NEUROTOXIC, CYTOTOXIC AND CARDIOVASCULAR EFFECTS OF SOME AUSTRALASIAN ELAPID VENOMS

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This thesis is dedicated to my beloved parents.

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MONASH UNIVERSITY

Declaration for thesis based or partially based on conjointly published or unpublished work

GENERAL DECLARATION

In accordance with Monash University Doctorate Regulation 17.2 Doctor of Philosophy and Research Master's regulations, the following declarations are made:

The research presented in this thesis was carried out while I was enrolled in a PhD program through the Department of Pharmacology, Faculty of Medicine, Nursing and Health Sciences, Monash University, Clayton.

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 experiments were conducted in accordance with the guidelines of the National Health and Medical Research Council Code of Practice for the Care and Use of Animal for Experimental Purposes in Australia and were approved by the Animal Ethics Committee (School of Biomedical Sciences, Faculty of Medicine, Nursing and Health Sciences, Monash University).

This thesis includes **3** original papers published in peer reviewed journals and **4** unpublished publications. The core theme of the thesis is **Neurotoxic, Cytotoxic and Cardiovascular effects of some Australasian elapid venoms**. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the candidate, working within the Department of Pharmacology and Department of Physiology, Monash University, Clayton, Australia under the supervision of **Professor Wayne C. Hodgson** and **Professor Helena C. Parkington**.

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.

In the case of Chapters 2 to 8 my contribution to the work involved the following:

Thesis Chapter	Publication Title	Publication Status	Nature and extent of candidate's contribution
2	Isolation and characterization of P-EPTX-Ap1a and P-EPTX-Ar1a: pre-synaptic neurotoxins from the venom of the northern (*Acanthophis praelongus*) and Irian Jayan (*Acanthophis rugosus*) death adders	Published	Conducted experiments, analyzed data and wrote the manuscript, with editorial assistance from the co-authors
3	Differential myotoxic and cytotoxic activities of pre-synaptic neurotoxins from Papuan taipan (*Oxyuranus scutellatus*) and Irian Jayan death adder (*Acanthophis rugosus*) venoms	Published	Conducted experiments, analyzed data and wrote the manuscript, with editorial assistance from the co-authors
4	Irian Jayan death adder (*Acanthophis rugosus*) neurotoxin activates rapid cytoplasmic Ca^{2+} influx after cationic channel activation in mouse neurons	Submitted	Conducted experiments, analyzed data and wrote the manuscript, with editorial assistance from the co-authors

Thesis Chapter	Publication Title	Publication Status	Nature and extent of candidate's contribution
5	*In vivo* and *in vitro* cardiovascular effects of Papuan taipan (*Oxyuranus scutellatus*) venom: Exploring "sudden collapse"	Published	Conducted experiments, analyzed data and wrote the manuscript, with editorial assistance from the co-authors
6	Hypotensive and vascular relaxant effects of phospholipase A_2 toxins from Papuan taipan (*Oxyuranus scutellatus*) venom	Submitted	Conducted experiments, analyzed data and wrote the manuscript, with editorial assistance from the co-authors
7	An examination of cardiovascular collapse induced by eastern brown snake (*Pseudonaja textilis*) venom	Accepted	Conducted experiments, analyzed data and wrote the manuscript, with editorial assistance from the co-authors
8	Prothrombin activator-like compound from *Pseudonaja textilis* venom induces early cardiovascular collapse	To be submitted	Conducted experiments, analyzed data and wrote the manuscript, with editorial assistance from the co-authors

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To a group of people in Thailand, my PhD would not have been possible without a scholarship from the National Science and Technology Development Agency (NSTDA) of the Royal Thai Government. Thank to A/P Borpit Klangkalya and A/P Chainarong Cherdchu for the opportunity to do this PhD.

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THESIS SUMMARY

A range of toxic components found in Australasian elapid venoms have been postulated to account for the clinical outcomes of envenoming. Depending on the species of snake, these outcomes may include neurotoxicity with flaccid paralysis, coagulopathy with spontaneous systemic hemorrhage, sudden cardiovascular collapse, and myotoxicity with skeletal muscle breakdown. In the present study, we investigated the toxins and mechanisms behind these often life-threatening symptoms from the venom of four species of Australasian elapid snakes (*Acanthophis praelongus*, *Acanthophis rugosus*, *Oxyuranus scutellatus* and *Pseudonaja textilis*) with emphasis on their neurotoxic, cytotoxic and cardiovascular activities.

