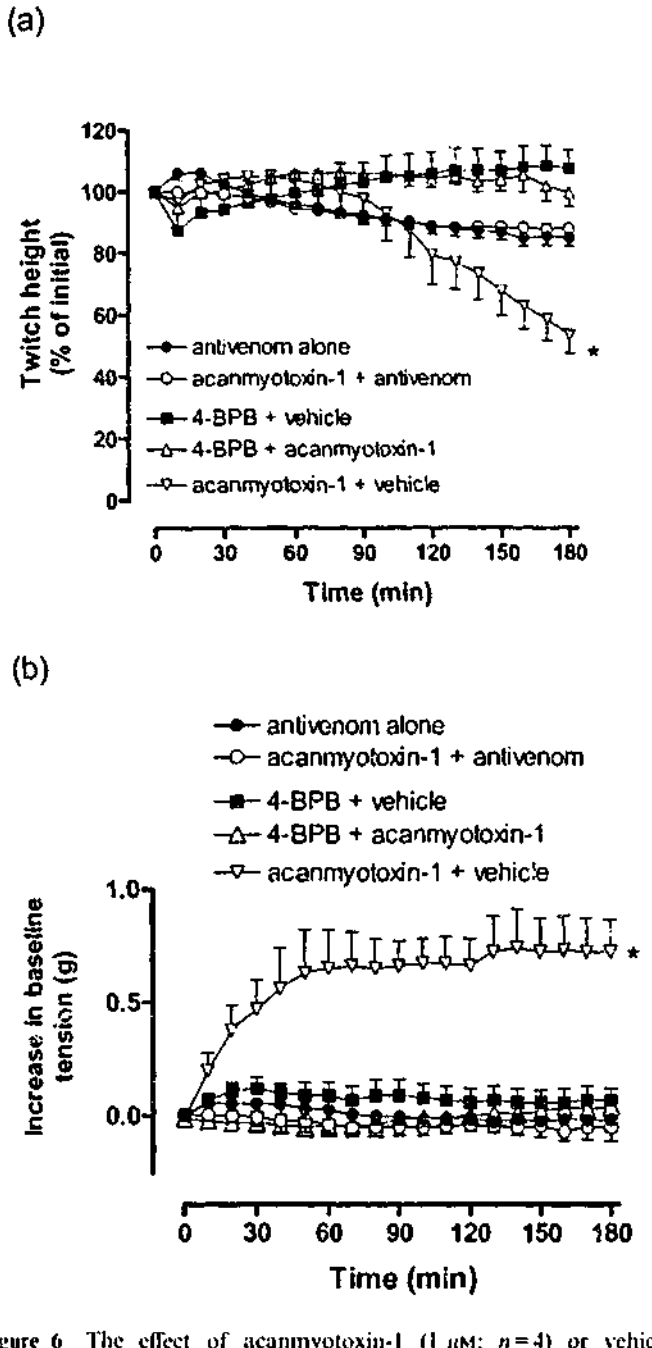


Figure 6 The effect of acanmyotoxin-1 (1 μM; $n=4$) or vehicle (BSA; $n=4$) in the presence of antivenom (5 units ml^{-1}), and effect of acanmyotoxin-1 (1 μM; $n=4$) or vehicle (sodium cacodylate; $n=6$) incubated in 4-BPB (1.8 mM) on (a) direct twitches or (b) baseline tension of the CBCNM preparation. Positive control was acanmyotoxin-1 (1 μM; $n=4$) incubated in vehicle (acetone). $^*P<0.05$, significantly different from 4-BPB plus vehicle, one-way ANOVA.

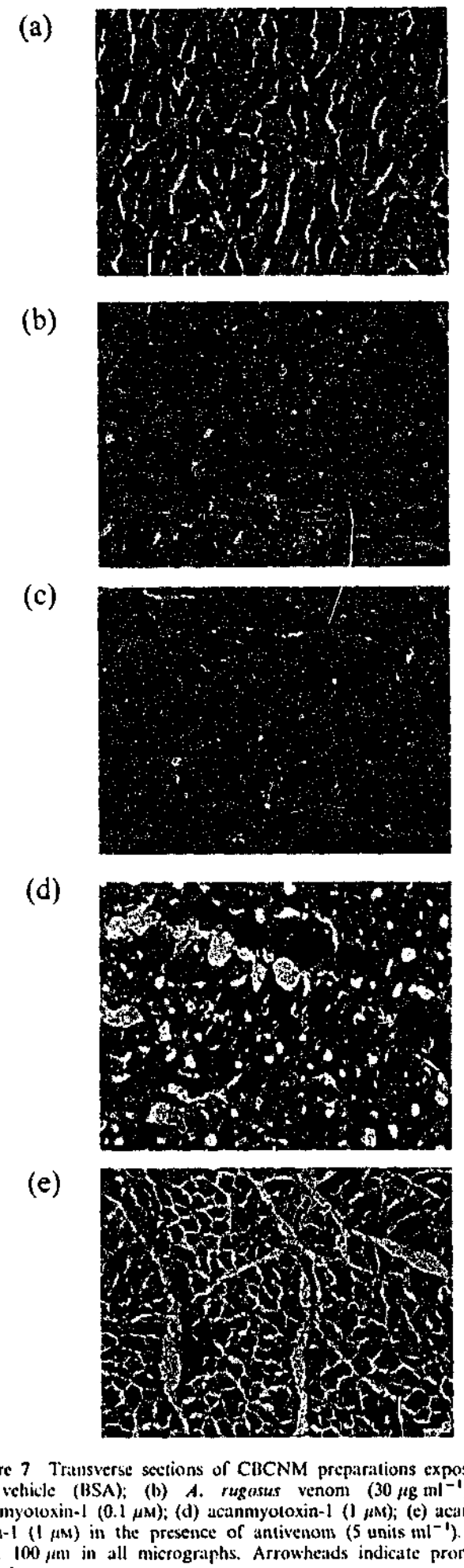


Figure 7 Transverse sections of CBCNM preparations exposed to (a) vehicle (BSA); (b) *A. rugosus* venom (30 $\mu g\ ml^{-1}$); (c) acanmyotoxin-1 (0.1 μM); (d) acanmyotoxin-1 (1 μM); (e) acanmyotoxin-1 (1 μM) in the presence of antivenom (5 units ml^{-1}). Scale bars, 100 μm in all micrographs. Arrowheads indicate prominent vacuoles.

acanmyotoxin-1 is also the last major peak to elute at the given conditions. Using electrospray mass spectrometry the molecular mass of acanmyotoxin-1 was determined to be 13811 daltons. It is well documented that elapid snake venom PLA_2 components usually have molecular mass in the range of 12–14 kDa (Dawson & Hemington, 1967; Sim, 1998). While the molecular mass of acanmyotoxin-1 is consistent with other snake venom PLA_2 components it is about 1 kDa bigger than PLA_2 components previously isolated from *A. antarcticus* and *A. praelongus* venoms. Comparison of the N-terminal sequences showed that acanmyotoxin-1 shared highest identity with taipoxin α chain (75%) and Pa-1G (65%). The taipoxin α chain is the subunit of a potent

presynaptic neurotoxin possessing myotoxic and PLA_2 activity (Harris & Maltin, 1982; Lind & Eaker, 1982), and Pa-1G is a myotoxic PLA_2 component (Geh *et al.*, 1992). Interestingly, lower sequence identity was seen with the antiplatelet active death adder PLA_2 components.

Due to the sequence homology and molecular mass resemblance of acanmyotoxin-1 to other elapid venom PLA_2 components, the specific activity of acanmyotoxin-1 was determined. High PLA_2 activity was detected in both *A. rugosus* venom and the isolated component. Given the high PLA_2 activity of *A. rugosus* whole venom it is possible that the whole venom may have other components with high PLA_2 activity.

A. rugosus venom and acanmyotoxin-1 were examined for *in vitro* myotoxicity using the directly stimulated CBCNM preparation. Both *A. rugosus* venom and acanmyotoxin-1 caused dose-dependent inhibition of direct twitches. Furthermore, both *A. rugosus* venom and acanmyotoxin-1 induced a dose-dependent increase in baseline tension. An inhibition of direct twitches and a rise in baseline tension is indicative of myotoxic activity (Harvey *et al.*, 1994). In addition to these results, light microscopy studies of tissues exposed to *A. rugosus* venom and acanmyotoxin-1 showed obvious morphological changes in skeletal muscle compared to tissues exposed to the vehicle. Together, these results suggest that both *A. rugosus* venom and acanmyotoxin-1 cause myotoxicity.

Several studies have shown that some elapid venom PLA_2 components, such as notexin and notechis II-5, are myotoxic as well as presynaptically neurotoxic (Harris & Johnson, 1978; Harris, 1991; Dixon & Harris, 1996). Therefore, acanmyotoxin-1 was examined for *in vitro* neurotoxicity using the indirectly stimulated CBCNM preparation. Acanmyotoxin-1 (0.1 μM) had no significant inhibitory effect on the indirect twitch height compared to the vehicle control, thus, suggesting it to be lacking in any detectable neurotoxic activity at this concentration. However, acanmyotoxin-1 (0.1 μM) caused a significant increase in baseline tension compared to vehicle. Given the myotoxic activity, it was not possible to further examine the neurotoxic activity of acanmyotoxin-1 at a higher concentration. In contrast to acanmyotoxin-1 (0.1 μM), paradoxin (0.07 μM) caused almost full inhibition of the indirect twitches over 3 h. Electrophysiological studies are required to further examine the neurotoxic activity of acanmyotoxin-1.

Due to habitat destruction and consequential decrease in species population levels, death adder envenomations are a rare occurrence in Australia, although these are still a significant health problem in Papua New Guinea (Currie *et al.*, 1991; Sutherland, 1992; Lalloo *et al.*, 1995; 1996). CSL death adder antivenom is indicated for use in envenomation by any death adder species (AMH, 1998; White, 1998). Since *A. antarcticus* venom lacks myotoxic activity, and given that death adder antivenom has been raised against *A. antarcticus* venom, it was of clinical relevance to examine the efficacy of death adder antivenom against the *in vitro* myotoxicity of acanmyotoxin-1. Previously, we have studied the efficacy of CSL death adder antivenom against the *in vitro* neurotoxicity of *A. rugosus* venom (Fry *et al.*, 2001). Prior incubation of antivenom (5 units ml^{-1}) prevented the inhibition of direct twitches and the increase in baseline tension caused by acanmyotoxin-1 (1 μM). Furthermore, antivenom prevented morphological changes from occurring due to acanmyotoxin-1 (1 μM). Thus, CSL death adder antivenom is effective in neutralizing the *in vitro* myotoxic activity of acanmyotoxin-1.

In order to determine whether the PLA_2 activity of acanmyotoxin-1 is necessary for the myotoxic action, acanmyotoxin-1 was subjected to 4-BPB modification. Many studies have shown that PLA_2 activity can be inhibited by selective acylation of His-48 using 4-BPB (Volwerk *et al.*, 1974; Abe *et al.*, 1977). When acanmyotoxin-1 was incubated with 4-BPB, the enzymatic activity as well as myotoxic activity was abolished. Thus, suggesting that PLA_2 activity is essential for the myotoxic activity of acanmyotoxin-1. In contrast to acanmyotoxin- 1, some studies have shown that Lys-49 PLA_2 components lack catalytic activity on artificial substrates (Soares *et al.*, 2000; 2001). However, 4-BPB modification of these Lys-49 PLA_2 components prevented some of their pharmacological effects (Soares *et al.*, 2000; 2001). Thus, it was suggested that inhibition of the pharmacological effects by 4-BPB modification were not due to the inhibition of enzymatic activity (Soares *et al.*, 2000). Recently, it has been shown that some Lys-49 PLA_2 components are catalytically active on biological substrates (Soares *et al.*, 2002). Therefore, the observed reduction in pharmacological effects after 4-BPB modification of Lys-49 PLA_2 components may still be the result of inhibition of the catalytic activity. However, it is possible that His-48 may be important in the pharmacological site of PLA_2 components.

In conclusion, *A. rugosus* venom caused dose-dependent *in vitro* myotoxicity in the CBCNM preparation. Acanmyotoxin-1 is the first myotoxic component to be isolated from any death adder venom. Although CSL death adder antivenom has been raised against *A. antarcticus* venom it is effective in neutralizing the myotoxic activity of acanmyotoxin-1. Furthermore, studies with 4-BPB suggest that PLA_2 activity is essential for the myotoxic activity of acanmyotoxin-1. Given the results of this study clinicians may need to be mindful of possible myotoxicity following death adder envenomation in Irian Jaya. In light of this finding, other death adder venoms should be examined for myotoxic activity.

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CHAPTER 5

Species-Dependent Variations in the *in Vitro* Myotoxicity of Death Adder (*Acanthophis*) Venoms

Declaration for Thesis Chapter 5

This chapter is made up of the following publication:

Species-dependent variations in the *in vitro* myotoxicity of death adder (*Acanthophis*) venoms

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I/we declare that over 90 % of this work has been done by the candidate. This manuscript has been written solely by the candidate taking into consideration the advice and recommendations of co-authors. Dr. Bryan Fry has supplied several of the death adder venoms for this study. All experiments were performed by the candidate.

The original data are stored at the Department of Pharmacology, Monash University, Clayton Campus, Australia and will be held for at least seven years from the date of publication.

Janith C. Wickramaratna : [redacted] Date: 15-8-03.

Bryan G. Fry : [redacted] Date: 13/8/03

Wayne C. Hodgson : [redacted] Date: 15.8.03

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Species-Dependent Variations in the *in Vitro* Myotoxicity of Death Adder (*Acanthophis*) Venoms

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Based on early studies on *Acanthophis antarcticus* (common death adder) venom, it has long been thought that death adder snake venoms are devoid of myotoxicity. However, a recent clinical study reported rhabdomyolysis in patients following death adder envenomations, in Papua New Guinea, by a species thought to be different to *A. antarcticus*. Subsequently, a myotoxic phospholipase A_2 component was isolated from *A. rugosus* (Irian Jayan death adder) venom. The present study examined the venoms of *A. praelongus* (northern), *A. pyrrhus* (desert), *A. hawkei* (Barkly Tableland), *A. wellsi* (black head), *A. rugosus*, *A.* sp. Seram and the regional variants of *A. antarcticus* for *in vitro* myotoxicity. Venoms (10–50 μg/ml) were examined for myotoxicity using the chick directly (0.1 Hz, 2 ms, supramaximal V) stimulated biventer cervicis nerve-muscle preparation. A significant contracture of skeletal muscle and/or inhibition of direct twitches were considered signs of myotoxicity. This was confirmed by histological examination. All venoms displayed high phospholipase A_2 activity. The venoms (10–50 μg/ml) of *A.* sp. Seram, *A. praelongus*, *A. rugosus*, and *A. wellsi* caused a significant inhibition of direct twitches and an increase in baseline tension compared to the vehicle ($n = 4–6$; two-way ANOVA, $p < 0.05$). Furthermore, these venoms caused dose-dependent morphological changes in skeletal muscle. In contrast, the venoms (10–50 μg/ml; $n = 3–6$) of *A. hawkei*, *A. pyrrhus*, and regional variants of *A. antarcticus* were devoid of myotoxicity. Prior incubation (10 min) of CSL death adder antivenom (5 U/ml) prevented the myotoxicity caused by *A.* sp. Seram, *A. praelongus*, *A. rugosus*, and *A. wellsi* venoms (50 μg/ml; $n = 4–7$). In conclusion, clinicians may need to be mindful of possible myotoxicity following envenomations by *A. praelongus*, *A. rugosus*, *A.* sp. Seram, and *A. wellsi* species.

Key Words: Acanthophis; *A. antarcticus*; antivenom; death adder; myotoxic; phospholipase A_2; *A. praelongus*; rhabdomyolysis; *A. rugosus*; venom.

Death adders (genus *Acanthophis*) are unique among Australian snakes in both morphology and behavior. Although classified into the Elapidae family of snakes they are viper-like in appearance and habit (Campbell, 1966; Cogger, 2000). Death adders 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 (Hoser, 1998), considerable debate remains about species identification (Wuster *et al.*, 1999). Of these, only the venom of the common (*A. antarcticus*) death adder has been studied in detail.

Acanthophis antarcticus venom has previously been examined for lethality, neurotoxicity, myotoxicity, and its effects on blood coagulation, both experimentally and clinically (Broad *et al.*, 1979; Campbell, 1966; Kellaway, 1929a,b; Mebs and Samejima, 1980; Sutherland *et al.*, 1981; Wickramaratna and Hodgson, 2001). In addition, five postsynaptic neurotoxins and four phospholipase A_2 (PLA_2) components have been isolated and sequenced from *A. antarcticus* venom (Chow *et al.*, 1998; Kim and Tamiya, 1981a,b; Sheumack *et al.*, 1979, 1990; Tyler *et al.*, 1997; van der Weyden *et al.*, 1997). However, no myotoxic components have been isolated from this venom.

Previously, using the chick isolated biventer cervicis nerve-muscle (CBCNM) preparation, we studied the venoms of the northern (*A. praelongus*), desert (*A. pyrrhus*), Barkly Tableland (*A. hawkei*), black head (*A. wellsi*), Irian Jayan (*A. rugosus*), and *A.* sp. Seram for *in vitro* neurotoxicity (Fry *et al.*, 2001). All venoms (1–10 μg/ml) caused dose-dependent neurotoxicity, which was postsynaptic in nature. In the same study, CSL death adder antivenom (1 U/ml), which is raised against *A. antarcticus* venom, prevented the neurotoxic effects of *A. pyrrhus*, *A. praelongus*, and *A. hawkei* venoms. However, it was markedly less effective against the venoms of *A. rugosus*, *A. wellsi*, and *A.* sp. Seram (Fry *et al.*, 2001). At a higher concentration, antivenom (5 U/ml) was effective against all venoms. In another study, the venoms of major species and regional variants of death adders were investigated by liquid chromatography/mass spectrometry (Fry *et al.*, 2002). This study revealed a great diversity in venom composition.

Based on early studies on *A. antarcticus* venom it was thought that death adder venoms were devoid of myotoxic

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activity (Sutherland *et al.*, 1981). *A. antarcticus* venom displayed no myotoxic activity *in vivo* in Rhesus monkeys (*Macaca fascicularis*; Sutherland *et al.*, 1981). In another study, Mebs and Samejima (1980) fractionated *A. antarcticus* venom by ion-exchange chromatography. None of the isolated fractions caused myoglobinuria in mice after sc injection. However, a clinical study reported myotoxic activity following death adder envenomations, in Papua New Guinea, by a species thought to be different to *A. antarcticus* (Lalloo *et al.*, 1996). In this study one patient developed renal failure following delayed presentation and two-thirds of envenomed patients had significantly elevated creatine kinase levels (Lalloo *et al.*, 1996). This is suggestive of rhabdomyolysis and the possible presence of myotoxic activity in the venom (Sutherland *et al.*, 1981). Recently, we have shown that venom of the Irian Jayan death adder (*A. rugosus*) causes dose-dependent *in vitro* myotoxicity, and subsequently isolated the first myotoxic PLA_2 from a death adder venom (Wickramaratna *et al.*, 2003). However, no studies have been performed to determine the effectiveness of CSL death adder antivenom, which has been raised against *A. antarcticus* venom, in neutralizing the myotoxic activity of *A. rugosus* venom.

The first aim of this study was to examine the venoms of *A. praelongus*, *A. pyrrhus*, *A. hawkei*, *A. wellsi*, *A.* sp. Seram, and the regional variants of *A. antarcticus* for *in vitro* myotoxic activity. The second was to determine the effectiveness of CSL death adder antivenom in neutralizing the myotoxic activity of death adder venoms.

MATERIALS AND METHODS

Venom preparation and storage. *A. antarcticus* venoms were obtained from populations in New South Wales (NSW), Queensland (Qld), South Australia (SA), and Western Australia (WA). *A. praelongus* venom was from populations in Cairns, Queensland; *A. pyrrhus* venom from Alice Springs, Northern Territory; *A. wellsi* venom from the Pilbarra region of Western Australia; *A. hawkei* venom from the Barkly Tableland region of Northern Territory; *A. rugosus* venom from Irian Jaya (West Papua), and *A.* sp. Seram from the island of Seram, Indonesia. Venoms were either purchased from Venom Supplies Pty. Ltd., South Australia, or milked from specimens caught by Dr. Bryan Fry. For each geographic variant or species; venoms were collected and pooled to minimize the effects of individual variations (Chippaux *et al.*, 1991). Freeze-dried venoms and stock solutions of venoms prepared in 0.1% bovine serum albumin (BSA) in 0.9% saline were stored at −20°C until required.

Determination of phospholipase A_2 activity. The PLA_2 activity of death adder venoms was determined using a secretory PLA_2 colorimetric assay kit (Cayman Chemical, Ann Arbor, MI). The assay uses the 1,2-dithio analogue of diheptanoyl phosphatidylcholine as a substrate. Free thiols generated upon hydrolysis of the thio ester bond at the *sn*-2 position by PLA_2 are detected using DTNB (5,5'-dithiobis(2-nitrobenzoic acid)). Color changes were monitored by the CERES900C microplate reader (Bio-Tek Instruments, Winooski, VT) at 405 nm, sampling every min for a 5 min period. PLA_2 activity was expressed as micromoles of phosphatidylcholine hydrolysed per min per mg of enzyme.

Inactivation of PLA_2 activity with 4-bromophenacyl bromide. The PLA_2 activity of *A. rugosus*, *A.* sp. Seram, and *A. praelongus* venoms were inhibited by alkylation with 4-bromophenacyl bromide (4-BPB). *A. rugosus*, *A.* sp. Seram, and *A. praelongus* venoms (10,000 μg/ml) were made up in sodium cacodylate-HCl buffer (25 μl, 0.1 M, pH 6.0), and 4-BPB made up in acetone was added to give a final concentration of 1.8 mM (Abe *et al.*, 1977; Bell *et al.*, 1998; Crachi *et al.*, 1999b). Each vial containing the above solution was then incubated at 30°C for 16 h. As a positive control, *A. rugosus*, *A.* sp. Seram, and *A. praelongus* venoms (10,000 μg/ml) made up in sodium cacodylate-HCl buffer were incubated with acetone. As a negative control, sodium cacodylate-HCl buffer was incubated with 1.8 mM 4-BPB in acetone.

Chick isolated biventer cervicis nerve-muscle preparation. Male White leg horn chicks aged between 9 and 11 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; and glucose, 11.1. The Krebs solution was bubbled with carbogen (95% O_2 and 5% CO_2) and maintained at 34°C. Direct twitches were evoked by stimulating the muscle directly every 10 s with pulses of 2 ms duration at a supramaximal voltage (Harvey *et al.*, 1994) using a Grass S88 stimulator. After a 30-min equilibration period, to ensure selective stimulation of muscle, d-tubocurarine (10 μM) was added and left in the organ bath for the duration of the experiment. Death adder venoms (10–50 μg/ml), 4-BPB modified *A. rugosus*, *A.* sp. Seram, and *A. praelongus* venoms (50 μg/ml) or relevant controls were left in contact with the preparations for a 3 h period. A significant contracture of skeletal muscle (i.e., a rise in baseline) and/or inhibition of direct twitches were considered signs of myotoxicity (Harvey *et al.*, 1994). Where indicated, CSL death adder antivenom (5 U/ml) was added 10 min prior (Barfaraz and Harvey, 1994; Crachi *et al.*, 1999a; Fry *et al.*, 2001; Wickramaratna and Hodgson, 2001) to the addition of death adder venoms (50 μg/ml).

Morphological studies. After the conclusion of the functional myotoxic experiments, the tissues were quickly placed in Tissue Tek and frozen with liquid nitrogen. The tissues were stored at −80°C until required. Using a Leica CM1800 cryostat, tissues were cut into transverse sections (14 μm) and placed onto gelatin-coated slides. Tissue sections were post fixed for 15 min in a solution containing 4% paraformaldehyde in phosphate buffered saline (PBS; [mol/l] NaCl, 0.137; KH_2PO_4, 0.002; and Na_2HPO_4, 0.008). Tissue sections were routinely stained with haematoxylin and eosin, and examined under a light microscope (Olympus BX 51, Olympus Optical Co., Japan). Areas exhibiting typical pathological changes were photographed using an Olympus C-4040ZOOM (Olympus Optical Co., Japan) digital camera.

Chemicals and drugs. The following drugs and chemicals were used: 4-bromophenacyl bromide (4-BPB), BSA, cacodylic acid (sodium cacodylate), d-tubocurarine chloride, eosin, Mayer's Hematoxylin solution (Sigma Chemical Co., St. Louis, MO). Except where indicated, stock solutions were made up in distilled water. 4-BPB was made up in acetone. Death adder antivenom, which is raised against *A. antarcticus* venom in horses, was obtained from CSL Ltd. (Melbourne, Australia).

Analysis of results and statistics. For isolated tissue experiments, responses were measured via a Grass force displacement transducer (FT03) and recorded on a MacLab System. The twitch height was expressed as a percentage of the initial twitch height (i.e., prior to the addition of venom or vehicle). Full data (i.e., response curves over a 3 h period) are shown for twitch height and baseline tension at 50 μg/ml venoms. However, for brevity full data are not shown at venom concentrations of 10 μg/ml and 30 μg/ml. Instead, data for all venom concentrations are summarized in Figures 2a and 2b using only the twitch height and baseline tension values at the 180-min time point. Statistical difference was determined by a two-way ANOVA on the twitch heights, at the 180-min time point, at different concentrations of venoms. Likewise, a two-way ANOVA was performed on the contractile responses induced by the venoms at different concentrations at the 180-min time point (i.e., only the data at the 180-min time point have been statistically analyzed). For 4-BPB modified venom studies, statistical difference was determined by a two-way ANOVA on the data at the 180-min time point. Statistical difference between the PLA_2 activity of 4-BPB treated and untreated venom was determined by a Student's unpaired *t*-test. 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 (ver. 1.0) software package.

RESULTS

Phospholipase A_2 Activity

High PLA_2 activity was detected in all death adder venoms (Table 1). While there was a large variation in the PLA_2 activity of death adder venoms, *A. pyrrhus* venom had the highest specific activity, 476.4 ± 12.4 μmol/min/mg ($n = 12$). The positive control, bee venom PLA_2, had a specific activity of 287.5 ± 17.5 μmol/min/mg ($n = 4$). 4-BPB treated *A. rugosus*, *A.* sp. Seram, and *A. praelongus* venoms had significantly reduced PLA_2 activities of 2.0 ± 0.9, 1.7 ± 1.1, and 1.8 ± 1.0 μmol/min/mg ($n = 8$) compared to their untreated venoms with specific activities of 140.2 ± 10.4, 420.4 ± 10.8, and 255.0 ± 8.6 μmol/min/mg, respectively ($n = 6$–8; Student's unpaired *t*-test, $p < 0.05$).

Chick Isolated Directly-Stimulated Biventer Cervicis Nerve-Muscle Preparation

Myotoxic studies. The venoms (10–50 μg/ml) of *A. antarcticus* (NSW, Qld, SA, WA), *A. hawkei*, *A. pyrrhus*, and *A. wellsi* had no significant inhibitory effect on the direct twitches compared to the vehicle ($n = 3$–6; two-way ANOVA, $p < 0.0001$; Figs. 1a and 2a). In contrast, *A.* sp. Seram venom (10–50 μg/ml) caused a significant inhibition of direct twitches compared to the vehicle ($n = 4$–6; Figs. 1a and 2a). However, this effect was not concentration-dependent as there was no significant difference in the twitch inhibition caused by *A.* sp. Seram venom at 10 μg/ml and 50 μg/ml ($n = 4$; Fig. 2a). Both *A. praelongus* venom (30–50 μg/ml) and *A. rugosus* venom (30–50 μg/ml) caused a significant inhibition of direct twitches compared to the vehicle ($n = 4$–6; Figs. 1a and 2a). This effect was concentration-dependent with *A. praelongus* (50 μg/ml) and *A. rugosus* (50 μg/ml) venoms causing a significantly greater inhibition of direct twitches compared to *A. praelongus* and *A. rugosus* venom at 10 μg/ml, respectively ($n = 5$–6; Fig. 2a). When taking all concentrations into consideration *A.* sp. Seram venom was significantly more potent in causing direct twitch inhibition than either *A. praelongus* venom or *A. rugosus* venom ($n = 4$–6). In contrast, there was no significant difference between *A. praelongus* venom and *A. rugosus* venom ($n = 5$–6; Fig. 2a).

The venoms (10–50 μg/ml) of *A. antarcticus* (NSW, Qld, SA, WA), *A. hawkei*, and *A. pyrrhus* had no significant effect on the baseline tension compared to the vehicle ($n = 3$–6; two-way ANOVA, $p < 0.0001$; Figs. 1b and 2b). While *A. wellsi* venom (10–30 μg/ml) had no significant effect on the baseline tension, *A. wellsi* venom (50 μg/ml) induced a significant increase in baseline tension compared to the vehicle ($n = 4$–6). The venoms (10–50 μg/ml) of *A.* sp. Seram, *A. praelon-*

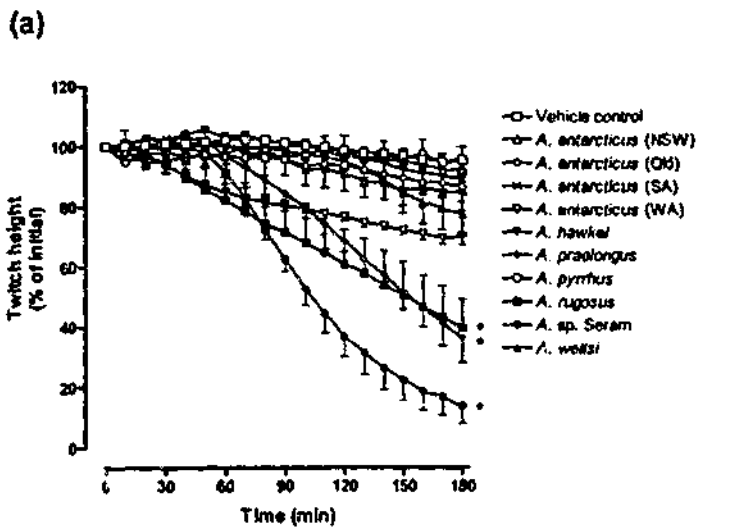


FIG. 1. The effect of *Acanthophis* venoms (10–50 μg/ml; $n = 3$–6) or vehicle ($n = 6$) on (a) direct twitches or (b) baseline tension of the CBCNM preparation at the 180-min time point. *$p < 0.05$, significantly different from vehicle, two-way ANOVA.

TABLE 1
Phospholipase A_2 Activity of Death Adder Venoms

Venoms	PLA_2 activity (μmol/min/mg)[a]
A. antarcticus (NSW)	145.8 ± 8.4
A. antarcticus (Qld)	142.1 ± 1.6
A. antarcticus (SA)	125.0 ± 6.5
A. antarcticus (WA)	344.8 ± 14.0
A. hawkei	318.0 ± 6.3
A. praelongus	255.0 ± 8.6
A. pyrrhus	476.4 ± 12.4
A. rugosus	140.2 ± 10.4[b]
A. sp. Seram	420.4 ± 10.8
A. wellsi	119.8 ± 6.2

[a]Data represent the mean ± SEM ($n = 6$–12).
[b]From Wickramaratna *et al.* (2003).

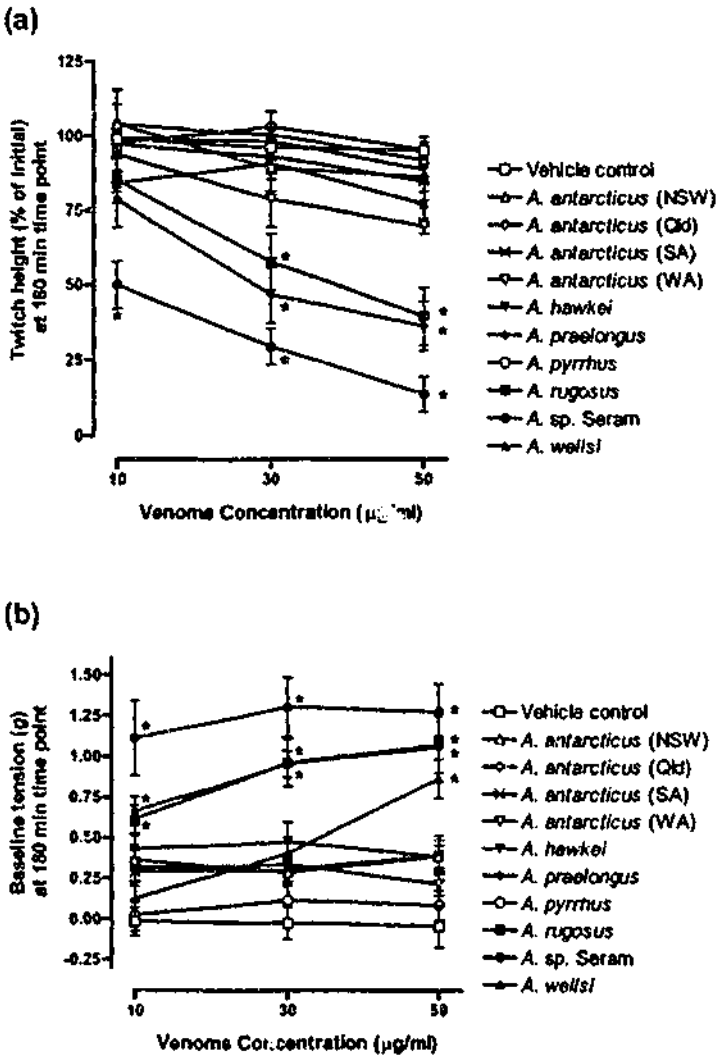
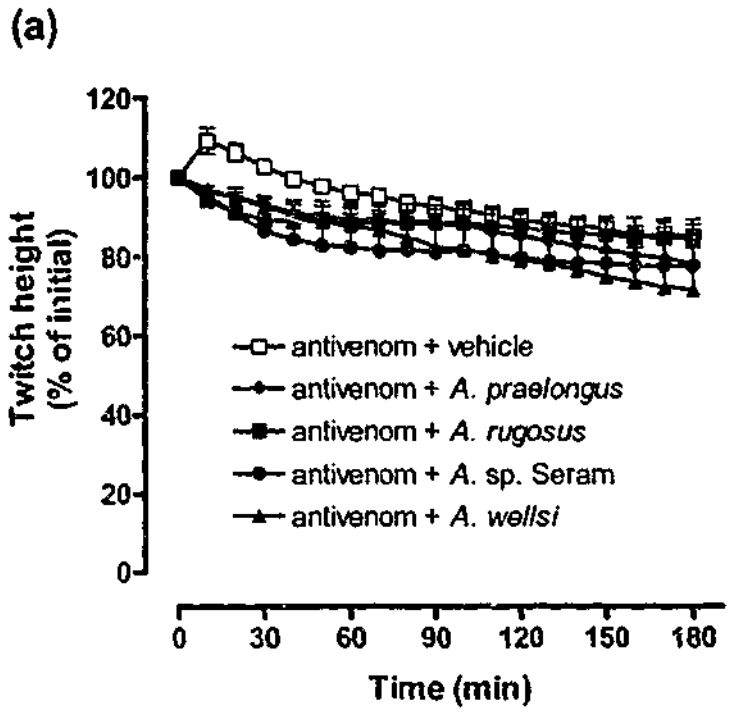


FIG. 2. The effect of *Acanthophis* venoms (50 µg/ml; n = 3–6) or vehicle (n = 6) on (a) direct twitches or (b) baseline tension of the CBCNM preparation. *$p < 0.05$, significantly different from vehicle, two-way ANOVA.

gus, and *A. rugosus* induced a significant increase in baseline tension compared to the vehicle (n = 4–6). However, there was no significant difference in the baseline contraction caused by *A.* sp. Seram venom at 10 µg/ml and 50 µg/ml (n = 4; Fig. 2b). This was also the case with *A. praelongus* and *A. rugosus* venoms.

Antivenom studies. Prior incubation (10 min) of CSL death adder antivenom (5 U/ml) prevented the inhibition of direct twitches and the increase in baseline tension caused by *A.* sp. Seram, *A. praelongus*, *A. rugosus*, and *A. wellsi* venoms (50 µg/ml; n = 4–7; Figs. 3a,b). *A.* sp. Seram, *A. praelongus*, *A. rugosus*, and *A. wellsi* venoms (50 µg/ml) in the presence of antivenom (5 U/ml) had no significant inhibitory effect on the direct twitches compared to the antivenom control (n = 4–7; Fig. 3a; one-way ANOVA, p = 0.49). Furthermore, *A.* sp. Seram, *A. praelongus*, *A. rugosus*, and *A. wellsi* venoms (50 µg/ml) in the presence of antivenom (5 U/ml) had no significant effect on the baseline tension compared to the antivenom control (n = 4–7; Fig. 3b; one-way ANOVA, p = 0.40).

4-BPB modified venom studies. *A. rugosus*, *A.* sp. Seram, and *A. praelongus* venoms (50 µg/ml) incubated with 4-BPB had no significant inhibitory effect on direct twitches compared to 4-BPB plus vehicle (n = 4–6; two-way ANOVA, $p < 0.0001$; Fig. 4a). However, *A. rugosus* *A.* sp. Seram, and *A. praelongus* venoms (50 µg/ml) incubated with vehicle (ace-



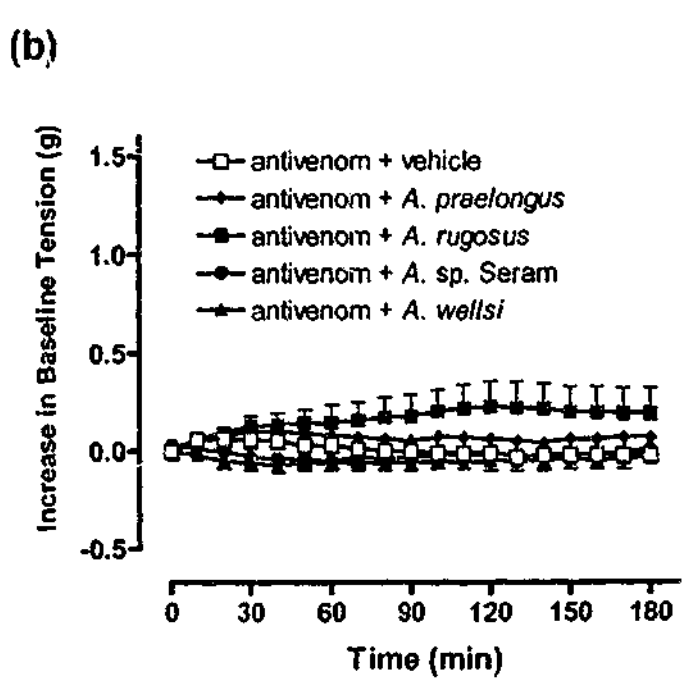


FIG. 3. The effect of *A. praelongus*, *A. rugosus*, *A.* sp. Seram, and *A. wellsi* venoms (50 µg/ml; n = 4–7) or vehicle (BSA; n = 4) in the presence of antivenom (5 U/ml) on (a) direct twitches or (b) baseline tension of the CBCNM preparation. *$p < 0.05$, significantly different from antivenom control, one-way ANOVA.