Previously, post-synaptic neurotoxins were postulated to be the primary components responsible for neurotoxicity following death adder envenoming in humans. However, neurotoxicity is often poorly reversed by antivenom or anticholinesterase suggesting that death adder venoms may contain pre-synaptic neurotoxins that do not respond, as well as post-synaptic neurotoxins, to antivenom. In this study, phospholipase A_2 (PLA_2) neurotoxins, P-EPTX-Ap1a and P-EPTX-Ar1a, were isolated from the venoms of *Acanthophis praelongus* (Northern death adder) and *Acanthophis rugosus* (Irian Jayan death adder), respectively. P-EPTX-Ap1a (20-100 nM) and P-EPTX-Ar1a (20-100 nM) inhibited indirect twitches of the chick biventer cervicis nerve-muscle preparation without affecting contractile responses to nicotinic receptor agonists. Pre-incubation of 4-bromophenacyl bromide (1.8 mM) markedly reduced the effect of both toxins on twitch height suggesting that PLA_2 activity plays an important role to induce pre-synaptic neurotoxicity.

Some snake pre-synaptic PLA_2 neurotoxins have been reported to also possess myotoxic activity and promote cell death. Therefore, pre-synaptic neurotoxins from *Oxyuranus scutellatus* (Papuan taipan) and *A. rugosus* venoms, cannitoxin and P-EPTX-Ar1a, respectively, as well as the whole venoms, were examined for myotoxic and cytotoxic activities. Based on size-exclusion high performance liquid chromatography (HPLC) analysis, cannitoxin represents 16% of *O. scutellatus* venom, while P-EPTX-Ar1a represents 6% of *A. rugosus* venom. *A. rugosus* venom induced significantly higher myotoxic activity than that of *O. scutellatus* venom in the chick biventer cervicis nerve-muscle preparation. In a rat skeletal muscle cell line (L6), *A. rugosus* venom and

P-EPTX-Ar1a induced significantly greater cytotoxicity than *O. scutellatus* venom and cannitoxin. Thus, *A. rugosus* and *O. scutellatus* venoms possess different myotoxic and cytotoxic activities. These activities were independent of the proportion of pre-synaptic neurotoxin and PLA_2 activity in the whole venoms.

The cytotoxicity and disruption in neurotransmitter (i.e. acetylcholine) release induced by snake pre-synaptic neurotoxins have been postulated to be due to an increase in intracellular Ca^{2+}. In the current study, P-EPTX-Ar1a, a pre-synaptic neurotoxin from *A. rugosus* venom, caused an increase in cytoplasmic calcium and membrane depolarization in primary dorsal root ganglion (DRG) neurons. Influx of Ca^{2+} did not occur in Ca^{2+}-free Hank's solution with EGTA. Ca^{2+} influx was also significantly reduced in the presence of nifedipine or agatoxin indicating that L-type and P/Q-type voltage-gated calcium channels, respectively, are involved in Ca^{2+} influx. Patch clamp studies in whole cell mode demonstrated that P-EPTX-Ar1a evoked inward currents in DRG neurons which were blocked by SKF96365, a cationic channel blocker, suggesting that P-EPTX-Ar1a induced-Ca^{2+} influx may be related to membrane depolarization through the activation of cationic channels.

Sudden cardiovascular collapse following envenoming by some Australasian elapids (e.g. *Oxyuranus* spp. or *Pseudonaja* spp.) is a poorly understood cause of mortality and morbidity. Previous studies showed that administration of *O. scutellatus* or *Pseudonaja textilis* (eastern brown snake) venoms cause sudden cardiovascular collapse in anaesthetized animals. In the current study, *O. scutellatus* venom failed to affect force of contraction and conductivity in rat isolated heart preparations. In anaesthetized rats, sub-lethal doses of *O. scutellatus* venom (5-10 µg/kg, i.v.) produced transient hypotension while 20 or 50 µg/kg (i.v.) of venom produced cardiovascular collapse in all animals tested. The administration of *P. textilis* venom (10 or 20 µg/kg, i.v.) to anaesthetized rats also induced sudden collapse. Interestingly, cardiovascular collapse induced by *O. scutellatus* or *P. textilis* venoms was attenuated by prior administration of small 'priming' doses of some Australasian elapid venoms (i.e. *O. scutellatus*, *P. textilis* or *A. rugosus* venoms) or a venom from an exotic snake i.e. *Daboia russelii limitis*. Prior administration of polyvalent snake antivenom or heparin also protected against sudden collapse induced by *O. scutellatus* or *P. textilis* venoms.