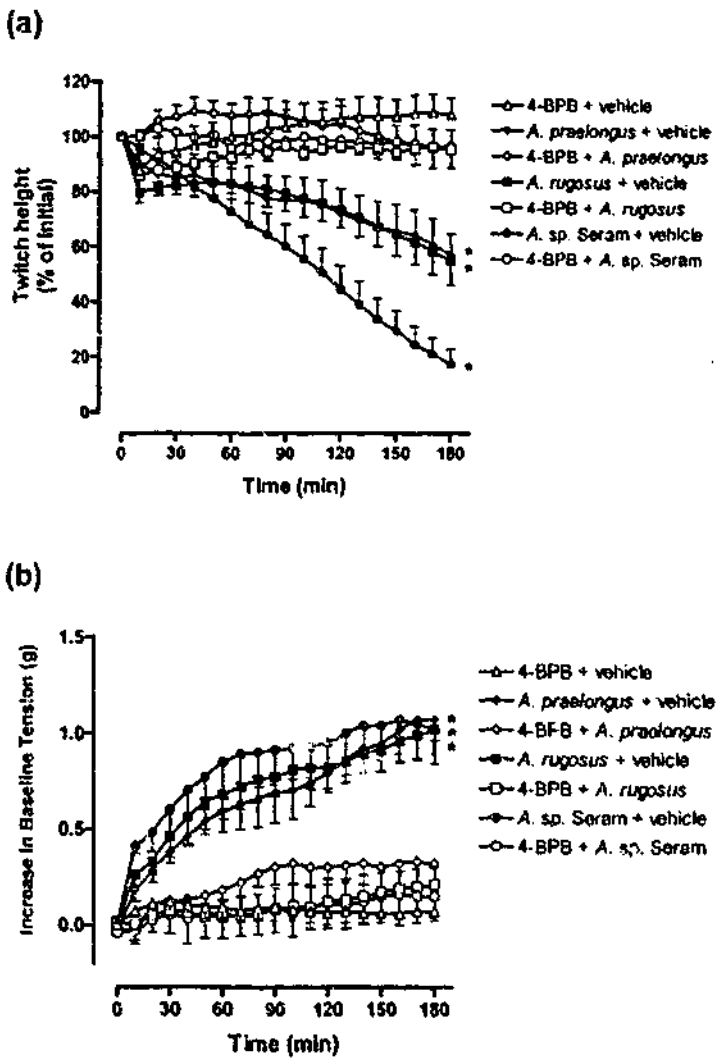


FIG. 4. The effect of *A. rugosus*, *A.* sp. Seram, and *A. praelongus* venoms (50 μg/ml; $n = 4–6$) or vehicle (sodium cacodylate; $n = 6$) incubated with 4-BPB (1.8 mM) on (a) direct twitches or (b) baseline tension of the CBCNM preparation. Positive control was *A. rugosus*, *A.* sp. Seram, and *A. praelongus* venoms (50 μg/ml; $n = 5$) incubated in vehicle (acetone). $^*p < 0.05$, significantly different from 4-BPB plus vehicle, two-way ANOVA.

tone) significantly inhibited direct twitches compared to 4-BPB plus vehicle (sodium cacodylate; $n = 4–6$; Fig. 4a). *A. rugosus*, *A.* sp. Seram, and *A. praelongus* venoms (50 μg/ml) incubated with 4-BPB had no significant effect on the baseline tension compared to 4-BPB plus vehicle ($n = 4–6$; two-way ANOVA, $p < 0.0001$; Fig. 4b). However, *A. rugosus*, *A.* sp. Seram, and *A. praelongus* venoms (50 μg/ml) incubated with vehicle induced a significant increase in baseline tension compared to 4-BPB plus vehicle ($n = 4–6$; Fig. 4b).

Morphological studies. Light microscopy studies of tissues exposed to *A.* sp. Seram, *A. praelongus*, *A. rugosus*, and *A. wellsi* venoms (10–50 μg/ml) showed dose-dependent morphological changes in skeletal muscle compared to the vehicle control tissues (Figs. 5a–e; data not shown for other venoms). These changes included the appearance of necrotic cells, vacuoles, edema, and cellular infiltrate. In contrast, tissues exposed to *A. antarcticus* (NSW, Qld, SA, WA), *A. hawkei*, and *A. pyrrhus* venoms (10–50 μg/ml) were similar in morphology to the vehicle control tissues (Fig. 5f; data not shown for other venoms). Prior incubation of CSL death adder antivenom (5 U/ml) prevented most of the morphological changes from occurring due to *A.* sp. Seram, *A. praelongus*, *A. rugosus*, and *A. wellsi* venoms. In the case of *A. rugosus* venom (50 μg/ml) a few vacuoles were evident in some tissues even in the presence of antivenom (5 U/ml; Fig. 5g). There were no detectable morphological changes in tissues equilibrated with antivenom alone (data not shown). *A. rugosus*, *A.* sp. Seram, and *A. praelongus* venoms (50 μg/ml) incubated with vehicle (i.e., acetone) induced morphological changes similar to the corresponding venom (50 μg/ml). However, no detectable morphological changes were seen in tissues exposed to *A. rugosus* (Fig. 5h), *A.* sp. Seram and *A. praelongus* venoms (50 μg/ml) incubated with 4-BPB or vehicle incubated with 4-BPB (data not shown).

DISCUSSION

Based on earlier studies on *A. antarcticus* venom it was thought that death adder venoms were devoid of myotoxic activity (Mebs and Samejima, 1980; Sutherland *et al.*, 1981). However, a recent clinical study reported evidence of rhabdomyolysis in patients following death adder envenomations, in Papua New Guinea, by a species different to *A. antarcticus* (Lalloo *et al.*, 1996). More recently, a myotoxic PLA_2 from *A. rugosus* venom was isolated (Wickramaratna *et al.*, 2003). Consequently, the present study examined the venoms of *A. praelongus*, *A. pyrrhus*, *A. hawkei*, *A. wellsi*, *A.* sp. Seram, and the regional variants of *A. antarcticus* for *in vitro* myotoxic activity. In addition, this study examined the effectiveness of CSL death adder antivenom in neutralizing the myotoxic activity of death adder venoms.

Death adder venoms were examined for *in vitro* myotoxicity using the directly stimulated CBCNM preparation. *A. antarcticus* (NSW, Qld, SA, WA), *A. hawkei*, and *A. pyrrhus* venoms did not cause a significant inhibition of the direct twitch height or an increase in the baseline tension. Furthermore, light microscopy studies indicated that tissues treated with these venoms had morphology similar to vehicle control tissues. Thus, these studies have shown that *A. antarcticus* (NSW, Qld, SA, WA), *A. hawkei*, and *A. pyrrhus* venoms are devoid of *in vitro* myotoxic activity. While several previous studies have shown that *A. antarcticus* venom is devoid of myotoxic activity none have examined the regional variations of this venom (Mebs and Samejima, 1980; Sutherland *et al.*, 1981; Wickramaratna and Hodgson, 2001). Liquid chromatography–mass spectrometry studies have shown variations in venom composition among the venoms of *A. antarcticus* regional variants (Fry *et al.*, 2001, 2002). Furthermore, functional studies have shown variations in neurotoxicity among the venoms of *A. antarcticus* regional variants (Fry *et al.*, 2001).

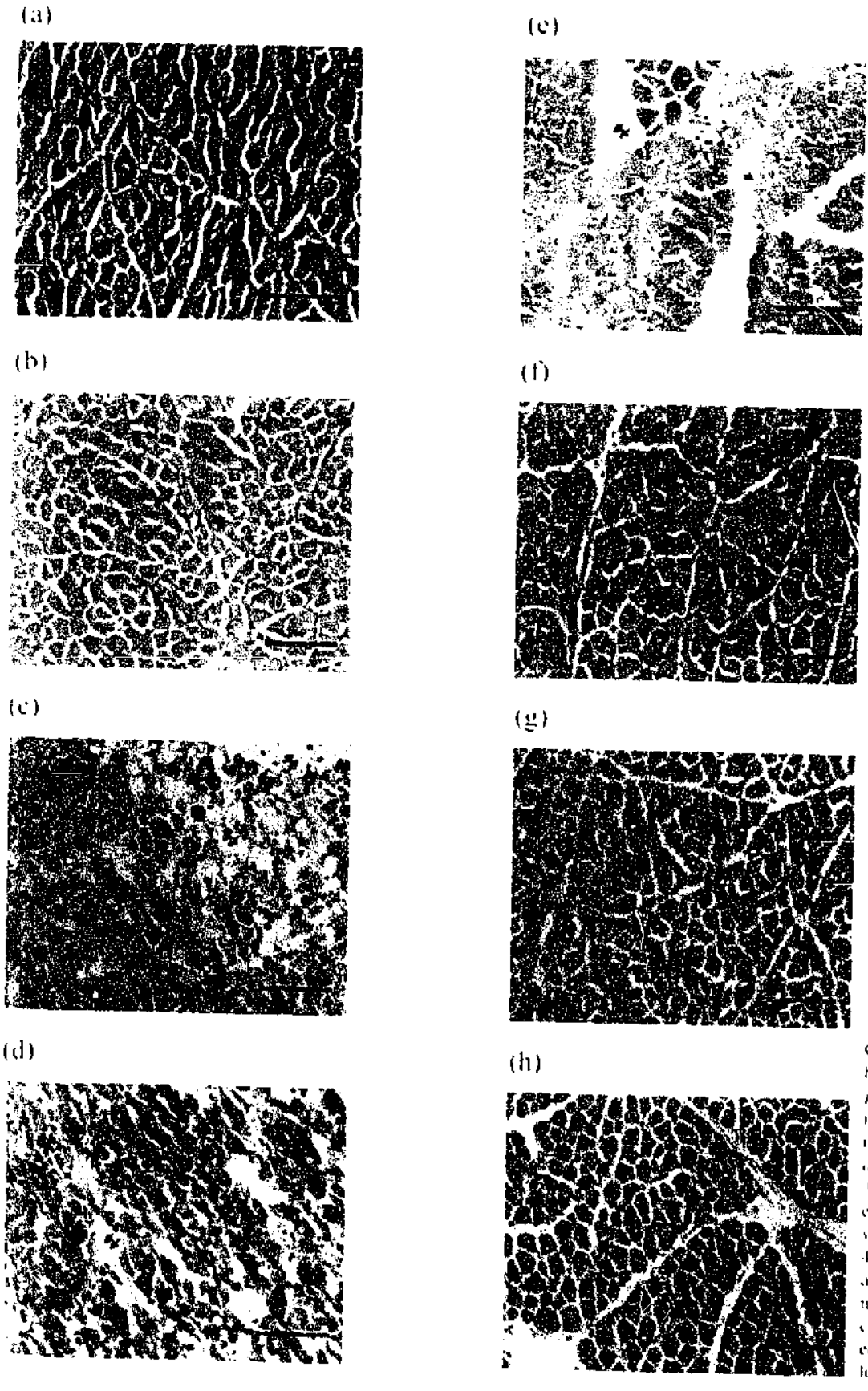


FIG. 5. Transverse sections of CBCNM preparations exposed to (a) vehicle (BSA); (b) *A. rugosus* venom (40 μg/mL); (c) *A. rugosus* venom (50 μg/mL); (d) *A. sp.* Setan venom (50 μg/mL); (e) *A. wellsi* venom (50 μg/mL); (f) *A. antarcticus* venom (WA; 50 μg/mL); (g) *A. rugosus* venom (50 μg/mL) in the presence of antivenom (5 U/mL); (h) *A. rugosus* venom (50 μg/mL) incubated with 4-BPB (1.8 mM). Scale bars, 100 μm in all micrographs. Arrows indicate prominent vacuoles, arrowheads indicate necrotic cells, double arrows indicate edema, double arrowheads indicate cellular infiltrate

Although *A. wellsi* venom had no effect on the direct twitch height it induced a dose-dependent increase in baseline tension. At the higher concentration, *A. wellsi* venom also caused morphological changes in skeletal muscle. Thus, suggesting that at higher concentrations this venom causes *in vitro* myotoxic activity. Both *A. praelongus* and *A. rugosus* venoms caused concentration-dependent inhibition of direct twitches, and an increase in baseline tension. Inhibition of direct twitches and a rise in baseline tension have been postulated to be indicative of myotoxic activity (Harvey *et al.*, 1994). Light microscopy studies showed that tissues exposed to *A. praelongus* and *A. rugosus* venoms caused dose-dependent morphological changes. Although we have previously shown that *A. rugosus* venom causes *in vitro* myotoxic activity (Wickramaratna *et al.*, 2003), this venom was included in the present study to allow for a comparison between venoms. In contrast to this study, a previous study showed that *A. praelongus* venom at 30 μg/ml did not cause a significant inhibition of direct twitches compared to the vehicle control (Wickramaratna and Hodgson, 2001). However, in that study the venom did cause a significant increase in baseline tension (Wickramaratna and Hodgson, 2001). This previous study neither examined a higher concentration of *A. praelongus* venom nor the morphology of exposed tissues. The use of younger chicks in the previous study may have contributed to this variability between the two studies (Harris, 1991).

At all concentrations tested, *A.* sp. Seram venom caused a significant inhibition of direct twitches and an increase in baseline tension. However, the twitch inhibition and the increase in baseline tension were not dose-dependent. Perhaps, had lower concentrations been tested, a dose-dependent effect may have been observed. Morphological studies however, showed dose-dependent skeletal muscle changes in tissues exposed to *A.* sp. Seram venom. Clearly, of all death adder venoms tested, *A.* sp. Seram venom was the most myotoxic.

While death adder envenomations have been uncommon in Australia in recent times due to habitat destruction and consequent decimation of populations, they are still significant health problem in Papua New Guinea and Irian Jaya (Currie, 2000; Currie *et al.*, 1991; Lalloo *et al.*, 1995, 1996; Sutherland, 1992). CSL death adder antivenom is the principal therapy for envenomation by any death adder species (AMH, 2003; White, 1998). Since *A. antarcticus* venom lacks myotoxic activity, and given that death adder antivenom has been raised against *A. antarcticus* venom, it was of clinical relevance to examine the efficacy of death adder antivenom against the *in vitro* myotoxicity of *A. praelongus*, *A. rugosus*, *A.* sp. Seram, and *A. wellsi* venoms. Prior incubation of antivenom totally prevented the inhibition of direct twitches and the increase in baseline tension caused by *A. praelongus*, *A. rugosus*, *A.* sp. Seram, and *A. wellsi* venoms. In addition, antivenom prevented most of the morphological changes from occurring due to these venoms. Therefore, CSL death adder antivenom is effective in neutralizing the *in vitro* myotoxic activity of death adder venoms. Previously, we have shown that death adder antivenom was effective in neutralizing the *in vitro* myotoxic activity of acanmyotoxin-1 (Wickramaratna *et al.*, 2003).

Since the most important clinical symptoms of death adder envenomations are due to postsynaptic neurotoxicity, anticholinesterase therapy has been suggested to supplement death adder antivenom (Currie *et al.*, 1988). Indeed, several clinicians have used anticholinesterases successfully to reduce the amount of antivenom administered (Currie *et al.*, 1988; Lalloo *et al.*, 1996; Little and Pereira, 2000). Anticholinesterase therapy has proven especially useful in Papua New Guinea and Irian Jaya to reduce the high costs associated with the use of death adder antivenom (Currie, 2000). However, given the results of the present study, clinicians may need to be mindful of possible myotoxicity following envenomations from *A. praelongus*, *A. rugosus*, *A.* sp. Seram, and *A. wellsi* species. With concomitant anticholinesterase therapy the neurotoxicity of death adder envenomations may resolve, however, unchecked myotoxicity could cause myoglobinuria and then renal failure.

Previously it was shown that acanmyotoxin-1, a myotoxic component from *A. rugosus* venom, contained high PLA_2 activity (Wickramaratna *et al.*, 2003). Studies have also shown that myotoxic fractions from other Australian elapid venoms contain PLA_2 activity (Harris and MacDonell, 1981; Mebs and Samejima, 1980). Liquid chromatography–mass spectrometry studies have shown that death adder venoms contain numerous components with molecular weights representative of PLA_2s (Fry *et al.*, 2002). Therefore, death adder venoms were examined for PLA_2 activity. While high PLA_2 activity was detected in all death adder venoms, *A. pyrrhus* venom had the highest specific activity. In order to examine whether the PLA_2 activity of *A. rugosus*, *A.* sp. Seram, and *A. praelongus* venoms is necessary for the myotoxic action, these venoms were subjected to 4-BPB modification. Although a myotoxic PLA_2 component has previously been isolated from *A. rugosus* venom this venom was subjected to 4-BPB modification to determine the presence of other components that may cause myotoxicity but are not mediated by PLA_2 activity. Studies have shown that PLA_2 activity can be inhibited by acylation using 4-BPB (Abe *et al.*, 1977; Volwerk *et al.*, 1974). 4-BPB treated *A. rugosus*, *A.* sp. Seram, and *A. praelongus* venoms had significantly reduced PLA_2 activity and no myotoxic activity. Thus, suggesting that PLA_2 activity is necessary for the myotoxic activity of these death adder venoms. However, no direct relationship was found between the degree of PLA_2 activity and the myotoxic activity of death adder venoms. For example, while *A. pyrrhus* venom had the highest PLA_2 activity it was devoid of myotoxic activity. This suggests the presence of other non-myotoxic PLA_2 components in those non-myotoxic death adder venoms. In fact, several PLA_2 components with antiplatelet activity have been isolated from *A. antarcticus* and *A. praelongus* venoms (Chow *et al.*, 1998; Sim, 1998). Similarly, it is possible that other non-myotoxic

PLA_2 components may also contribute to the PLA_2 activity of myotoxic death adder venoms.

In conclusion, *A.* sp. Seram, *A. praelongus*, *A. rugosus*, and *A. wellsi* venoms caused *in vitro* myotoxicity in the CBCNM preparation. In contrast, *A. antarcticus* (NSW, Qld, SA, WA), *A. hawkei*, and *A. pyrrhus* venoms were devoid of myotoxic activity. Although CSL death adder antivenom has been raised against *A. antarcticus* venom it is effective in neutralizing the myotoxic activity of *A. praelongus*, *A. rugosus*, *A.* sp. Seram, and *A. wellsi* venoms. Given the results of this study clinicians need to be mindful of possible myotoxicity following envenomations by *A. praelongus*, *A. rugosus*, *A.* sp. Seram, and *A. wellsi* death adder species.

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CHAPTER 6

General Discussion

The venom of the common death adder (*A. antarcticus*) was first studied pharmacologically in 1929 (Fairley, 1929a,b; Kellaway, 1929a,b). However, other death adder venoms have been poorly studied. In fact, no pharmacological studies have been undertaken on venoms from the Barkly Tableland (*A. hawkei*), Pilbarra (*A. wellsi*), or Irian Jayan (*A. rugosus*) death adders, nor from death adders from the Indonesian island of Seram (*A.* sp. Seram). Furthermore, previous studies did not make a distinction between the four geographic populations of *A. antarcticus*. In the current study, venoms from *A. hawkei*, *A. praelongus*, *A. pyrrhus*, *A. rugosus*, *A.* sp. Seram, *A. wellsi* and four geographic variants of *A. antarcticus* (NSW; Qld; SA; WA) were studied for *in vitro* neurotoxicity and myotoxicity. In addition, the efficacy of CSL death adder antivenom against the *in vitro* neurotoxicity and myotoxicity of these venoms was examined. The first ever myotoxin from a death adder venom, as well as two neurotoxins from *A. rugosus* and *A.* sp. Seram venoms were isolated and characterised.

Clinically, the most important symptoms of death adder envenomations are those relating to neurotoxicity, such as ptosis, generalized paralysis and respiratory failure (Campbell, 1966; Lalloo *et al.*, 1996). Hence, the above mentioned venoms were examined for *in vitro* neurotoxicity using the chick biventer cervicis nerve-muscle preparation. All venoms (0.3 – 10 μg/ml) caused concentration-dependent inhibition of nerve mediated twitches, and blocked contractile responses to exogenous acetylcholine and carbachol, suggesting postsynaptic neurotoxicity.

Given the obvious ethical concerns and complex regulatory requirements involved in gaining approval for murine LD_{50} studies in many countries, including Australia, they have been largely superseded by *in vitro* studies. As discussed in chapter two, one method of ranking venom neurotoxicity is by using isolated skeletal muscle preparations and comparing the time taken to cause 90% inhibition of nerve mediated twitches (ie. t_{90}

value). Table 6.1 compares the t_{90} values of death adder venoms with those of other Australasian and Asian-Pacific snake venoms that have been previously examined in our laboratory. Based on t_{90} values, the rank order of neurotoxicity of the snake venoms was: sea snake(s) > sea krait > death adder > tiger snake ≥ yellow (Stephen's) banded snake > Australian copperhead > taipan venoms. While sea snakes and sea kraits seem to contain the most neurotoxic venom, some death adder venoms are not far behind. In fact, among the terrestrial snakes death adder venoms are the most neurotoxic. However, as previously discussed in chapter two, it is likely that t_{90} values and LD_{50} values do not equate to the same parameters of toxicity. The rank order of lethality based on murine LD_{50}s of snake venoms was: taipan > tiger snake > beaked sea snake > common death adder > Australian copperhead > yellow (Stephen's) banded snake (Broad *et al.*, 1979). While murine LD_{50} determinations are based on "quantity" (ie. what concentration of venom kills 50% of mice usually over a 24 – 48 hour period), t_{90} values are based on how "quick" a venom acts. Therefore, it is possible to have an extremely "lethal" venom (based on LD_{50} values) that takes a long time to produce its effects. However, venoms with potent postsynaptic neurotoxins, which act rapidly, are likely to rank highly using the *in vitro* technique. Therefore, knowledge of both parameters is still desirable.

Given that murine LD_{50} studies are based on whole animals they take into account the lethality of numerous toxic components (i.e. neurotoxins, cardiotoxins, myotoxins, haemolytic factors, coagulant and anticoagulant factors), acting via different mechanisms, present in crude venom. Obviously, this is not the case with *in vitro* neurotoxicity studies and is a limitation of this procedure. However, neuromuscular paralysis is one of the most important effects of Australasian elapid envenomations. Therefore, in the absence of LD_{50} studies *in vitro* neurotoxicity studies provide valuable information.

Table 6.1 A comparison of the lethality (LD_{50}) and neurotoxicity (t_{90} values) of a range of Australasian / Asian-Pacific snake venoms

Common Name	Scientific Name	LD_{50} (mg/kg, s.c.)ψ	t_{90} @ 3µg/ml (min)	t_{90} @ 10µg/ml (min)
Beaked sea snake (Malaysia)*	*Enhydrina schistosa*	0.173	10.5 ± 0.7†	ND
Dusky sea snake	*Aipysurus fuscus*	ND	13.0 ± 1.4†	ND
Beaked sea snake (Weipa, Australia)*	*E. schistosa*	ND	13.1 ± 1.6†	ND
Banded sea krait	*Laticauda colubrine*	ND	14.6 ± 0.5†	ND
Olive sea snake	*Aipysurus laevis*	ND	15.5 ± 1.6†	ND
Common death adder (New South Wales)*	*Acanthophis antarcticus*	0.338	15.8 ± 1.3‡	10.2 ± 0.6‡
Leaf scaled sea snake	*Aipysurus foliosquamatus*	ND	18.0 ± 1.4†	ND
Common death adder (Queensland)*	*A. antarcticus*	0.338	20.7 ± 1.8[a]	9.6 ± 0.9‡
Seram death adder	*A.* sp. Seram	ND	21.1 ± 1.0‡	7.7 ± 0.5‡
Pilbarra death adder	*A. wellsi*	ND	23.7 ± 5.4‡	12.7 ± 1.8‡
Common death adder (South Australia)*	*A. antarcticus*	0.338	28.2 ± 2.5‡	11.4 ± 1.4‡
Desert death adder	*A. pyrrhus*	ND	28.5 ± 1.8[a]	18.9 ± 4.7‡
Common death adder (Western Australia)*	*A. antarcticus*	0.338	29.8 ± 5.0‡	9.8 ± 1.1‡
Irian Jayan death adder	*A. rugosus*	ND	30.6 ± 3.5‡	10.5 ± 0.5‡
Northern death adder	*A. praelongus*	ND	32.6 ± 3.0[a]	20.0 ± 2.3‡
Hardwick's sea snake (Weipa, Australia)*	*Lapemis hardwickii*	ND	33.5 ± 3.6†	ND
Hardwick's sea snake (Malaysia)*	*L. hardwickii*	ND	36.5 ± 4.7†	ND
Tiger snake	*Notechis scutatus*	0.118	38.1 ± 6.1§	21.7 ± 1.6§
Barkly tableland death adder	*A. hawkei*	ND	40.9 ± 5.5‡	17.4 ± 1.9‡
Yellow banded snake	*Hoplocephalus stephensi*	1.44	46.7 ± 6.7§	19.7 ± 1.7§
Australian copperhead	*Austrelaps superbus*	0.500	89.2 ± 9.8§	25.6 ± 2.5§
Inland taipan	*Oxyuranus microlepidotus*	0.010	ND	29 ± 3[b]
Coastal taipan	*O. s. scutellatus*	0.064	ND	43 ± 3[b]
Papuan taipan	*O. s. canni*	0.051	ND	45 ± 4[b]

Snake venoms are ranked by t_{90} at 3µg/ml. Data shown are mean ± SEM.
ND: not determined.
*Geographical variants; location indicated in parentheses.
ψData from Broad *et al.* (1979)
† Unpublished data from our laboratory.
‡ Data from Chapter 2
§ Data from Hodgson *et al.* (2003)
[a] Data from Wickramaratna & Hodgson (2001)
[b] Data from Crachi *et al.* (1999)
This table has been published in a similar form in Hodgson & Wickramaratna (2002).

A previous study in our laboratory showed that CSL death adder antivenom, while very effective against *A. praelongus* and *A. pyrrhus* venoms, was significantly less effective against the neurotoxicity of *A. antarcticus* (Qld) venom (Wickramaratna & Hodgson, 2001). Therefore, it was hypothesised that although death adder antivenom was raised against *A. antarcticus* venom it is possible that it may not have been raised against a pool of *A. antarcticus* venoms representative of all geographic variations. Thus, the antivenom may lack the ability to neutralise some neurotoxic components of venoms from sub-populations of *A. antarcticus* species (Schenberg, 1963; Wickramaratna & Hodgson, 2001). To investigate this hypothesis the efficacy of CSL death adder antivenom was examined against the *in vitro* neurotoxicity of venoms from *A. antarcticus* geographic variants. It was also of clinical relevance to determine the effectiveness of death adder antivenom against the *in vitro* neurotoxicity of those unstudied death adder venoms (Currie, 2000).

Studies described in chapter two showed that CSL death adder antivenom (1 unit/ml) was very effective against the neurotoxicity of *A. hawkei*, *A. praelongus* and *A. pyrrhus* venoms, while markedly less effective against *A. antarcticus* (NSW; SA; WA), *A. rugosus* and *A.* sp. Seram venoms. It was surprising that antivenom raised against *A. antarcticus* venom was markedly less effective against the neurotoxicity of all venoms from *A. antarcticus* geographical variants compared to venoms from *A. hawkei*, *A. praelongus* and *A. pyrrhus*. It is possible that death adder antivenom may neutralise some neurotoxins within the venoms better than others. This was confirmed by studies described in chapter three, utilising isolated neurotoxins from *A.* sp. Seram and *A. rugosus* venoms. Antivenom (1 unit/ml) had no significant effect on the twitch inhibition caused by acantoxin IVa. However, under the same conditions, antivenom totally prevented the twitch inhibition of acantoxin Va. This suggests that acantoxin IVa is one of the neurotoxic components

present in *A.* sp. Seram venom that is not readily neutralised by death adder antivenom. The same cannot be said of acantoxin Va. A higher concentration of antivenom (i.e. 5 units/ml) was required to prevent the twitch inhibition caused by acantoxin IVa. Similarly, the higher concentration of antivenom was effective against all death adder venoms. Therefore, it can be said that the effectiveness of death adder antivenom is dependent on its ability to neutralise the different neurotoxins within the whole venoms. Indeed, it has been shown that subtle changes in the primary sequence of neurotoxins can confer protection against antivenom (Abe & Tamiya, 1979; Menez *et al.*, 1982; Menez, 1991).

Liquid chromatography - mass spectrometry (LC-MS) studies revealed that a molecular mass representing acantoxin IVa is not found in any other death adder venom other than *A.* sp. Seram venom (refer chapter two & appendix; Fry *et al.*, 2002). However, it is likely that those death adder venoms less susceptible to neutralisation by antivenom may also contain neurotoxins that confer some protection against antivenom. LC-MS profiles of venoms from the *A. antarcticus* geographic variants showed a lesser degree of variability than observed between species. Based on these variations, Fry *et al.* (2002) suggested taxonomic relationships among death adder species. LC-MS studies indicated that venoms of *A. hawkei*, *A. rugosus* and *A.* sp. Seram are distinctive from other death adder venoms examined. However, caution must be shown when interpreting variations in venom since it has been suggested that venom composition may change as a result of feeding on local prey (Daltry *et al.*, 1996).

Given that the most important clinical symptoms of death adder envenomations are due to postsynaptic neurotoxicity, anticholinesterase therapy has been suggested as a supplement to death adder antivenom (Currie *et al.*, 1988). A marked recovery of neurotoxicity in death adder envenomed patients was observed after anticholinesterase treatment (Currie *et al.*, 1988; Hudson, 1988; Lalloo *et al.*, 1996; Little & Pereira, 2000).

However, Sutherland and Tibballs (2001) cautioned that anticholinesterase treatment was no substitute for antivenom therapy as the effects are transient. They also suggested that a neurophysiological study should be undertaken to confirm the effectiveness of anticholinesterases against death adder envenomation. Studies described in chapter two showed that the recovery of neurotoxicity due to the anticholinesterase neostigmine was transient in nature. Furthermore, the anticholinesterase had no significant effect on the overall neurotoxicity of any death adder venom as determined by the t_{90} values. Neostigmine had a very similar effect on both acantoxin IVa and acantoxin Va (refer chapter 3). Given the pseudo-irreversible nature of the antagonism caused by neurotoxins present in death adder venoms, it is no surprise that the neurotoxicity is not easily reversed. Perhaps, a more convincing reversal with neostigmine may have been obtained had a much lower concentration of these venoms and toxins been examined. Furthermore, it is possible that death adder neurotoxins may bind to nicotinic acetylcholine receptors in the chick biventer preparation more tightly than they do at mammalian and human skeletal muscle receptors. However, unpublished studies in our laboratory utilising the mouse phrenic nerve-diaphragm preparation also provided very similar results. Future studies could be carried out examining the effectiveness of a combination of anticholinesterase and antivenom against the *in vitro* neurotoxicity of death adder venoms.

Studies described in chapter two showed that the neurotoxicity of death adder venoms is predominately postsynaptic in nature. Prior to the current study, neurotoxins had not been isolated from any other death adder venom except those from *A. antarcticus* venom. Furthermore, the neurotoxins isolated from *A. antarcticus* venom have been poorly characterised pharmacologically. Hence, acantoxin IVa and acantoxin Va represent the first neurotoxins isolated from *A.* sp. Seram and *A. rugosus* death adder venoms, respectively. Although acantoxin IVa has a similar potency to α-bungarotoxin for skeletal

muscle nAChR it is approximately 25,000 fold less potent at α7-type nAChR. While acantoxin Va was several fold less potent than acantoxin IVa at skeletal muscle nAChR, it was approximately 350 fold more potent than acantoxin IVa at α7-type neuronal nAChR. However, in contrast to long-chain neurotoxins, acantoxin IVa completely inhibited specific [^{3}H]-MLA binding in rat hippocampus homogenate. Clearly, long-chain neurotoxin resistant [^{3}H]-MLA binding requires further investigation. While MLA is thought to be a selective antagonist of α7-type neuronal nAChR, a recent study found that MLA may also interact with presumed α3/α6β2β3-type neuronal nAChR in the rat striatal dopaminergic nerve terminals at low concentrations (Mogg *et al.*, 2002). Therefore, this opens up the possibility that long-chain neurotoxin resistant [^{3}H]-MLA binding may represent another subtype of nAChR. However, as mentioned in chapter three, detailed autoradiographical analysis has shown that the distribution of [^{125}I]-α-bungarotoxin and [^{3}H]-MLA binding sites correlate highly. Therefore, it is unlikely that long-chain neurotoxin resistant [^{3}H]-MLA binding would represent another type of nAChR (Davies *et al.*, 1999; Whiteaker *et al.*, 1999). Theories most favoured by Davies *et al.* (1999) and Whiteaker *et al.* (1999) for explaining 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. For the first time studies described in chapter three have 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 hippocampus homogenate.

A short-chain neurotoxin, as opposed to long-chain neurotoxins, was capable of blocking nicotine-evoked release of dopamine in the rat striatum (Dajas-Bailador *et al.*,

1998). Therefore, acantoxin IVa and acantoxin Va were examined for activity at other neuronal nAChR. Neither acantoxin IVa nor acantoxin Va displayed activity at α4β2 subtype neuronal nAChR or cytisine-resistant [^{3}H]-epibatidine binding sites. Furthermore, acantoxin IVa had no activity at ganglionic nAChR. In future, it would be interesting to determine whether acantoxin IVa is capable of blocking nicotine-evoked release of dopamine in the rat striatum. Furthermore, [^{125}I]-acantoxin IVa could be utilised to study whether short-chain neurotoxins preferentially bind to another neuronal nAChR compared to long-chain neurotoxins.

Based on earlier studies on *A. antarcticus* venom it was thought that death adder venoms were devoid of myotoxic activity (Mebs & Samejima, 1980; Sutherland *et al.*, 1981). A clinical study reported myotoxic activity *in vivo* following death adder envenomations, in Papua New Guinea, by a species thought to be different to *A. antarcticus* (Lalloo *et al.*, 1996). Guided by LC-MS studies the first myotoxic PLA_2 component was isolated from death adder venom. Acanmyotoxin-1, isolated from *A. rugosus* venom, caused dose-dependent myotoxicity in the chick biventer preparation. Antivenom studies showed that CSL death adder antivenom was effective in neutralising the myotoxic activity of acanmyotoxin-1. Studies described in chapter four also indicated that PLA_2 activity is essential for the myotoxic activity of acanmyotoxin-1.

Following the isolation of acanmyotoxin-1 death adder venoms were examined for *in vitro* myotoxicity. Studies showed that *A.* sp. Seram, *A. praelongus*, *A. rugosus* and *A. wellsi* venoms are myotoxic, while *A. antarcticus* (NSW; Qld; SA; WA), *A. hawkei* and *A. pyrrhus* venoms are devoid of myotoxic activity. Although CSL death adder antivenom has been raised against *A. antarcticus* venom it was effective in neutralising the myotoxic activity of *A. praelongus*, *A. rugosus*, *A.* sp. Seram and *A. wellsi* venoms. Given the results of this study clinicians need to be mindful of possible myotoxicity following

envenomations by *A. praelongus*, *A. rugosus*, *A.* sp. Seram and *A. wellsi* death adder species. Further studies based on an *in vivo* animal model would be useful to determine whether the myotoxins in death adder venoms cause systemic effects away from the site of injection.

In conclusion, it is anticipated that these *in vitro* neurotoxicity and myotoxicity studies on death adder venoms will be of potential clinical relevance. Furthermore, the first ever myotoxin from a death adder venom, as well as two neurotoxins from *A. rugosus* and *A.* sp. Seram venoms, were isolated and characterised. It is anticipated that future studies may use acantoxin IVa and acantoxin Va as research tools to further study neuronal nicotinic acetylcholine receptors.

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Appendix

Other Papers in Support of the Thesis

Electrospray liquid chromatography / mass spectrometry fingerprinting of *Acanthophis* (death adder) venoms: taxonomic and toxinological implications

Published in Rapid Communications in Mass Spectrometry (2002), vol. 16, pp. 600 – 608.

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RAPID COMMUNICATIONS IN MASS SPECTROMETRY
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RCM

Electrospray liquid chromatography/mass spectrometry fingerprinting of *Acanthophis* (death adder) venoms: taxonomic and toxinological implications

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Death adders (genus *Acanthophis*) are unique among elapid snakes in both morphology and venom composition. Despite this genus being among the most divergent of all elapids, the venom has been historically regarded as relatively quite simple. In this study, liquid chromatography/mass spectrometry (LC/MS) analysis has revealed a much greater diversity in venom composition, including the presence of molecules of novel molecular weights that may represent a new class of venom component. Furthermore, significant variation exists between species and populations, which allow for the LC/MS fingerprinting of each species. Mass profiling of *Acanthophis* venoms clearly demonstrates the effectiveness of this technique which underpins fundamental studies ranging from chemotaxonomy to drug design. Copyright © 2002 John Wiley & Sons, Ltd.

Death adders (genus *Acanthophis*) are unique among elapids in both morphology and venom composition. They are the only elapids that are viper-like in appearance and habit, with all species characterised by a somewhat flattened, triangular head, short stout body and thin rat-like tail ending in a curved spine.[1] The tail is used as a caudal lure to attract birds and reptiles. In addition, death adders are the only elapids with semi-mobile fangs. This, in some respects, makes them the most evolved of all elapids, and morphologically intermediate between the Elapidae and the Viperidae. The species-level taxonomy of these animals is poorly resolved, with a recent amateur revision[2] only serving to further muddy the waters.[3-5]

Death adders 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 through the Indonesian islands such as Seram, Halmahera, Obi and Tanimbar. In addition, due to the cryptozoic nature of these snakes, great differences in morphology can be observed even between geographically adjacent populations. This is particularly true of island forms, since these snakes are far less adept swimmers than other elapids, and gene flow between islands may thus be very restricted.

Death adder venoms have long been considered unique in being made up overwhelmingly by alpha postsynaptic neurotoxins.[6] Only recently have phospholipase A_2 toxins been isolated.[7-9] The peptidic neurotoxins found in Australian elapids all bind with high affinity to skeletal nicotinic acetylcholine receptors. However, two subgroups exist, differing in size, from an average of slightly above 60 amino acids (short chain) versus an average of 73 amino acids (long chain), and having either four or five disulfide bridges, respectively. These structural differences are due to the long-chain toxins having a C-terminal extension.[10] The majority of the Australian elapid PLA_2s are basic, 118 amino acids, have seven disulfide bonds and molecular weights in the range 12–14 kDa.

Death adder envenomations are a rare occurrence in Australia, but remain a significant health problem in Papua New Guinea.[11-13] Clinical symptoms of envenomation by *Acanthophis* spp. include those relating to the paralysis of bulbar and ocular muscles, and death occurs through inhibition of respiration due to paralysis of the voluntary muscles.[14,15] However, these studies also unexpectedly reported rhabdomyolysis in rodent assays and bleeding/coagulopathy in proven *Acanthophis* victims in Port Moresby (Papua New Guinea). These effects indicate that in some populations significant amounts of toxins other than neurotoxins are present. A previous study also showed

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significant differences in the ability of antivenom to reverse the effects of venom *in vitro*.[16]

The current study focused upon the major *Acanthophis* variants from continental Australia and islands in the Indo-Pacific region. Venom was profiled using liquid chromatography/mass spectrometry (LC/MS), a technique previously used to fingerprint a diverse array of venoms ranging from the *Brachypelma* genus of tarantula[17] through cone snails.[18] Further, mass spectrometric analysis was uniquely utilised to examine evolutionary trends in amphibians through the examination of skin toxins.[19] This study used LC/MS to profile the venoms to determine if patterns between species can be distinguished, and whether such profiles can contribute to the elucidation of the taxonomy of these animals. Particular attention was paid to the relationships between the *A. praelongus* populations.