A prothrombin activator-like compound, PTV3, was partially purified from *P. textilis* venom. Protein bands of PTV3 displayed homology to catalytic and non-catalytic subunits of prothrombin activator 'pseutarin C' of *P. textilis* venom. Administration of PTV3 (10 and 20 μg/kg, i.v.) induced rapid cardiovascular collapse which was abolished by prior administration of small priming doses of PTV3 (2 and 5 μg/kg, i.v.) or heparin. This indicates that the prothrombin activator-like compound, PTV3 may contribute to sudden cardiovascular collapse in anaesthetized rats.

In isolated rat mesenteric arteries, *P. textilis* venom but not PTV3 induced endothelium-dependent relaxation. *O. scutellatus* venom also induced both endothelium-dependent and -independent relaxation in pre-contracted rat mesenteric arteries which were inhibited by indomethacin, IbTX or Rp-8-CPT-cAMPs suggesting that vascular relaxation induced by the venom may be due to a combination of release of dilator autacoids and a direct relaxing effect on vascular smooth muscle involving the cAMP/protein kinase A (PKA) cascade. We subsequently isolated a PLA_2 fraction (OSC3) from *O. scutellatus* venom and examined the hypotensive and vascular relaxant responses. OSC3 displayed high PLA_2 activity and caused endothelium-dependent and -independent relaxation in pre-contracted rat mesenteric artery rings. Indomethacin and Rp-8-CPT-cAMPs markedly attenuated vascular relaxation induced by OSC3 on endothelium-denuded mesenteric arteries. Reverse-phase HPLC analysis of OSC3 indicated the presence of 2 major components, i.e. OSC3a and OSC3b. Both components induced a hypotensive effect in anaesthetized rats which was attenuated by prior administration of indomethacin. The amino acid sequencing indicated that the active components of OSC3 showed homology to PLA_2 toxins from *O. scutellatus* (coastal taipan) venom. This finding indicates that PLA_2 of *O. scutellatus* venom contains indirect relaxant and hypotensive effects that involve other vasoactive compounds e.g. PGI_2 or PKA. It can be concluded that Australasian elapid induced-early cardiovascular collapse involves a combination of mediator-induced relaxation and prothrombin activator-like compound.

In conclusion, this study examined the mechanisms behind snake pre-synaptic neurotoxin-induced neurotoxic and cytotoxic activities and the contributing factors to early cardiovascular collapse following Australasian elapid envenoming. These data provide useful insights for the clinical management of snake envenomed patients.

ABBREVIATIONS

ACh	acetylcholine
ACN	acetonitrile
BAPTA-AM	1,2-Bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid tetrakis(acetoxymethyl ester)
BK_{ca}	large-calcium activated potassium channel
4-BPB	4-bromophenacyl bromide
$[Ca^{2+}]_i$	intracellular calcium
cAMP	Cyclic adenosine monophosphate
CBCNM	chick biventer cervicis nerve-muscle
cGMP	cyclic guanosine monophosphate
CGN	cerebellar granule neuron
ChTX	Charybdotoxin
CK	Creatine kinase
CNS	central nervous system
DHP	Dihydropyridine
DRG	dorsal root ganglion
EDHF	endothelium-derived hyperpolarizing factor
EDTA	Ethylenediaminetetra-acetic acid
EGTA	Ethylene glycol tetra-acetic acid
ER	endoplasmic reticulum
FAs	fatty acids
GC	guanylate cyclase
GPCR	G protein-coupled receptor
H-89	N-[2-(p-bromocinnamyl-amino)ethyl]-5-iso-quinolinesulfonamide
HPLC	high-performance liquid chromatography
5-HT	5-hydroxytryptamine
IbTX	iberiotoxin
i.p.	intra peritoneal
IP_3	inositol trisphosphate
i.v.	intra venous
kDa	kilodalton
K_V	voltage-gated K^+ channel
LC-MS	liquid chromatography-mass spectrometry
L-NAME	nitro-L-arginine methylester
LVA	low-voltage-activated