MATERIALS AND METHODS

Species examined

Venoms from a number of populations of death adder were examined. In view of the confusion surrounding the systematics of this group, a discussion of the affinities of the venoms involved is crucial to allow the interpretation of our results in the light of future systematic findings.[4] *Acanthophis antarcticus* (common death adder) is the widest ranging species, with two disjunct geographic ranges, New South Wales/Queensland and South Australia/Western Australia. Venom was obtained from populations in New South Wales (Eden), Queensland (Gold Coast), South Australia (Eyre Penninsula), and Western Australia (Darling Range). The Seram death adder (*Acanthophis* sp.) is an unnamed species from the island of Seram. Similar populations may be found on the islands of Halmahera, Obi and Tanimbar. The Barkly Tableland death adder is an isolated form from the Barkly Tableland region of the Northern Territory. It was described as a new species, *Acanthophis hawkei*.[20] However, this was not recognised by most subsequent authors,[1] and its status remains to be resolved. *Acanthophis praelongus* (northern death adder) is a wide ranging species found in northern Queensland and the Northern Territory, and may in fact be a species complex, but its status at this time is far from resolved (K. Aplin, Western Australian Museum, personal communication). Our venoms are represented by the four main geographical variations, referred to by Hoser[2] as *A. praelongus* (Cairns, Queensland), *A. woolfi* (Mt. Isa, Queensland), *A. lancasteri* (Hayes Creek, Northern Territory) and *A. cummingi* (Humpty Doo, Northern Territory). However, in view of the lack of evidence for this taxonomic arrangement, the *praelongus* populations will be referred to in this text simply by geographical region, i.e., Cairns (pra-cai), Mt. Isa (pra-isa), Hayes Creek (pra-hay) and Humpty Doo (pra-hum). *Acanthophis pyrrhus* (desert death adder) is found in desert regions from western to central Australia. The venoms used in this study originate from specimens from the Alice Springs area. The Irian Jaya death adder is a problematic taxon, which was first described as *Acanthophis antarcticus rugosus*.[21] This taxon is not widely recognised in the literature, and is usually regarded as a synonym of *A. antarcticus* or *A. praelongus*. However, in the absence of clear evidence of conspecificity with any of the Australian mainland *Acanthophis*, we tentatively treat this form as a full species, *A. rugosus*. Our venoms come from the Merauke area of Irian Jaya. *Acanthophis wellsi* (Pilbara death adder, also called Black Head death adder) is found in the Pilbara region of Western Australia. The validity of this species has recently been confirmed.[22]

Venom collection

Pooled venoms for particular populations or geographic ranges were used for all species to minimise the effects of individual variations,[23] with at least six unrelated adults of both sexes making up each pool. Collection of venom from snakes utilised the naturally aggressive nature of these species, where venom is collected from the fang tips of an irritated specimen through the specimen biting down on a latex covered specimen bottle and injecting the venom. Polyethylene materials (polyethylene pipette tips, polyethylene Eppendorf tubes, specimen bottles) were used to handle and contain the venom due to the strong affinity some peptides possess for glass and polystyrene. The venom was dissolved in 0.1% trifluoroacetic acid (TFA)/H_2O solution for sample transfer and then lyophilised for storage. Venoms were collected by the first author with the exception of *A. rugosus* and *A.* sp. Seram, which were purchased from Venom Supplies, Tanunda, South Australia.

Liquid chromatography/mass spectrometry

On-line LC/MS of venom samples dissolved in 0.1% TFA to a concentration of ~1 mg/mL was performed on a Phenomenex C^{18} analytical column (1 × 150 mm, 5 µ particle size, 300 Å) with solvent A (0.05% TFA) and solvent B (90% acetonitrile in 0.045% TFA) at a flow rate of 50 µL/min. The solvent delivery and gradient formation was a 1% gradient from 0 to 60% acetonitrile/0.05% TFA over 60 min. Electrospray mass spectra were acquired on a PE-SCIEX API 300 LC/MS/MS system with an Ionspray atmospheric pressure ionisation source. Samples (10 µL) were injected manually into the LC/MS system and analysed in positive ion mode. Full scan data was acquired at an orifice potential of 80 V over the ion range 600–3000 *m/z* with a step size of 0.2 u. Data processing was performed with the aid of the software package Biomultiview (PE-SCIEX, Canada).

RP-HPLC

The column used was a Vydac Preparative C18 column (20 × 250 mm, 10 µ, 300 Å) on a Waters 600 HPLC system and UV absorbance was monitored at 214 nm by a Waters 486 tuneable absorbance detector. Samples were dissolved in 2 mL of buffer A (0.1% TFA), manually injected and then the column run at 100% A to wash off the concentrated salts present. The following gradient conditions of buffer B (90% acetonitrile, 0.09% TFA) were then used: 0 to 60% over 60 min (1% gradient) and then 60 to 80% in 5 min (4% gradient).

Edman degradation sequencing

Reduction/alkylation

Pure peptide (0.1 mg) was dissolved in 100 µL of 0.1 M ammonium bicarbonate at pH 7.0. Ten µL of 0.1 M tris(carboxyethyl)phosphine HCl (TCEP; pH 7) was added and incubated at 50°C for 20–30 min. Thirty µL of 0.1 M

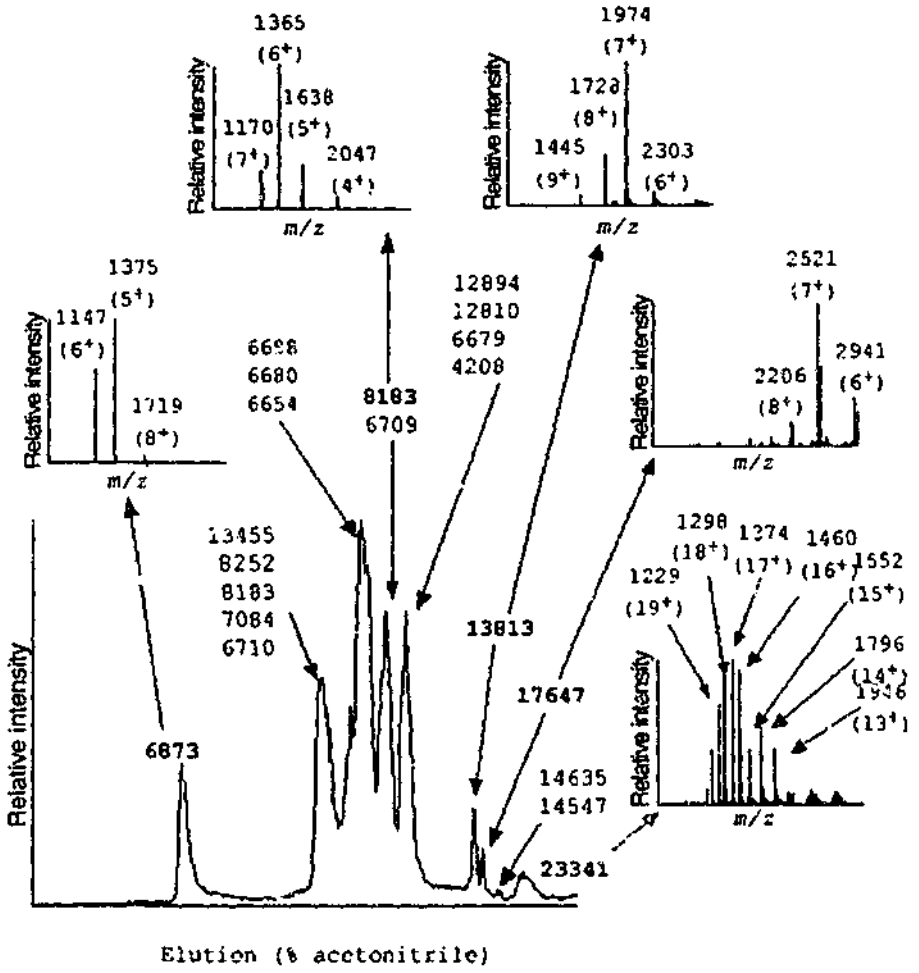

Figure 1. Representative LC/MS profiles of *Acanthophis* venom. Shown is the Western Australian population of *Acanthophis antarticus*. Mass (in Da) is given above each peak for each component present. A 1%/min gradient of acetonitrile was used.

maleimide were then added and incubated at 50 °C for a further 20–30 min. Maleimide added 97 u per cysteine and mass spectroscopy showed an increase of 194 Da per disulfide bond. The reduced/alkylated peptides were N-terminally sequenced using Edman degradation chemistry on an Applied Biosystems 477A protein sequencer.

Data analysis

In order to visualise the pattern of variation in venoms, all components present were scored as present or absent (0 or 1) for each venom. Results were analysed for a tolerance of 1–2 Da for interpretation of the results. Both phenetic and phylogenetic methods of analysis were employed: principal coordinates analysis and UPGMA were implemented using the MVSP (multivariate statistical package) software (Kovach Computing Services, Pentraeth, Anglesey, UK). The phenetic analyses were run on a matrix of Gower general similarity coefficients, which emphasise the shared presence rather than the shared absence of components.

An unrooted parsimony network was constructed using PAUP*4.0b8, using branch-and-bound searching. Due to the somewhat isolated and uncertain phylogenetic position of *Acanthophis* among Australasian elapids,[24,25] choosing an outgroup venom turned out to be impractical. Branch support was assessed by means of bootstrap analysis, using 10000 pseudoreplicates and branch-and-bound searching.

The above analyses were carried out for the combined data of all venom peaks, and separately for the three major classes of toxins, with molecular weights of 6–7, 7–8 and 13 kDa. Other toxin classes were represented by too few peaks to allow a meaningful analysis.

The interpretation of the phylogenetic and phenetic analyses is somewhat complicated by the fact these methods assume independence between characters, i.e., the presence/absence of one peak is assumed to be independent of the presence/absence of another. This is unlikely to apply fully to venom proteins when the homologies of different peaks are unclear: in some cases, different peaks in different venoms are likely to represent alleles of the same homologous locus, so that their presence/absence cannot be independent.

RESULTS

Venoms were profiled using LC/MS to determine differences in venom composition. All venoms had essentially the same generalizsed elution profile, consisting of an early eluting component but with the vast majority of eluting in the mid part of the gradient in Fig. 1). Significant similarities and differences as to the composition of individual molecules were evident in total numbers, molecular weights and retention times of the toxins (Table 1). Representatives of

Table 1. Toxins identified in *Acanthophis* venoms. Toxins are organised by elution and by molecular weight within each time block. Underlined components are the major components present in the venoms

% ACn	A. ant NSW	A. ant Qld	A. ant SA	A. ant WA	A. sp Ser	A. haw	A. pra-Cai	A. pra-Hum	A. pra-Mt. Is a	A. pra-Hay	A. pyr	A. rug	A. wel
8	6873	6873	6873	6873	6912 6816	6785	6853 6802	6854 6799	6854 6786	6952 6853	6913 6866	6913 6884	
11													6747
12										6973			
14	8750	8758	8752	8751			7050	6763	8659 6942	8659	7020 6942		7047
15		8290				8785 7991	8784 7014 7992	8791 7993	7992	8784 8558	7999 6914	7991 6844	6916
16		7291						13498 13456			6763	7656	7769
17	13456 8252 8183 7084	13456 8252 8183 6651	8252 8183 7084	13456 8252 8183 7084	13494 8196 8113	8834 8150	13496	8840	8820 8756 7032	8225 7055	8140	8829	8400 7575
18	6710 6703 5044	6710 5044	6710 5044	6711 5044	6703 5044	5044 7044	6709 5044	7044 5044	14651 8791 8130 6709 5044			6619 5044	6700 5044
19	8125 7073	7070		7070	7091	8148, 7027							7044
20	8635 6687 6651	8375 6686 6652	8376 6677 6652	6688 6680 6654	6535	6653 6623	8784 7269	13511	8784 6758 6654 6744			13151	8737 8509
21	6709	8291 6710	6709	6709	7296	8250 6709 6675	6698 6613	8839 6736 6665	7295 6709 6672	6695 6666 6635		13286	6765 6637
22	8186	6612	8183	8183	8196	6693	6676		7260		6725 6675	7217	6678
23	8635	6676	6676	6679	6704	6737	6709		6622	13510 6737	13485 6753	13048 12983 6657	6733
24	12897 12846	12841	12896	12898	13051	13427 13263 6753		13433 13288 7221	13182	13433 13265 12951 12799 7221	6755	13399	12994
25	12810 4207 4093	12810 4209	12854 12848 6461 4207	12810 4208	4344	8140 4238	13080 8385		12846 8130 4352	13545 6705 4351	12878 8171 4332	4223	13543 8143 7737 7322 4330
26		7280	7219			12879 13111 7219	12957 4353	13111 4351	12798	13037	7573	13084	
27			7272			13251 13139		13081		13126	8172 7507		8173 7505
28	13810	13809	13811	13809		13856 13779	13824	13828		13887	7257	13813	
29	17843 17646 14634 14547	17843 17646 14634 14547	17843 17646 14634 14547	17843 17646 14634 14547	14587 14523	17820 17611 14600 14515	17820 17157 14635 14548	17608 17153 14636 14600 14549 14505 14418	17810 17157	17850 17165 14637	7603	14635 14600 14548	
32	23349	23346	23286 23346	23346	23532	23340	23372 23292	23326	23359 23309	23326	23307	23370	23920

Table 2. N-Terminal sequences and identification of isolated toxins from the different classes in the venom

MW (Da)	Source	N-Terminal sequence	Snake venom toxin similarity[a]
4223	*A. rugosus*	EKPDSTGNGCFGFPIDRIGS	Natriuretic peptide
6617	*A. rugosus*	KNRPHFCHLPAYPGPCNAFV	Kunitz-type protease inhibitor
6654	*A. antarticus*	KDRPVFCNLPAYTGPCKNVL	Kunitz-type protease inhibitor
6814	*A.* sp Seram	MQCCNQQSSQPKTTTTCPGG	Short-chain alpha-neurotoxin
6854	*A. praelongus* Mt. Isa	MQCCNQQSSQPKTTTTCPGG	Short-chain alpha-neurotoxin
6873	*A. antarticus* WA	MQCCNQQSSQPKTTTTCPGG	Short-chain alpha-neurotoxin
7055	*A. praelongus* Hayes Creek	MQCYNQQPTQPKTTTTCPGG	Short-chain alpha-neurotoxin
7295	*A. sp* Seram	KTCFKTPYNKSEPCPDGQDL	Long-chain alpha-neurotoxin
7991	*A. rugosus*	VICYLGYNYAQPCPPGENVC	Long-chain alpha-neurotoxin
8125	*A. antarticus*	VICYRGYNNPQTCPPGENVC	Long-chain alpha-neurotoxin
8377	*A. antarticus*	VICYRKYTNNKTCPDGENVC	Long-chain alpha-neurotoxin
8751	*A. antarticus* WA	VICYVGYNNPQTCPPGGNVC	Long-chain alpha-neurotoxin
13408	*A. rugosus*	NLAQFGFMIKCAINGGSRPVV	Phospholipase A_2
13060	*A. rugosus*	NLAQFGFMIKCAANKGSRPV	Phospholipase A_2
13898	*A.* sp. Seram	NLLQFAFMIECANKMIQPVE	Phospholipase A_2
17843	*A. ant* SA	SIPKPSKNFEQFGNMIQCTI	Taipoxin-gamma chain

[a] Snake venom toxin type as determined by database sequence homology searching.

different toxin classes were sequenced and subsequent database searching revealed the type of toxin (Table 2).

The representative of the 4-kDa class was shown to be a member of the natriuretic peptides. While natriuretic peptides have been previously isolated from venoms ranging from elapids[26] to the platypus,[27] this is the first publication showing the widespread presence within a genus and they also represent the largest natriuretic peptides reported to date. The identity of the peptide of mass 5044 remains to be confirmed but it appears too large for it to be a natriuretic peptide and it does not fall within the range of any previously reported toxins from Australian elapids.[6] As such, it may represent a new class of venom peptide.

The putative peptidic neurotoxins accounted for the vast majority of the venom components present, consistent with the *in vitro* as well as clinical effects of the venoms.[16] As expected, the short-chain neurotoxins displayed strong homology with other members of this class isolated from Australian elapids.[6] The 7.2–8.8 kDa toxins were shown to be long-chain neurotoxins and displayed strong homology with previously isolated toxins. The 6.6–7 kDa class also contained kunitz-type protease inhibitors. Kunitz-type protease inhibitors have been previously isolated from numerous venoms and have been shown to be potent inhibitors of blood chemistry enzymes.[28] Accordingly, these inhibitors may be responsible in part for the bleeding disorders that have been reported from severe *Acanthophis* envenomations or in experimental models.[15,16]

Proteins with molecular weights corresponding to PLA_2 toxins (12–14 kDa) were also present in all the venoms, in greater quantities and molecular weight diversities than expected. N-Terminal sequencing of isolated components confirmed their identity as PLA_2s. As both anti-platelet and presynaptically active neurotoxic PLA_2s have been reported from these venoms,[8,9,29] the large abundance in some of the populations indicates that some envenomations may produce more complex symptoms than others.

In each venom analysed, LC/MS profiling revealed components of unusual size. Interestingly, the 17.6–17.8 kDa components were found only in the continental species *A. antarcticus*, *A. praelongus* and *A. hawkei*, being absent entirely from the island forms as well as in the continental species *A. pyrrhus* and *A. wellsi*. N-Terminal sequencing revealed them to be highly homologous to the gamma chain of taipoxin, an extremely potent neurotoxin complex from the *Oxyuranus scutellatus* (Coastal Taipan).[6] This raises the interesting question as to whether a similar complex is found in *Acanthophis* venom. The 23-kDa components were ubiquitous in the venoms, at least one isoform being present in each venom. As these components appear to be N-terminally blocked, they were not successfully sequenced for this study. However, these components are consistent in molecular weight and retention time with a group of toxins that share significant homology to the mammalian CRISP (cysteine rich secretory protein) family. These components have been widely reported in reptilian venoms, having been isolated from the gila monster[30] as well as in all the major families of venomous snakes.[31–33] Due to their virtual ubiquity, these toxins almost deserve the name 'fundamental toxin', and their evolution would represent a fascinating study topic from a venom evolution standpoint.

The first eluting component in all species had a mass similar to that of the short-chain peptidic neurotoxin Toxin Aa-c previously isolated from *A. antarcticus* venom.[34] Significantly, the molecular weight of each of the first eluting toxins was unique to each species, which may have uses in 'fingerprinting' the venoms (Table 3). Phenetic and phylogenetic analyses were carried out on a matrix of 192

Table 3. Diagnostic *m/z* fingerprint of each venom based upon the first major eluting peak

Species	A. ant	A. sp Ser	A. haw	A. pra-cai	A. pra-hum	A. pra-isa	A. pra-hay	A. pyr	A. rug	A. wel
m/z	6873	6816	6785	6854	6854	6854	6854	6866	6884	6747

Table 4. Number of components by molecular weight class identified by LC/MS analysis of *Acanthophis* venoms

Species	3–5 kDa	6.6–7.2 kDa	7.3–8.8 kDa	~14 kDa	17 kDa	~23 kDa	Total
A. ant NSW	3	8	7	7	2	1	28
A. ant QLD	2	10	9	6	2	1	30
A. ant SA	2	8	7	9	2	2	31
A. ant WA	2	8	4	6	2	1	23
A. sp. Seram	2	6	4	4	0	1	17
A. hawkei	2	10	5	12	2	1	32
A. pra-cai	2	9	8	6	2	2	29
A. pra-hum	2	6	4	12	2	1	27
A. pra-isa	2	11	10	4	2	2	31
A. pra-hay	2	9	5	9	2	1	28
A. pyrrhus	3	10	8	2	0	1	24
A. rugosus	3	7	4	9	0	1	24
A. wellsi	2	9	12	3	0	1	27

individual peaks and the 13 taxa represented in the study. Sixty-six compounds were shared between two or more venoms, the remainder were unique to one venom. The number of bands for each molecular weight class is shown in Table 4. The *Acanthophis antarcticus* populations share >70% of their components, whereas, in most other species, <60% of components are shared (except in pra-cai and pra-hum).

The parsimony analysis of the combined data of all peaks resulted in 53 equally most parsimonious trees of 245 steps (consistency index 0.7796; c.i. excluding uninformative characters 0.5462) and the parsimony network in Fig. 2(a). The four venoms of *Acanthophis antarcticus* cluster together, as do those of *A. wellsi* and *A. pyrrhus*. Both these nodes are supported by high bootstrap values. The relationships among the remaining taxa are unresolved. Reweighting all characters by the maximum value of their rescaled consistency indices resulted in considerably higher resolution (Fig. 2(b)). However, bootstrap support for the newly supported nodes was low, except for the node linking pra-hay and pra-hum.

The ordination of the individual venoms along the first three axes of the principal coordinates analysis is shown in Fig. 3, and the UPGMA phenogram in Fig. 4. UPGMA and

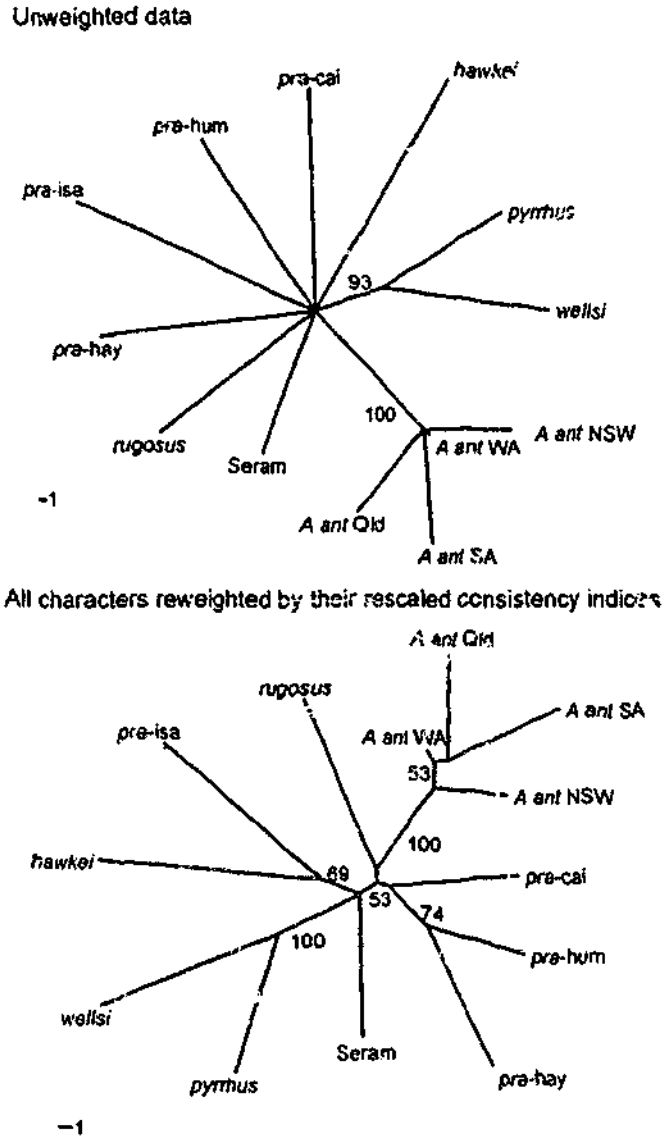

Figure 2. (a) Consensus of 53 equally most parsimonious networks of the 13 *Acanthophis* venoms. Numbers indicate bootstrap support for nodes, branch lengths correspond to the inferred number of evolutionary changes along each branch. (b) Parsimony network of the 13 *Acanthophis* venoms, each character being reweighted by the maximum value of its rescaled consistency index.

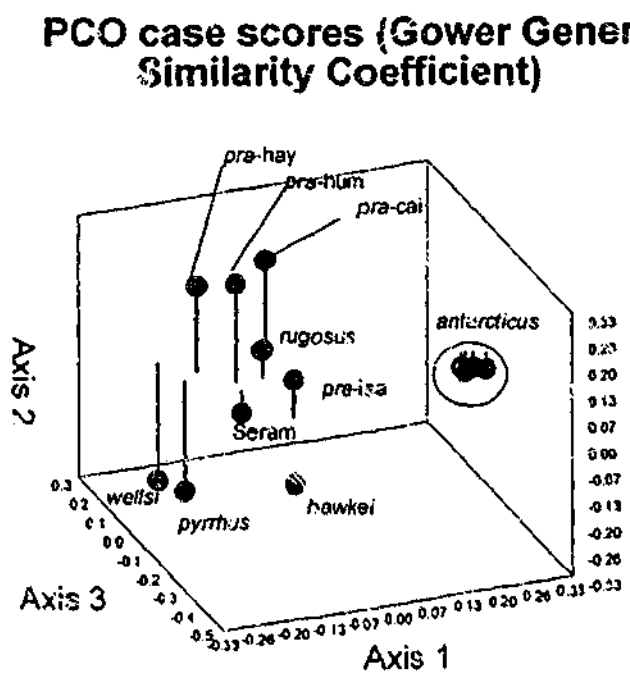

Figure 3. Ordination of *Acanthophis* venoms along the first three axes of the principal coordinate analysis. Axes 1, 2 and 3 summarise 24.9, 17.6 and 14.5% of the total variance, respectively.

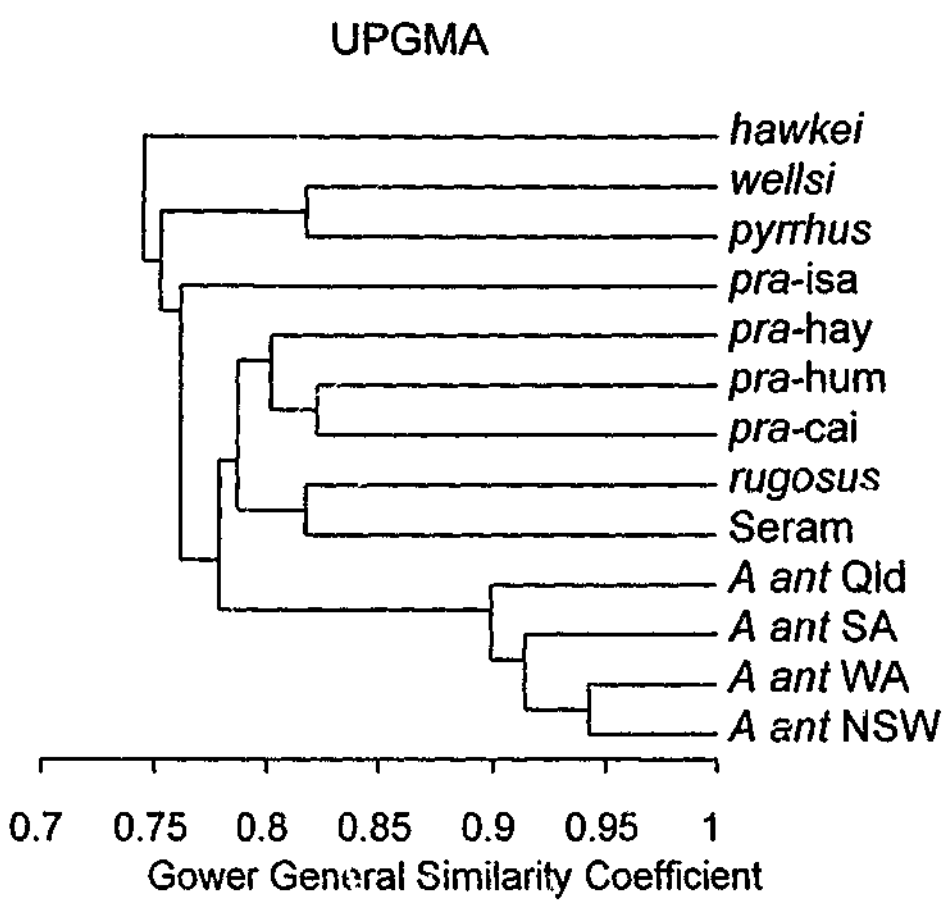

Figure 4. UPGMA phenogram of the 13 *Acanthophis* venoms included in this study.

PCO scatter plots of the three major toxin classes differed considerably from the plots of all peaks combined, and from each other. Both phenetic and phylogenetic analyses of the three major toxin classes revealed considerable incongruence between these toxin classes and the combined database. With the exception of the consistent clustering of the *A. antarcticus* samples, there were no consistent features of either dendrograms or scatter plots between toxin classes (data not shown).

Parsimony networks for three size classes of toxins (6–7 kDa; 7–8 kDa; PLA_2s) agree in joining the four *Acanthophis antarcticus*. However, they differ considerably in terms of the arrangement of the remaining taxa, to the extent where a consensus tree between them would be entirely unresolved except for the grouping of *A. antarcticus* venoms. Reweighting of characters adds little to any of the individual size class networks. The same applies for UPGMA dendrograms and PCO plots for the individual toxin classes: *A. antarcticus* venoms are consistently grouped together, and clearly distinct from venoms of the other taxa (data not shown).

DISCUSSION

The most significant revelation from the LC/MS studies was the diversity of venom molecules not predicted by previous studies.[16] However, the mass spectrometer used in this study was able to scan at a much greater range and sensitivity that that previously used (*m/z* 3000 versus a maximum scan of *m/z* 2300). This allowed for the determination of larger masses, components present in lesser amounts, and also more hydrophobic components. This increased range was particularly important in the revealing of a great diversity of PLA_2s as well as the discovery of new molecular weight classes of components in these venoms. Further, improved chromatography techniques allowed for better separation of the components and thus reduced the potential for ion suppression.

Also significant was the minimal level of conservation of individual molecules. The first eluting components have masses corresponding to isoforms of the short-chain neurotoxin Toxin Aa-c.[6] The characteristic masses for each species of this first peak allow for preliminary *m/z* fingerprinting. This is particularly notable in the case of the *A. praelongus* venoms. Despite these venoms showing far greater variance than the *A. antarticus* venoms, the first eluting peak always contained the 6854 toxin, which may suggest that these snakes are closely related. Another interesting feature revealed by the LC/MS profiling of the venom was components of unusual size. Natriuretic peptides of ~4.2 kDa are present in each venom and a component of mass 5044 is also present in all venoms but does not correspond in molecular weight to other isolated components from elapids and thus may represent a new class of venom molecule.

A. antarcticus crude venom has previously been examined for lethality, neurotoxicity, myotoxicity and its effects on blood coagulation, both experimentally and clinically.[14,35–39] Recently, *A. antarticus*, *A. praelongus* and *A. pyrrhus* venoms were studied for *in vitro* neurotoxicity, myotoxicity and phospholipase A_2 activity[29,40] and the entire genus studied for neurotoxicity and relative neutralisation by antivenom.[16]

Species variations in chromatographic profiles have been previously observed for *A. antarcticus*, *A. praelongus* and *A. pyrrhus* venoms.[29] In this study, venoms were profiled using on-line liquid chromatography/mass spectrometry to determine basic biochemical differences. As previously detailed, all venoms had essentially the same generalised elution profile. Given that these venoms are from snakes belonging to the same genus this is not surprising. However, close examination and comparison of each profile showed many differences in peak distribution and complexity between venoms from different species of death adder.

Previous reports suggest that variations in venom composition as a result of geographic location or differences in species are not unique to death adders.[41–44] This suggests that, potentially, LC/MS venom profiles may be of value in illuminating taxonomic relationships among death adder species, as has been previously suggested for some spider venoms.[17] Preliminary work on the LC/MS profiles of *Acanthophis* species showed the venoms of one species (*A. praelongus*) to be much more complex than the other species examined. It was hypothesised that this was due to *A. praelongus* actually being a species complex.[16]

Our analyses tend to agree with other studies on several points of potential taxonomic relevance[22] while differing from others.[2,22,20] The four samples of *Acanthophis antarcticus* invariably cluster together, and their separation from the remaining venoms is supported by high bootstrap values in the parsimony analysis. Moreover, the different *A. antarcticus* venoms have relatively few unique components. Among all *A. antarcticus* venoms, less than 30% of the compounds are unique to any one venom. In none of the analyses did the *A. antarcticus* samples fall into categories corresponding to the two geographically isolated populations (SA and WA vs. Qld and NSW), unlike the allozyme study of Aplin and Donnellan.[22] The venom data presented here thus do not support the recognition of western *A. acanthophis* as a separate subspecies or species, as has been suggested previously.[2,20]

Among the remaining species, each venom is made up to a far greater extent of unique compounds not found in other venoms examined: between 30 and 65% of observed peaks in any one venom are unique to that venom. These other venoms are all highly divergent from each other, and both phenetic and phylogenetic relationships between them are poorly defined (Figs 2–4), except that *A. pyrrhus* and *A. wellsi* cluster together in all analyses of the combined data, with high levels of bootstrap support in the parsimony analysis. Populations currently assigned to *Acanthophis praelongus* differed far more from each other than the even more widespread *A. antarticus* populations did, and did not form a cohesive cluster in any of our analyses. The greater complexity of the pooled *A. praelongus* venom in a previous study[16] was thus the result of the heterogeneity of the included venoms. Further work is needed to confirm whether the variations in venom are indicative of differences in genetic relatedness.

The consistent clustering of the four *A. antarcticus* samples, and that of *A. pyrrhus* with *A. wellsi*, are in agreement with the allozyme analysis presented previously.[22] The remaining venoms are all highly different from each other. Taken at face value, this validates the hypothesis that these different forms may constitute different species. Our results thus provide some evidence for the species status of *A. hawkei* and *A. rugosus*. The position of the undescribed Seram death adder requires further investigation. Its venom shows no particular affinities with any of the venoms included in this study, and differs considerably from that of its nearest neighbor (*A. rugosus*). However, several further *Acanthophis* 'forms' of uncertain status are found in New Guinea,[45] and the relationship between the Seram death adder and these forms requires further investigation. While it is beyond the scope of this paper to propose species status for the Seram death adder, the data presented here clearly marks this population as a potential candidate for being a new species but probably with strong affinities to *A. laevis* from New Guinea.

The congruence between venom profiles and allozyme evidence suggests that the LC/MS profiles may be of systematic usefulness, and that venom composition in these snakes may be associated with their phylogeny. Nevertheless, the taxonomic interpretation of venom data requires caution, as considerable differences in venom can occur between taxonomically undifferentiated populations,[46] perhaps as a result of natural selection for different prey in different regions.[47] There are no published studies of geographic variation in the diet of *Acanthophis*. Consequently, it is not presently possible to separate the effects of phylogeny and natural selection for different prey types on the pattern of variation in venom composition of these snakes.

The present study illustrates the usefulness of LC/MS profiles in the study of variation in snake venoms at low taxonomic levels. In this study, the LC/MS technique has helped develop a fingerprint for the venoms of different populations and species of death adder, as well as revealing the existence of classes of toxin in quantities greater than those reported for this genus in the past or revealing entirely new classes to be found in these venoms. This is in line with previous clinical reports as well as the *in vitro* studies undertaken in which the venoms were shown to have significant differences in activities and relative neutralisation by antivenom.[16] Furthermore, the venom profiles have revealed considerable potential to contribute towards the resolution of taxonomic problems within the *Acanthophis* genus.

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Invited Paper: Animal Toxins of Asia and Australia

IN VITRO NEUROMUSCULAR ACTIVITY OF SNAKE VENOMS

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SUMMARY

1. Snake venoms consist of a multitude of pharmacologically active components used for the capture of prey. Neurotoxins are particularly important in this regard, producing paralysis of skeletal muscles. These neurotoxins can be classified according to their site of action (i.e. pre- or post-synaptic).

2. Presynaptic neurotoxins, which display varying phospholipase A_2 activities, have been identified in the venoms of the four major families of venomous snakes (i.e. Crotalidae, Elapidae, Hydrophiidae and Viperidae). The blockade of transmission produced by these toxins is usually characterized by a triphasic effect on acetylcholine release. Considerable work has been directed at identifying the binding site(s) on the presynaptic nerve terminal for these toxins, although their mechanism of action remains unclear.

3. Post-synaptic neurotoxins are antagonists of the nicotinic receptor on the skeletal muscle. Depending on their sequence, post-synaptic toxins are subdivided into short- and long-chain toxins. These toxins display different binding kinetics and different affinity for subtypes of nicotinic receptors. Post-synaptic neurotoxins have only been identified in venoms from the families Elapidae and Hydrophiidae.

4. Due to the high cost of developing new antivenoms and the reluctance of many companies to engage in this area of research, new methodologies are required to test the efficacy of existing antivenoms to ensure their optimal use. While chicken eggs have proven useful for the examination of haemorrhagic venoms, this procedure is not suited to venoms that primarily display neurotoxic activity. The chick biventer cervicis muscle has proven useful for this procedure, enabling the rapid screening of antivenoms against a range of venoms.

5. Historically, the lethality of snake venoms has been based on murine LD_{50} studies. Due to ethical reasons, these studies are being superseded by *in vitro* studies. Instead, the time taken to produce 90% inhibition of nerve-mediated twitches (i.e. t_{90}) in skeletal muscle preparations can be determined. However, these two procedures result in different rank orders because they are measuring two different parameters. While murine LD_{50} determinations are based on 'quantity', t_{90} values are based on how 'quick' a venom acts. Therefore, knowledge of both parameters is still desirable.

6. *In vitro* neuromuscular preparations have proven to be invaluable tools in the examination of snake venoms and isolated neurotoxins. They will continue to play a role in further elucidating the mechanism of action of these highly potent toxins. Further study of these toxins may provide more highly specific research tools or lead compounds for pharmaceutical agents.

Key words: antivenom, neuromuscular junction, neurotoxin, phospholipase A_2, post-synaptic, presynaptic, snake venom.

INTRODUCTION

Snake venoms are a cocktail of toxins and enzymes that have evolved to assist in the capture and digestion of prey, as well as for use in defence against predators. Human systemic envenomation is associated with a number of adverse effects, the nature and severity of which depends on the species of snake, the quantity of venom administered and the time period between envenomation and the administration of appropriate medical treatment. These effects may include paralysis, myolysis, blood coagulation disturbances and renal damage.[1,2] Since the development of antivenoms and the introduction of efficient first-aid procedures, envenomation in Australia rarely results in death, with an average of two fatalities per year.[2] However, worldwide snake envenomation remains a major clinical problem, particularly throughout much of the Asian region. The accuracy of the figures is debatable, with a study in the 1950s suggesting an annual mortality rate of 25 000–35 000.[3] However, it is believed that these figures markedly underestimate the extent of the problem,[1] with more recent reports of an annual mortality of 100 000 in Asia.[4]

One of the major targets of snake venom is the somatic nervous system, in particular the skeletal neuromuscular junction. Inhibition of neurotransmission at this site results in the paralysis of bulbar and ocular muscles, as well as paralysis of respiratory muscles,[5,6] the latter often resulting in death. Therefore, much research has been directed at increasing our understanding of the action of snake venoms and isolated toxins at the neuromuscular junction.

NEUROMUSCULAR TRANSMISSION

The majority of neurotoxins act on the peripheral nervous system because they do not cross the blood–brain barrier.[7,8] However,

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before the action of venoms and toxins at the skeletal neuromuscular junction can be discussed, it is important to have a basic understanding of neuromuscular transmission at this site. Acetylcholine, the principle neurotransmitter at the skeletal neuromuscular junction, is synthesized and stored in the nerve endings. The majority is stored in vesicles (approximately 80%),[9] with the remainder being in solution in the axoplasm. Due to single vesicles spontaneously fusing with the presynaptic membrane, small amounts of acetylcholine are released continuously, resulting in small and transient depolarizations of the post-junctional membrane (i.e. miniature end-plate potentials; MEPP). These do not result in a twitch. However, in response to an action potential, depolarization of the nerve terminal results in the release of a sufficient quantity of acetylcholine to produce an end-plate potential (EPP), eventually resulting in a twitch response.[9,10] Acetylcholine, released either spontaneously or by depolarization of the nerve terminal, binds to a recognition site on either of the two α-subunits of the nicotinic receptor located in the post-synaptic membrane before being enzymatically degraded by acetylcholinesterase. Activation of the nicotinic receptor by acetylcholine requires both recognition sites to be occupied.[9]

Snakes have evolved potent toxins that are capable of inhibiting neuromuscular transmission at presynaptic (β-neurotoxins) and post-synaptic (α-neurotoxins) sites. The presynaptic neurotoxicity is associated with phospholipase A_2 activity found in the venoms. Many of these phospholipases A_2 have also been shown to be myotoxic, hypotensive, cardiotoxic and haemorrhagic, among other activities.[11] However, the present review will focus on their neurotoxic effects.