LysoPL(s)	lysophospholipid(s)
MAP	mean arterial pressure
MLCK	myosin-light-chain kinase
mN	millinewton
MW	molecular weight
nAChRs	nicotinic acetylcholine receptors
$Na_V s$	voltage-gated Na^+ channels
NMDA	N-methyl-D-aspartate
NO	nitric oxide
NOS	nitric oxide synthase
NPs	natriuretic peptides
PE	phenylephrine
PGI_2	prostacyclin
PKA	protein kinase A
PL	phospholipid
PLA_2	phospholipase A_2
PVDF	polyvinyl difluoride
RP-HPLC	Reverse-phase high-performance liquid chromatography
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
sp.	species
SR	sarcoplasmic reticulum
STX	saxitoxin
TFA	trifluoroacetic acid
TRP	transient receptor potential
TTX	Tetrodotoxin
VICC	venom-induced consumption coagulopathy
VOCCs	voltage-operated calcium channels

PUBLICATIONS

Original articles

Chaisakul J, Konstantakopoulos N, Smith AI, Hodgson WC (2010). Isolation and characterisation of P-EPTX-Ap1a and P-EPTX-Ar1a: pre-synaptic neurotoxins from the venom of the northern (*Acanthophis praelongus*) and Irian Jayan (*Acanthophis rugosus*) death adders. *Biochem Pharmacol* 80: 895-902.

Chaisakul J, Isbister GK, Konstantakopoulos N, Tare M, Parkington HC, Hodgson WC (2012). *In vivo* and *in vitro* cardiovascular effects of Papuan taipan (*Oxyuranus scutellatus*) venom: Exploring "sudden collapse". *Toxicol Lett* 213: 243-248.

Chaisakul J, Parkington HC, Isbister GK, Konstantakopoulos N, Hodgson WC (2013). Differential myotoxic and cytotoxic activities of pre-synaptic neurotoxins from Papuan taipan (*Oxyuranus scutellatus*) and Irian Jayan death adder (*Acanthophis rugosus*) venoms. *Basic Clin Pharmacol Toxicol* 112: 325-334.

Communications

Chaisakul J, Konstantakopoulos N, Smith AI, Isbister GK and Hodgson WC (2009). Isolation and pharmacological characterization of a presynaptic phospholipase A_2 neurotoxin complex (β-Acanthoxin 2) from the venom of the northern death adder (*Acanthophis prealongus*). *The 43rd Annual Scientific Meeting 2009 of Australasian Society of Clinical and Experimental Pharmacologists and Toxicologists (ASCEPT)*, Sydney, Australia, November-December 2009.

Chaisakul J, Konstantakopoulos N, Smith AI and Hodgson WC (2010). Isolation and characterisation of P-EPTX-Ap1a and P-EPTX-Ar1a: pre-synaptic neurotoxins from the venom of the northern (*Acanthophis praelongus*) and Irian Jayan (*Acanthophis rugosus*) death adders. *Venom Adelaide 2010*, Adelaide, Australia, June 2010.

Chaisakul J, Tonta MA, Coleman HA, Konstantakopoulos N, Hodgson WC and Parkington HC (2010). Calcium influx-activating action of P-EPTX-Ar1a: an isolated neurotoxin from the venom of Irian Jayan death adder. *Adelaide 2010 joint meeting of Australian Physiological Society/ Australian Society for Biophysics (AuPS/ASB Symposia*), Adelaide, Australia, November-December 2010.

Chaisakul J, Parkington HC, Tare M, Isbister GK, Konstantakopoulos N and Hodgson WC (2011). Self protective effect of Papuan taipan (*Oxyuranus scutellatus canni*) venom on cardiovascular collapse. *The 17th Congress of the European Section of the International Society on Toxinology (IST)*, Valencia, Spain, September 2011.

Chaisakul J, Isbister GK, Konstantakopoulos N, Tare M, Parkington HC and Hodgson WC (2012). Vascular effects of Australasian elapid snake venoms. *The 20th National Scientific Conference of Australian Vascular Biology Society (AVBS)*, Gold Coast, Australia, September 2012.