IN VITRO PREPARATIONS

Skeletal muscle preparations from the mouse, chick, rat and toad have been used previously for examination of the effects of venoms and toxins on neuromuscular transmission.[12-19] However, the mouse phrenic nerve–diaphragm and chick biventer cervicis nerve–muscle preparations have been more widely used and, therefore, provide a greater opportunity for comparisons between studies. For the former, diaphragms with intact phrenic nerves are dissected free from mice, while the chick preparation involves removal of the biventer muscles from the neck of young chicks.[20] The associated tendon encloses the nerve supply of the muscle. Both preparations are mounted under a resting tension of approximately 1 g in organ baths, with twitches being evoked by stimulating the motor nerve every 10 s with pulses of 0.2 msec duration at a supramaximal voltage. Addition of the competitive skeletal muscle nicotinic receptor antagonist (+)-tubocurarine and subsequent abolition of twitches can be used to confirm that the stimulation parameters chosen are selective for nerves.[15-18]

An additional advantage of the chick biventer cervicis nerve–muscle preparation is that it contains both focally and multiply innervated muscle fibres. The former mediate the electrically evoked twitch, whereas the latter can be stimulated by the addition of exogenous nicotinic agonists, such as carbachol or acetylcholine.[12,15-18] This enables prejunctional effects of venoms/toxins to be distinguished from post-junctional effects.

PRESYNAPTIC INHIBITION

A number of presynaptic neurotoxins have been isolated from the venoms of snakes. Indeed, they have been identified in the venoms of the four major families of venomous snakes, namely Crotalidae, Elapidae, Hydrophiidae and Viperidae, indicating the importance of their activity.[8] These toxins display phospholipase A_2 activity and have presumably evolved from having primarily a role in the digestion of prey to one that includes the immobilization and killing of prey. Interestingly, their neurotoxic activity does not appear to be related directly to their phospholipase activity and the subsequent hydrolysis of membrane phospholipids.[21] In humans, the paralysis associated with these toxins may be long lasting, up to 3 weeks, with some envenomed patients requiring prolonged assisted ventilation for survival.[8]

The presynaptic neurotoxins can be single-chain polypeptides (e.g. notexin) or toxins consisting of multiple subunits. For example, crotoxin, taipoxin and textilotoxin consist of two, three and five subunits, respectively (Table 1). Most snake venoms contain multiple isoforms of a particular neurotoxin that differ in their amino acid sequence.[8] While there does not appear to be a direct correlation between chain structure and potency, it has been postulated that there is a relationship between chain structure and binding.[22] Some of the presynaptic neurotoxins have been well characterized, while others (e.g. paradoxin) require further investigation. In general, these toxins produce neuromuscular blockade by inhibiting the release of acetylcholine from the nerve terminal. In doing so, they do not significantly alter the sensitivity of the motor endplate to acetylcholine. *In vitro*, the rate of onset of neuromuscular blockade is dependent on the temperature of the bathing solution and on the frequency of nerve stimulation, being markedly decreased as the temperature and/or frequency of stimulation is lowered.[23]

However, their activity is generally characterized by a triphasic effect on acetylcholine release (i.e. a decrease, followed by a transient increase and then complete blockade).[8,24,25] The initial two phases appear to be independent of phospholipase activity[7] and are

Table 1 Some representative presynaptic (β)-neurotoxins isolated from snake venoms

Toxin	Subunit composition	Snake		References
		Common name	Scientific name	
Notexin	Single chain	Australian tiger snake	*Notechis scutatus*	73
Taipoxin	Three subunits	Australian coastal taipan	*Oxyuranus scutellatus*	24, 26, 72
Paradoxin	Three subunits	Australian inland taipan	*Oxyuranus microlepidotus*	27, 71
Crotoxin	Two subunits	South American rattlesnake	*Crotalus durissus terrificus*	24, 26, 70, 74
Textilotoxin	Four subunits	Australian common brown snake	*Pseudonaja textilis*	14, 28
β-Bungarotoxin	Two subunits*	Asian krait	*Bungarus multicinctus*	24, 26, 74

*Covalently linked.

particularly evident when the safety factor of transmission is lowered by reducing the Ca^{2+} or increasing the Mg^{2+} content of the bathing medium.[26] However, there is some variation between β-neurotoxins with regard to this triphasic activity and, in particular, the response observed in different muscle preparations. For example, although paradoxin produces marked facilitation of twitch height, the initial inhibitory phase appears to be absent in the mouse phrenic nerve–diaphragm muscle preparation.[27] This activity is similar to that of notexin, where the initial inhibitory phase is also absent in mouse preparations,[10] rather than the more closely related taipoxin, although it has been reported that the initial inhibitory phase to taipoxin and β-bungarotoxin is markedly less pronounced in the rat phrenic nerve–diaphragm muscle preparation.[26]

Species differences in the sensitivity of nerve–muscle preparations to nerve-mediated twitch blockade by presynaptically acting phospholipase A_2 neurotoxins have been well documented.[25,26] We have shown that the mouse diaphragm is markedly more sensitive to paradoxin than the chick biventer cervicis.[27] The same order of sensitivity has been shown for taipoxin, textilotoxin and notexin, which are all β-neurotoxins from Australian elapids (Table 1). In contrast, crotoxin and β-bungarotoxin are far more potent on chick preparations.[25] Textilotoxin is reputed to be the most potent presynaptic neurotoxin isolated from snake venom.[25] Su *et al.* have shown that textilotoxin abolishes nerve-mediated twitches of the mouse phrenic nerve diaphragm muscle preparation in 194 min at a concentration of 0.3 μg/mL and in 110 min at a concentration of 1 μg/mL[28] However, we have shown that 0.3 μg/mL paradoxin abolished twitches in 110 min, indicating that, at least in this tissue, paradoxin is more potent than textilotoxin.[27] A similar time (i.e. 103 min) for complete inhibition in the mouse preparation has been reported for taipoxin, although this was recorded at a concentration of 1 μg/mL. Despite this apparent difference in the mouse phrenic nerve–diaphragm muscle preparation, the rate of twitch blockade produced by paradoxin (3 μg/mL) and taipoxin (3 μg/mL) in the chick biventer cervicis was found to be almost identical (i.e. 160 min).[16]

Interestingly, we found that increasing the concentration of paradoxin 10-fold in the mouse phrenic nerve–diaphragm muscle preparation had no significant effect on the time taken to abolish twitch responses.[27] This may indicate that there is saturation of binding sites for the neurotoxin. Alternatively, due to the mechanism of action of the toxin, it may not be possible to produce complete inhibition in a shorter time frame.

Of interest clinically is the finding that, after a relatively short time period (i.e. approximately 30 min), the inhibitory effects of the β-neurotoxins cannot be reversed by the addition of antivenom, although we have shown that antivenom can prevent the *in vitro* inhibition caused by both paradoxin and taipoxin if given prior to the addition of the neurotoxins.[16]

Considerable work has been undertaken to try and identify the binding site(s) on the presynaptic nerve terminal for these phospholipase neurotoxins (for a review on binding sites for phospholipases A_2 see Lambeau and Lazdunski[29]). Selective binding sites, which appear to be common to most of the phospholipases A_2, have been identified in a number of tissues, including rat brain synaptosomes (N-type) and rabbit cultured skeletal muscle cells (M-type).[30–32] Affinity for these binding sites displayed good correlation with their toxicity.[30] However, there were marked differences between the binding sites in different tissues, suggesting the existence of a family of receptors for neurotoxic phospholipases.[32] Additional work has shown that while the binding of notexin to receptor sites on the mouse hemidiaphragm was substantially reversible, the binding of crotoxin was only slightly reversible and that of β-bungarotoxin, taipoxin and textilotoxin was poorly reversible.[22]

In contrast with the early changes on neurotransmitter release produced by β-neurotoxins, the late block of release appears to be due to their phospholipase A_2 activity and the hydrolysis of membrane phospholipids.[24] Whether the late block of acetylcholine release produced by the β-neurotoxins is due to an internal or external action at the presynaptic membrane has been the topic of much debate. Previous studies have suggested that the neurotoxins are not internalized.[22] However, an internal action where the neurotoxins enter the lumen of the synaptic vesicles following endocytosis and hydrolyse the phospholipids of the inner leaflet of the membrane has recently been suggested.[33] This hypothesis appears to account for the majority of actions reported for these toxins. This has been supported by work showing that calmodulin, which is generally considered to be an intracellular protein, is an acceptor for the presynaptic toxin ammodytoxin C.[34]

In experiments using perineural recording in the mouse triangularis sterni, many of the β-neurotoxins have been shown to block K^+ currents.[27,35,36] It has been suggested that the transient facilitatory phase produced by β-neurotoxins (see above) is due to this activity, with the resulting slowing of repolarization following an action potential allowing a greater influx of calcium, which, in turn, results in increased release of acetylcholine.[10,37] Interestingly, the results of a recent study examining a range of presynaptic neurotoxins, using patch clamping and several types of cloned voltage-gated K^+ channels stably expressed in mammalian cell lines, suggest that the facilitation observed is unlikely to be due to blockade of these K^+ channels.[38] However, as the authors indicated, they cannot exclude the possibility that the cloned voltage-gated K^+ channels used in their study did not express the binding site for PLA_2 neurotoxins or that they lack important regulatory proteins.

Studies examining the longer-term neuropathological effects of the presynaptic neurotoxins notexin and taipoxin in the hind limb of rats have shown that after producing depletion of transmitter from the motor nerve terminals the toxins cause degeneration of the terminal and axonal cytoskeleton.[39,40] This was characterized by 70% of muscle fibres being completely denervated within 24 h, with almost complete regeneration and functional re-innervation by 5 days. However, collateral innervation, which persisted for at least 9 months, was common in regenerated muscles.[39] Similar findings had previously been reported for β-bungarotoxin.[40]

MYOTOXICITY

Many of the phospholipase A_2 toxins found in snake venoms also display potent myotoxicity. However, there is considerable variability in the degree of myotoxicity displayed by these toxins.

While the examination of the neurotoxic components of snake venoms in isolated skeletal muscle preparations has been well established, the suitability of the same preparations for the examination of myotoxic components has been questioned. Myotoxicity is hard to define in the absence of a histological analysis of the affected muscle.[12,41] However, for the purpose of preliminary screening, monitoring the effect of venoms or toxins on electrically

evoked twitches of skeletal muscle preparations has proven useful.[12,15,18] These studies have focused on the inhibition of twitches and measurement of direct contracture (rise in the baseline tension). Twitches are evoked by stimulating the muscle directly, usually every 10 s with pulses of 2 msec duration at a supramaximal voltage. To achieve selective stimulation of muscle, the experiments may be performed in the continued presence of (+)-tubocurarine.[15,18]

Myotoxic phospholiase A_2 toxins have been isolated from the venom of the King brown snake (*Pseudechis australis*). All four components (i.e Pa-1G, Pa-5, Pa-12C and Pa-15) inhibited direct twitches of the mouse phrenic nerve–diaphragm muscle preparation and produced slow developing contractures and inhibited responses to KCl in the chick biventer cervicis.[42] In contrast, the venom of the Papuan taipan (*Oxyuranus scutellatus canni*)[15] and three death adders (*Acanthophis antarcticus, Acanthophis praelongus* and *Acanthophis pyrrhus*)[18] produced no significant effect on direct twitches of the chick biventer cervicis muscle. This indicates an apparent lack of myotoxicity at concentrations equal or greater than those producing complete abolition of indirect twitch contractions, although *A. praelongus* venom did produce a significant contracture.[18]

POST-SYNAPTIC INHIBITION

Post-synaptic or α-neurotoxins are antagonists of the nicotinic receptor on the skeletal muscle. They are widely referred to as 'curare-mimetic toxins' due to their similarity in action to the competitive nicotinic receptor antagonist (+)-tubocurarine.[43] These neurotoxins bind with high affinity ($K_D = 10^{-12}$ to 10^{-9} mol/L) and specificity to acetylcholine binding sites on skeletal muscle nicotinic receptors.[44] The skeletal muscle nicotinic receptor is a heteropentameric protein consisting of five membrane-spanning subunits with the stoichiometry of 2α1, 1β1, 1γ and 1δ.[45,46] The receptor consists of two acetylcholine binding sites located at the interfaces between the α1 and γ and the α1 and δ chains.[45,47] Given that these binding sites interact in a positively co-operative manner, by occupying one or both sites snake α-neurotoxins inhibit the opening of the ion channel associated with the receptor in response to cholinergic agonists.[46] Thus, α-neurotoxins block nicotinic transmission in skeletal muscle and cause paralysis in prey.

In contrast with β-neurotoxins, α-neurotoxins are only found in the venoms of snakes from the families Elapidae and Hydrophiidae. To date, more than 100 post-synaptic neurotoxins have been isolated and sequenced.[43] Depending on their sequence, post-synaptic neurotoxins are subdivided into short- or long-chain neurotoxins.[48] Short-chain neurotoxins consist of 60–62 amino acid residues and four disulphide bridges.[43] Long-chain neurotoxins have 66–74 amino acid residues and usually five disulphide bridges.[43] While the positions of four disulphide bridges are common to both long- and short-chain neurotoxins, the extra disulphide bridge found in long-chain neurotoxins is generally located between Cys-30 and Cys-34.[43] Until recently, the major functional difference between the two types of α-neurotoxins was thought to be in the kinetics of association and dissociation with the skeletal muscle nicotinic receptor.[49] It was shown that short-chain neurotoxins tend to associate with the receptor approximately six- to sevenfold faster and dissociate five- to ninefold faster than long-chain neurotoxins.[50] However, it has also been shown that long-chain α-neurotoxins bind to neuronal α7 nicotinic receptors with higher affinity than short-chain neurotoxins.[51] While lacking the extra disulphide bridge, *Laticauda colubrina* toxin has been considered to be a long-chain neurotoxin based on high sequence homology with other long-chain α-neurotoxins.[43,51] However, functionally it behaves as a short-chain neurotoxin at neuronal α7 nicotinic receptors.[51] Thus, functional classification of α-neurotoxins requires pharmacological characterization of toxins at both skeletal muscle and neuronal nicotinic receptors.

Many of the amino acid residues of erabutoxin a, a short-chain neurotoxin from the sea snake *Laticauda semifasciata*, necessary for high-affinity binding to skeletal muscle nicotinic receptors have been identified. Using site-directed mutagenesis, it has been shown that changing any one of Ser-8, Lys-27, Trp-29, Asp-31 or Arg-33 decreased the binding affinity of the toxin by several-fold.[52] In addition to these amino acid residues, Gln-7, Gln-10, Ile-36, Glu-38 and Lys-47 of erabutoxin a were shown to be important for high-affinity binding to the skeletal muscle nicotinic receptor.[53] Therefore, it seems that specific amino acid residues from all three loops of erabutoxin a contribute to the 'functional site', including both invariant and variant residues. It has been suggested that the invariant residues form a common 'functional core', whereas the variant residues allow for prey-specific high-affinity binding.[53,54]

Although more than 100 neurotoxins have been isolated and sequenced, only a few have undergone extensive pharmacological characterization. Many toxins have been classified as post-synaptic neurotoxins on the basis of their amino acid sequence and/or observation of flaccid paralysis in mice. While in the past neurotoxins have undergone LD_{50} studies (tabulated in Mebs and Claus[55]), these are becoming increasingly difficult to perform for obvious ethical and regulatory reasons. Other studies have undertaken binding experiments using membrane-bound nicotinic acetylcholine receptors from *Torpedo* electric organs.[50,56] While this assay provides important binding information on post-synaptic neurotoxins, it is not possible to determine whether the toxin is an agonist or antagonist at the receptor. However, agonist or antagonist activity can be determined using *in vitro* neuromuscular preparations. As described above, both the mouse phrenic nerve–hemidiaphragm and chick biventer cervicis muscle preparations have been used extensively to determine the neurotoxicity of snake venoms.[12,15,18] However, although the toad rectus abdominis and chick biventer cervicis preparations contain multiply innervated muscle fibres and are therefore able to respond to exogenous nicotinic agonists, to our knowledge these or other such preparations have not been used to determine the pA_2 value of post-synaptic neurotoxins. This is probably due to the widely known fact that the majority of snake post-synaptic neurotoxins, especially the long-chain neurotoxins, undergo almost irreversible binding to the skeletal muscle nicotinic receptor.[50] Given the pseudo-irreversible antagonism displayed by most post-synaptic neurotoxins, Schild plot analysis of concentration–response curves to nicotinic agonists in the presence of these neurotoxins is invalid. However, the 'modified Lew and Angus' method may be used to generate a reliable estimate of the pA_2 value in these cases.[57–59] In addition, given the pseudo-irreversible antagonistic nature of snake neurotoxins, such as α-bungarotoxin, one must question the appropriateness of referring to these neurotoxins as 'curare-mimetic toxins'.

ANTIVENOMS AND *IN VITRO* TESTING

The vast majority of antivenoms are produced in animals. For example, in Australia, the clinically available snake antivenoms are raised in horses by CSL (Melbourne, Victoria, Australia). Due to the high cost involved in the development and production of new antivenoms and the relative small return to the manufacturer, it is unlikely that many new antivenoms will become available using current technology. If recent history is an accurate guide, then the few new antivenoms that do end up on the market are likely to be cost prohibitive. For example, CroFab™ (Crotalidae Polyvalent Immune Fab (Ovine); Protherics, Nashville, TN, USA) received US Food and Drug Administration (FDA) approval in October 2000 for use in patients with minimal or moderate North American crotalid envenomation. CroFab™ is a lyophilized preparation of ovine Fab (monovalent) immunoglobulin fragments obtained from sheep immunized with one of four snake venoms.[60] The final product is a mixture of the four monospecific antivenoms. Each monospecific antivenom is obtained by fractionating the immunoglobulin from the ovine serum, digesting it with papain and isolating the venom-specific Fab fragments on ion exchange and affinity chromatography columns. The final product has proven to be very effective clinically, but an initial dose of six vials is recommended,[60] with additional administration as required.[61] This is likely to result in the combined administration of ≥ 12 vials. At a cost of US$9300 for 12 vials,[61] this is an expensive procedure and one that is unlikely to be extrapolated to the Asia–Pacific region where there is an acute shortage of specific (i.e. monovalent) antivenoms. Therefore, the lack of antivenoms for systemic envenomation by many snakes is likely to remain a clinical problem. In some cases, even when antivenoms are available, there is a high risk of adverse effects. Despite using state-of-the-art production procedures, clinical trials have shown that the use of CroFab™ still results in patients developing acute reactions or serum sickness.[60] In response to this problem, considerable research is being directed at alternative production methods, including hyperimmunizing adult chickens with snake venom and isolating antibodies from the eggs.[62,63] However, for the foreseeable future, it is likely that many cases of snake envenomation will be treated with non-species-specific antivenoms.[64] Therefore, there is a need for simple screening tests to examine the efficacy of antivenoms against a range of snake venoms. Current preclinical testing involves the median effective dose (ED_{50}) test in mice. This involves calculating the amount of antivenom that, when injected intravenously together with a defined lethal quantity of venom, results in the survival of 50% of animals over a 24 h period.[65] However, this test requires large numbers of animals. Therefore, alternative methods are being investigated.

One method that has been proposed involves the use of chicken eggs. Hatching eggs are used on the 6th day after fertilization, at which stage the embryo is alive but is insensate.[65–67] However, while this methodology appears promising for the examination of haemorrhagic venoms (e.g. Viperidae), it is not sensitive to venoms that primarily display neurotoxic activity (e.g. Elapidae and Hydrophidae). Although it may be overly simplistic to categorize the actions of venoms in this way,[1] it is snakes from these latter

Table 2 A comparison of the lethality (LD_{50}) and neurotoxicity (t_{90} values) for a range of Asian–Pacific snake venoms

Common name	Scientific name	LD_{50}(mg/kg, s.c.)§	t_{90} @3 μg/mL (min)	t_{90} @10 μg/mL (min)
Beaked sea snake (Malaysia)*	*Enhydrina schistosa*	0.173	10.5 ±0.7†	ND
Dusky sea snake	*Aipysurus fuscus*	ND	13.0 ±1.4†	ND
Beaked sea snake (Weipa, Australia)*	*E. schistosa*	ND	13.1 ±1.6†	ND
Banded sea krait	*Laticauda colubrina*	ND	14.6 ±0.5†	ND
Olive sea snake	*Aipysurus laevis*	ND	15.5 ±1.6†	ND
Common death adder (New South Wales)*	*Acanthophis antarcticus*	0.338	15.8 ±1.3‡	10.2 ±0.6‡
Leaf scaled sea snake	*Aipysurus foliosquamatus*	ND	18.0 ±1.4†	ND
Common death adder (Queensland)*	*A. antarcticus*	0.338	20.7 ±1.8‡	9.6 ±0.9‡
Seram death adder	*Acanthophis* sp. seram	ND	21.1 ±1.0‡	7.7 ±0.5‡
Black head death adder	*Acanthophis wellsi*	ND	23.7 ±5.4‡	12.7 ±1.8‡
Common death adder (South Australia)*	*A. antarcticus*	0.338	28.2 ±2.5‡	11.4 ±1.4‡
Desert death adder	*Acanthophis pyrrhus*	ND	28.5 ±1.8‡	18.9 ±4.7‡
Common death adder (Western Australia)*	*A. antarcticus*	0.338	29.8 ±5.0‡	9.8 ±1.1‡
Irian Jayan death adder	*Acanthophis rugosus*	ND	30.6 ±3.5‡	10.5 ±0.5‡
Northern death adder	*Acanthophis praelongus*	ND	32.6 ±3.0‡	20.0 ±2.3‡
Hardwick's sea snake (Weipa, Australia)*	*Lapemis hardwickii*	ND	33.5 ±3.6†	ND
Hardwick's sea snake (Malaysia)*	*L. hardwickii*	ND	36.5 ±4.7†	ND
Tiger snake	*Notechis scutatus*	0.118	38.1 ±6.1‡	21.7 ±1.6‡
Barkly and death adder	*Acanthophis hawkei*	ND	40.9 ±5.5‡	17.4 ±1.9‡
Yellow banded snake	*Hoplocephalus stephensi*	1.44	46.7 ±6.7‡	19.7 ±1.7‡
Australian copperhead	*Austrelaps superbus*	0.500	89.2 ±9.8‡	25.6 ±2.5‡
Inland taipan	*Oxyuranus microlepidotus*	0.010	ND	29 ±3‡
Coastal taipan	*Oxyuranus scutellatus scutellatus*	0.064	ND	43 ±3‡
Papuan taipan	*Oxyuranus scutellatus canni*	0.051	ND	45 ±4‡

Snake venoms are ranked by t_{90} at 3 μg/mL. Data shown are the mean±SEM. ND, not determined.

*Geographical variants; locations are indicated in parentheses.

§Data from Broad *et al.*[69]

†Unpublished data from our laboratory.

‡Data from Crachi *et al.*,[16] Fry *et al.*[17] and Hodgson and Eriksson.[76]

families that are responsible for many of the severe cases of envenomation throughout the Asia–Pacific region, in particular Australia, where all clinically important terrestrial snakes are elapids. *In vitro* neuromuscular preparations have proven to be extremely useful for screening antivenoms against the neurotoxic effects of venoms. In these experiments, antivenom is added prior to the addition of venoms.[13,16–18] The venoms are then left in contact with the preparations until twitch blockade occurs or for a defined time period. Antivenoms that prove to be effective in this test can then be further examined in 'reversal' studies that more closely mimic the clinical situation. In these tests, antivenom is added after there has been considerable inhibition of twitches. We have chosen to use the t_{90} (i.e. time taken to produce 90% inhibition of nerve-mediated twitches) as a standard time point.[17,18] As described above, venoms are then left in contact with the preparations for a defined period. Reversal of twitch inhibition is indicative of antivenom efficacy. How well the results of such *in vitro* tests extrapolate to the clinical situation is difficult to assess. However, such studies will certainly provide valuable information regarding the effectiveness of antivenoms against the neurotoxic components of snake venoms.

LETHALITY VERSUS NEUROTOXICITY

Determination of the lethality of snake venoms in whole animals has usually been performed by conducting murine LD_{50} experiments. A large study undertaken to rank order the lethality of venoms provided valuable information, but was far from complete because the venoms of many medically important snakes were not included.[68] Due to obvious ethical reasons and complex regulatory requirements involved in gaining approval for murine LD_{50} studies in many countries, including Australia, these studies have been largely superseded by *in vitro* studies. One method of 'ranking' venom neurotoxicity is by using isolated skeletal muscle preparations.[15,17,18] The time taken to cause 50% or 90% inhibition of nerve mediated twitches (i.e. t_{50} or t_{90}) can be calculated to compare neurotoxicity. Venoms from numerous Australasian/Asian–Pacific snakes have been examined in our laboratory using this technique (Table 2).

A comparison of t_{90} values indicates that death adder venoms are more neurotoxic than coastal (*Oxyuranus scutellatus scutellatus*), inland (*Oxyuranus microlepidotus*) and Papuan (*O. scutellatus canni*) taipan venoms. However, in terms of LD_{50} values, taipan venoms remain the most lethal of all snake venoms examined to date.[68] Some other interesting differences between t_{90} and LD_{50} 'rankings' have been observed. Based on t_{90} values, the rank order of neurotoxicity of the snake venoms was sea snake(s) > sea krait > death adder > tiger snake ≥ yellow (Stephen's) banded snake > Australian copperhead > taipan venoms (Table 2). The rank order of lethality based on murine LD_{50} values of snake venoms was taipan > tiger snake > beaked sea snake > common death adder > Australian copperhead > yellow (Stephen's) banded snake.[68] However, this difference in rank order is to be expected because murine LD_{50} and t_{90} values are measuring two different parameters of toxicity. Whereas murine LD_{50} determinations are based on 'quantity' (i.e. what concentration of venom kills 50% of mice, usually over a 24–48 h period), t_{90} values are based on how 'quick' a venom acts. Therefore, it is possible to have an extremely 'lethal' venom (based on LD_{50} values) that takes a long time to produce its effects, whereas venoms with potent post-synaptic neurotoxins, which act rapidly, are likely to rank highly using the *in vitro* technique. Therefore, knowledge of both parameters is desirable.

In addition, given that murine LD_{50} studies are based on whole animals, these take into account the lethality of numerous toxic components (i.e. neurotoxins, cardiotoxins, myotoxins, haemolytic factors, coagulant and anticoagulant factors), acting via different mechanisms, present in crude venom. Obviously, this is not the case with *in vitro* neurotoxicity studies and is a limitation of this procedure. However, neuromuscular paralysis is one of the most important effects of Australasian elapid envenomations.[69]

CONCLUSIONS

Although much has been learned regarding the neuromuscular activity of snake venoms, there is still considerable work to be done before we fully understand the complex interactions that occur between venom components, nerve and muscle tissue. *In vitro* neuromuscular preparations have proven to be invaluable tools in the examination of neurotoxins and will continue to play a role in further elucidating the mechanism of action of these highly potent toxins. Further study of these toxins may provide more highly specific research tools or lead compounds for pharmaceutical agents.

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Effectiveness of Snake Antivenom: Species and Regional Venom Variation and Its Clinical Impact

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ABSTRACT

The ubiquity of venom variation in snakes poses special problems for the manufacture of antivenom and has undermined the commercial attractiveness of this class of therapeutic agent. In particular, it has been amply documented that both interspecific and intraspecific variation in venom composition can affect the neutralisation capacity of antivenoms. This may be exacerbated by the selective use of tests of venom toxicity

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and antivenom efficacy, such as the lethal dose and ED_{50}, resulting in inadequate neutralisation of time, rather than dose, dependent toxins, particularly enzymes involved in defibrinogenating, haemorrhagic and necrotising venom activities. The clinical consequences can be reduced efficacy against some important venom activities or even complete treatment failure in critical envenomations. All these factors, combined with the ongoing reduction in the number of antivenom manufacturers world-wide, and concomitant contraction in the range of available antivenoms, present significant challenges for the treatment of snakebite in the 21st century.

INTRODUCTION

The debate about the clinical efficacy of snake antivenoms and the degree of variation in venom composition began soon after production of the first antivenoms in the late nineteenth century (Calmette, 1894; Frazer, 1895; Phisalix and Bertrand, 1894). The 'sérum antivenimeux', developed by Calmette using mainly monocled cobra (*Naja kaouthia*) venom, was initially promoted as a universal snake antivenom. This was a consequence of Calmette's belief in the single mode of snake venom action (Hawgood, 1992).

However, early attempts to neutralise two Australian snake venoms (that of the Red-bellied Black Snake, *Pseudechis porphyriacus*, and the Mainland Tiger Snake, *Notechis scutatus*) with this serum failed (Martin, 1897). Similarly, in 1898 serum from dogs immunised with Brazilian Pit Viper (*Bothrops jararaca*) venom was found to be ineffective at neutralising the venom of the South American Rattlesnake (*Crotalus durissus terrificus*) and vice-versa (Brazil, 1901). This and other work in India, Australia and North America (Lamb, 1904; Russell, 1988; Tidswell, 1902) provided clear evidence of antivenom specificity, undermining Calmette's 'universal' hypothesis. This ultimately led to the development of a series of regional antivenoms based on the venom of medically significant endemic species on the various continents.

Eventually Calmette accepted the concept of two modes of snake venom action (neurotoxic and haemorrhagic) emergent from this antivenom specificity work but he persisted in the view that his serum could neutralise all neurotoxic venoms (Calmette, 1908). The search for the minimal number of modes of ophidian venom action and its significance for antivenom manufacture and utility continues unabated (Mebs and Kornalík, 1981; Vogtman, 1950; Warrell, 1986; Warrell and Arnett, 1976). This review will discuss contemporary issues in interspecific and intraspecific variation in venom composition and their clinical significance.

Molecular Basis of Venom Evolution

The mutational change of genes is the primary basis for evolution. Genetic drift or natural selection will cause the spread of nucleotide substitutions, insertions/deletions, recombinations or gene conversions through a population, until their fixation within that population. As a result, is well documented that significant venom variation can occur between closely related species or even within a species itself (Assakura et al., 1992; Daltry et al., 1996; Fry et al., 2002; Glenn et al., 1983; Jiménez-Porras, 1964; Yang et al., 1991).

Toxin-encoding genes undergo frequent gene duplication, sometimes followed by diversification into different functions and structures (Kordiš and Gubenšek, 2000; Slowinski et al., 1997). In contrast to mitochondrial protein endoding genes, toxin-encoding genes do not favor one codon for an amino acid over another (Fry, B.G. et al. unpublished results). Also in contrast to mitochondria, mutations in codons are more likely to occur in position 1 rather than positions 2 or 3. In addition non-synonymous substitutions are as likely to occur as synonymous substitutions. These factors combine to lead to a state where mutations are as likely as not to change the amino acid encoded by the codon. A change of as little as a single amino acid can have profound effects upon not only the specificity and potency of a molecule but also upon its antigenicity and thus relative neutralisation by antivenom. This situation is greatly complicated by frequent duplication of toxin genes, with each duplicate evolving rapidly and independently of each other (Afifiyan et al., 1999; Chang et al., 1999). Thus, the fundamental molecular basis of venom evolution favors a multiplicity of actions and consequently a multiplicity of toxins that need to be counteracted by antivenom.

Snake Systematics

The problem of interspecific differences in venom composition can be overcome to some extent by paying scrupulous attention to the systematics of the snakes involved. Unfortunately, it is clear that many toxinologists do not pay sufficient attention to the systematic status of the snakes they are working with, and as a result, many venoms used in toxinology cannot be attributed to any known species (Wüster and McCarthy, 1996). In a clinical context, misidentification of physically similar species may result in the selection of the wrong antivenom type (Trinca, 1969; Winkel et al., 2001) and, consequently, a fatal outcome (Sutherland and Leonard, 1995).

Although attention to systematics can alleviate some of the problems caused by interspecific snake venom variation, the value of this is limited by our as yet inadequate or developing knowledge of the systematics of

many medically important groups of venomous snake. The systematics of many of the most medically significant groups of venomous snakes, such as Asian and African Cobras (*Naja*), Australian Death Adders, Brown snakes, and Black snakes (*Acanthophis*, *Pseudonaja* and *Pseudechis*), the South American Lanceheads (*Bothrops atrox* complex), Saw-scaled vipers (*Echis*) and Asian Green Pit vipers (*Trimeresurus* spp.) are either in a state of flux or remain poorly understood.

However, an understanding of the taxonomic status of different populations of venomous snakes alone cannot necessarily predict patterns of venom variation. There is ample evidence that venom composition can vary extensively even among populations which are unambiguously conspecific (e.g., *Crotalus scutulatus*—Wilkinson et al., 1991; *Daboia russelii*—Warrell, 1989; Wüster et al., 1992), perhaps as a result of natural selection for geographic differences in diet (Daltry et al., 1996). Such variation can have considerable implications for the effectiveness of antivenoms.

Even broad-spectrum polyvalent antivenoms may not be able to neutralize some of the venom variants present within species that are included in the manufacture of the antivenom: for instance, a broad-spectrum anti-*Bothrops* antivenom, raised from venoms of a number of species groups within the genus, was highly effective in neutralising the venoms of some populations of the *Bothrops atrox* species complex, but almost ineffective against that of other populations of the complex (Wüster et al., unpublished data).

Venom Toxicity and Antivenom Efficacy Tests

An additional issue impinging on antivenom effectiveness in clinical practice is the definition of snake venom toxicity and antivenom efficacy. The presence of venom variation in this context means that the 'standard' definition of venom toxicity and antivenom efficacy used by commercial manufacturers can be suboptimal when applied in clinical situations. For example, the traditional measure of venom toxicity, the lethal dose (Trevan, 1927), has the disadvantage of being biased towards the most potent venom component and/or those toxins having a maximum effect within the predetermined time frame (Chippaux, 1998). Studies of antivenom effectiveness typically involve the determination of the median effective antivenom dose (ED_{50}) at which a sample of experimentally envenomed animals survive within a predetermined observation period after an otherwise lethal injection of venom through a specified route (Chippaux and Goyffon, 1998). In any case, it is becoming increasingly difficult to perform LD_{50} studies for obvious ethical and regulatory requirements. However, venom toxicity and effectiveness of antivenom can be studied using in vitro (Barfaraz and Harvey, 1994; Fry et al., 2001) or insensate in vivo

(Sells et al., 2001) preparations. This enables the investigator to concentrate on a specific system (e.g. neuromuscular junction, blood, atria and blood vessels) that is targeted by a venom component. Furthermore, the effectiveness of antivenom against a toxic venom component acting at a specific system can be studied in detail.

Indeed, the overall toxicity of any venom is related to a variety of components interacting with a multitude of tissues, cells and receptors or substrates over time in a manner dependent on individual pharmacokinetics and dynamics. Consequently, in vitro toxicity and antivenom efficacy testing does not take these into account. In addition, such methods take no account of variable antivenom absorption and distribution, nor of the pharmacokinetics of different toxins, since the venom and antivenom are usually preincubated in vitro (Barfaraz and Harvey, 1994).

When compared to the reactions of the animals used in laboratory testing, it is worth noting the natural resistance of many animals to the venoms of the snakes that prey upon them. This classic tale of co-evolution has been well documented. Most notable of these studies was one that showed the resistance of eels to the venom of the sea kraits (Heatwole and Poran, 1995). The authors showed that moray eels of the genus *Gymnothorax* occuring sympatrically with sea kraits, and thus subject to predation from them, show dramatically more resistance to *Laticauda* venoms than populations from outside the range of the sea kraits. Comparative studies of ground squirrels (*Spermophilus beecheyi*) from areas with an without rattlesnakes yielded similar results (Poran et al., 1987). In the case of the mongoose, the resistance to the neurotoxicity comes down to a five residue difference in the acetylcholine receptor when compared to the equivalent receptor in the highly sensitive mouse (Barchan et al., 1992).

One example of the practical consequence of the focus on small animal survival experiments is the well-recognised limitation of the neutralising potential of the Australian brown snake (*Pseudonaja textilis*) antivenom (CSL Limited, Parkville). The efficacy of this antivenom is assessed in guinea pigs using an ED_{50} assay that does not specifically examine its activity against the fibrinolytic effects of *Pseudonaja* prothrombin activators nor other cardiovascular effects (Sutherland and Tibballs, 2001; Tibballs and Sutherland, 1991). It is of concern that there is growing evidence of the slow, limited and interspecifically variable neutralisation of the cardiovascular and haematological effects of *Pseudonaja* venom by the existing brown snake antivenom (Masci et al., 1998; Sprivulus et al., 1996; Tibballs and Sutherland, 1991).

Similar limitations have been identified regarding the efficacy of Australian tiger snake, but not taipan (*O. scutellatus*), antivenom (Sprivulus et al., 1996). Interestingly, another problem, more related to interspecific venom variation, arises with this latter product. In vitro analysis of

neurotoxicity suggests that, although taipan antivenom effectively neutralises the presynaptic neurotoxins of both the coastal and inland (*O. microlepidotus*) species, it is less effective against the postsynaptic toxicity of the latter (Crachi et al., 1999). The clinical consequences of the aforementioned procedural limitations can therefore be reduced efficacy against some venom activities (Henderson et al., 1993) or even complete antivenom failure in critical envenomations (Gillissen et al., 1994).

Global Antivenom Crisis

As the extent of clinically significant variation in snake venom composition undermined the possibility of a universal antivenom, so it erected a major barrier to the widespread availability of this product. The current global burden of snake bite is estimated at approximately 5 million bites and 100,000 deaths each year (Chippaux, 1998). Most of these are concentrated in the developing nations of the Indo-Pacific region and Africa, where there is little access to antivenom for the most at-risk populations (Cheng and Winkel, 2001a,b; Warrell, 1999). Even in affluent nations, the unattractive economics of antivenom development and production have resulted in pressure on existing manufacturers to withdraw from this market (Galli, 2001; Theakston and Warrell, 2000).

The need for further expenditure on procedural refinements to improve antivenom quality, as described here, will add to such pressures. This comes in the midst of an acute crisis in antivenom availability for Africa (Theakston and Reid, 1983) and a long-standing under-supply in Asia (McNamee, 2001). The ongoing reduction in the number of antivenom manufacturers world-wide, and concomitant contraction in the range of available antivenoms, present significant challenges for the treatment of snake bite. We therefore recently proposed a global strategy for snake bite control and procurement funding to overcome the inequality of antivenom supply (Cheng and Winkel, 2001a,b).

RECOMMENDATIONS

All these factors put together result in a somewhat complex picture for antivenom producers and policy makers. The optimization of antivenom production and effectiveness requires a number of preconditions:

- An understanding of the medically important species in any given region. This appears trivial, but a several studies have shown that the medical importance of a number of species has been greatly

underestimated in the past (e.g., *Bungarus candidus* in Thailand—Looareesuwan et al., 1988; Viravan et al., 1992), whereas that of others has been overestimated (e.g., *Bungarus fasciatus* and *Ophiophagus hannah* in Thailand—Looareesuwan et al., 1988; Viravan et al., 1992), resulting in a lack of antivenom in the case of the former and the production of largely superfluous antivenoms in the case of the latter. Clearly, if resources are limited, antivenom production should concentrate on species of appreciable public health importance. This requires extensive epidemiological studies, particularly community surveys and pharmacological characterisation of venoms.

- Scrupulous attention to the systematics and identification of the snakes concerned. Antivenom should be produced against the species responsible for the majority of bites, wherever they may occur, and not against similar species that happen to be conveniently available, but are not in fact responsible for bites.
- Antivenoms should be raised from venoms collected across the entire range of each species across the target region. Due to intraspecific geographic variation in venom composition, an antivenom raised against the venom of one population may be less effective against the venom of another population of the same species (Fry et al., 2001, Wüster et al., in prep.). This concern is particularly applicable to venom producers who rely on captive-bred stocks of venomous snakes: often, the captive stocks originate from a few specimens from a single locality, and are thus likely to contain only a fraction of the total number of antigens present in the species as a whole.

At the very least, antivenoms should be tested for neutralising ability against the venoms of all major populations of any species, with special emphasis on those occurring in regions with a high incidence of snakebite.

The World Health Organisation recommendations (W.H.O., 1981) regarding the specific assessment of defibrinogenating, haemorrhagic and necrotising venom activities should form a routine part of snake venom toxicity testing and antivenom efficacy assessment (Gutiérrez et al., 1990; Theakston and Reid, 1983).

A global strategy for snakebite control and procurement funding is required to overcome the inequality of antivenom supply and to improve existing antivenom deficiencies in neutralising interspecific and intraspecific venom variation. This should form part of global initiatives to secure access to essential drugs through partnerships between donors, the public sector and pharmaceutical industry (Cheng and Winkel, 2001a,b; Scholtz, 1999).

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