Chapter 1

General Introduction

1. Rationale and background

Snake envenoming is an occupational hazard in many countries throughout the world. The highest burden of snakebite is in tropical regions of Asia (i.e. South Asia and Southeast Asia), Papua New Guinea, almost all African countries, and Latin America (Gutierrez *et al.*, 2010). A study funded by the World Health Organization estimated that over 440,000 snake envenoming cases and 20,000 deaths occur globally each year (Kasturiratne *et al.*, 2008). Interestingly, Australia is home to the vast majority of snakes whose venom is 'ranked' in the top 25 for lethality based on LD_{50} values (i.e. 'dose' of venom required to kill 50% of a population of mice) (Broad *et al.*, 1979). However, morbidity and mortality from snakebite in Australia is relatively low compared to the geographical locations listed above. This is most likely due to developed first aid procedures (i.e. pressure immobilization technique), an extensive collection of high quality antivenoms and relatively easy access to clinical assistance from most parts of the country.

However, snake envenoming remains a significant life-threatening problem in Papua New Guinea and some Indonesian islands (i.e. Irian Jaya) (Currie, 2000), where access to antivenom and health care are far more limited. In Port Moresby General Hospital, Papua New Guinea, 12% of patients admitted following snake envenoming died from severe respiratory complications and/or intracerebral hemorrhage (McGain *et al.*, 2004).

Nearly all of the medically important venomous snakes of Australia, Papua New Guinea and the Indonesian islands belong to the family Elapidae (Currie, 2000). Elapid snakes are characterized by a pair of anterior fangs in their upper jaw (White, 1991), which contain an enclosed venom canal in contrast to the open groove found in the fangs of vipers (Parker and Grandison, 1977). This effective biting mechanism, as well as the presence of a highly potent venom, make elapid snakes extremely dangerous (Hodgson and Wickramaratna, 2006).

Envenoming by Australasian elapids can result in a range of clinical outcomes. Life-threatening symptoms may include progressive neuromuscular paralysis, sudden cardiovascular collapse, severe coagulopathy with fibrinogen depletion and bleeding in vital organs (i.e. intracranial hemorrhage) (Currie, 2000). Systemic envenoming can be fatal if first aid treatment

and, if indicated, antivenom is not administered early, especially in the presence of neurotoxicity. Neurotoxicity following envenoming by snakes of genus *Acanthophis* (death adders) is often poorly reversed even by large doses of antivenom or neostigmine (anticholinesterase) (Johnston *et al.*, 2012). This might be due to the fact that post-synaptic neurotoxins present in death adder venom are "pseudo-irreversible", and/or the venoms contain pre-synaptic neurotoxins which are resistant to antivenom. This has been reported in patients, with delayed-onset or gradually developing neurotoxicity (Gunja *et al.*, 2007). Recently, pre-synaptic neurotoxins from the venoms of death adders have been identified and characterized (Blacklow *et al.*, 2010a; Blacklow *et al.*, 2010b) indicating that pre-synaptic neurotoxins are highly likely to be responsible for delayed neurotoxicity.

Despite clinical managements, deaths resulting from rapid cardiovascular collapse after brown snake (genus *Pseudonaja*) envenoming have been reported in Australia (Allen *et al.*, 2012). A recent report also described a similar case of Indian Russell viper-induced pericardial hemorrhage with cardiac tamponade (Senthilkumaran *et al.*, 2012). However, the causes and mechanisms behind sudden cardiovascular collapse following snake envenoming remain unclear.

In this thesis, venoms of some Australasian elapids responsible for life-threatening symptoms (i.e. progressive neurotoxicity and cardiovascular collapse) have been characterized using pharmacological, biochemical and physiological techniques. These studies will hopefully be of significant benefit to clinicians treating envenomed patients both in Australia and overseas.

2. Australasian venomous snakes

Ten genera of elapids are found in the Australo-Papuan region. They are: *Notechis* spp. (tiger snakes), *Acanthophis* spp. (death adders), *Pseudonaja* spp. (brown snakes), *Pseudechis* spp. (black snakes), *Tropidechis* spp. (rough-scaled snakes), *Oxyuranus* spp. (taipans), *Austrelaps* spp. (copperheads), *Demansia* spp. (whip snakes), *Rhinoplocephalus* sp. or *Micropechis* sp. (small-eyed snakes) and *Hoplocephalus* spp. (broad-headed snakes) (Mirtschin and Davis, 1982).

In Australia, neurotoxicity and myotoxicity are the most common outcomes following taipan and/or tiger snake envenoming (White, 1998) while snakes of genus *Pseudonaja* (brown snakes) are responsible for a number of deaths due to early cardiovascular collapse (Allen *et al.*, 2012). Papuan taipan (*Oxyuranus scutellatus*), smooth-scaled death adder (*Acanthophis laevis*), and New Guinea small-eyed snake (*Micropechis ikaheka*) are three clinically important elapids responsible for significant cases of envenoming in Papua New Guinea (Vargas *et al.*, 2011).

In the current study, the venoms of four Australasian elapids from three different genera were examined i.e. *O. scutellatus*, *P. textilis*, *A. praelongus* and *A. rugosus*. Three distinct species of taipan are widely recognized. These are the Australian coastal taipan (*Oxyuranus scutellatus*; Figure 1a), inland taipan (*Oxyuranus microlepidotus*) and, more recently, the Western Desert taipan (*Oxyuranus temporalis*) (Doughty *et al.*, 2007). Although the Papuan taipan (previously known as *O. scutellatus canni*) was thought to be a subspecies of the Australian coastal taipan, taxonomic studies have indicated that there is no significant differentiation between the two populations (*O. scutellatus*) (Vargas *et al.*, 2011; Wuster *et al.*, 2005). Envenoming by taipan causes significant physiological disturbances, characterized by local tender lymphadenopathy, abdominal pain, coagulopathy, myotoxicity, neurotoxicity, renal toxicity and cardiac disturbances (Lalloo *et al.*, 1995a; Lalloo *et al.*, 1997; Trevett *et al.*, 1995).

Snakes in the genus *Pseudonaja* are widely distributed across mainland Australia. These include the Peninsula brown snake (*P. textilis inframacula*), Ingram's brown snake (*P. ingrami*), western brown snake (*P. nuchalis*), speckled brown snake (*P. guttata*), Dugite (*P. affinis affinis*), Tanners (*P. affinis tanneri*) and common or eastern brown snake (*P. textilis textilis*; Figure 2a) (Mirtschin and Davis, 1982). Eastern brown snakes are commonly found in the eastern half of Australia, with isolated populations in the Northern Territory (Figure 2b). The eastern brown snake population in Papua New Guinea is thought to have resulted from the transportation from Australia of eggs in military or agricultural equipment (O'Shea, 1996). Bites from snakes of genus *Pseudonaja* account for a considerable proportion of the morbidity and mortality from snakebite in Australia (Allen *et al.*, 2012). The most significant symptom following envenoming by brown

snakes is defibrination which may result in severe coagulopathy from the presence of potent procoagulant toxins.

The elapids of genus *Acanthophis* (death adders) are viper-like in appearance characterized by the presence of a broad triangular head, and a muscular body with a small thin rat-like tail terminating in a sharp cured spine (Mirtschin and Davis, 1982). Death adders are found in a variety of geographical areas in continental Australia, as well as eastern Indonesian islands and Papua New Guinea (Wickramaratna *et al.*, 2003b). Five Australian species have been reported; (1) *A. hawkei* (Barkly Tableland death adder), (2) *A. antarcticus* (common death adder), (3) *A. wellsi* (black head death adder), (4) *A. pyrrhus* (desert death adder) and (5) *A. praelongus* (northern death adder; Figure 3a). Two species have been identified in Papua New Guinea in Irian Jaya and on the island of Seram; i.e. *A. rugosus* (Irian Jayan death adder; Figure 4a) and *A. laevis*, respectively. Neurotoxicity, anticoagulation, myotoxicity and rhabdomyolysis are potential outcomes following death adder envenoming (Isbister *et al.*, 2010a; Lalloo *et al.*, 1996; Wickramaratna *et al.*, 2003b).

(a)

(b)

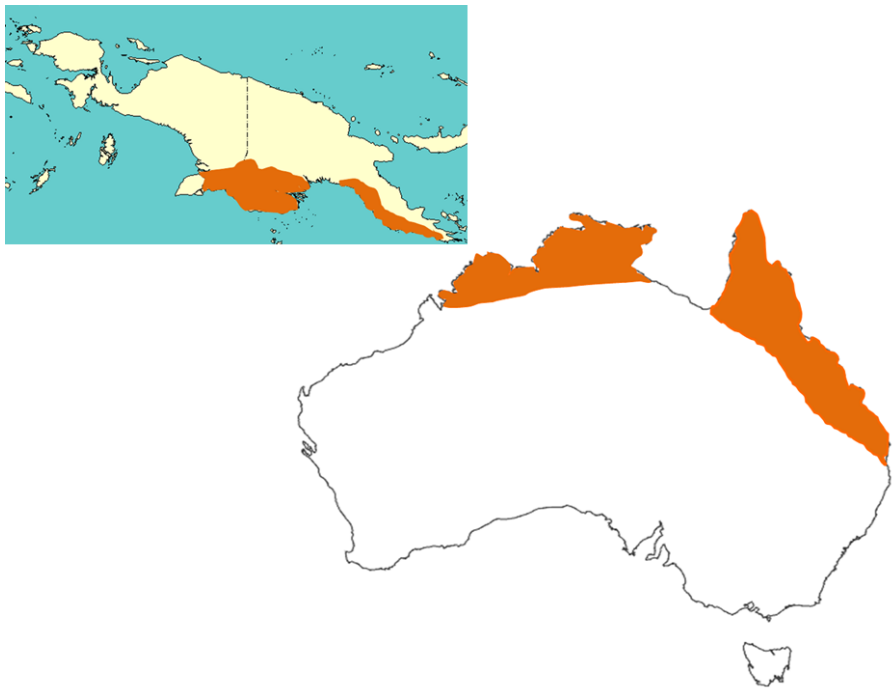

Figure 1: *(a) A photograph of Oxyuranus scutellatus (kindly supplied by Peter Mirtschin) and (b) map of Papua New Guinea and Australia showing its distribution in orange (adapted from White et al., 1998 and* http://www.avru.org/research/png_srp/implicated_spp/snakebite2.html).

(a)

(b)

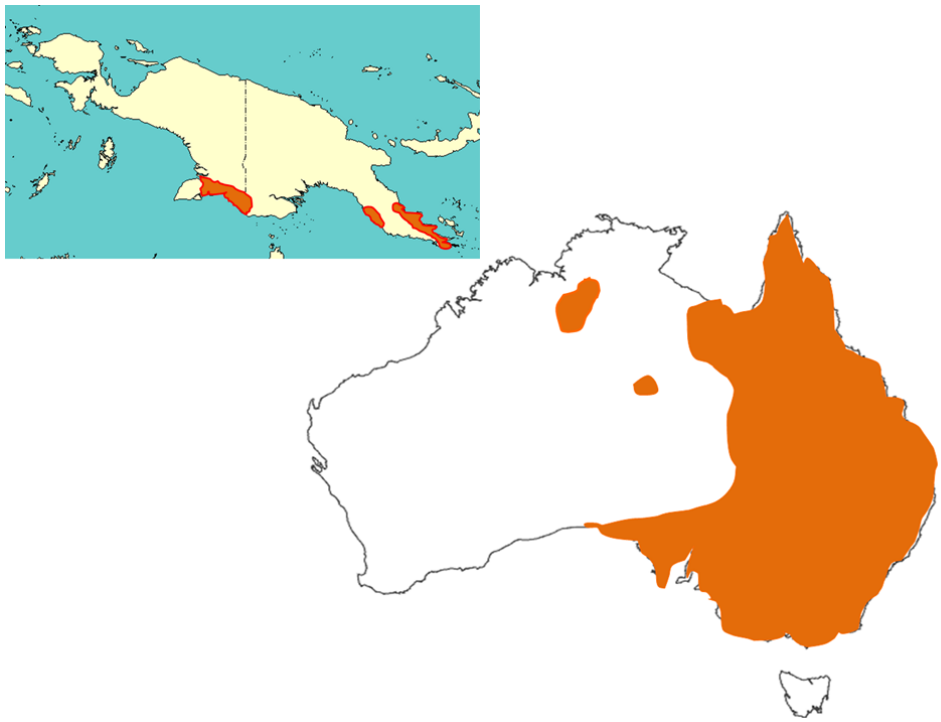

Figure 2: *(a) A photograph of Pseudonaja textilis (eastern brown snake) (kindly supplied by Peter Mirtschin) and (b) a map of Australia and Papua New Guinea showing its distribution in orange (White et al., 1998 and* http://www.avru.org/research/png_srp/implicated_spp/snakebite2.html).

(a)

(b)

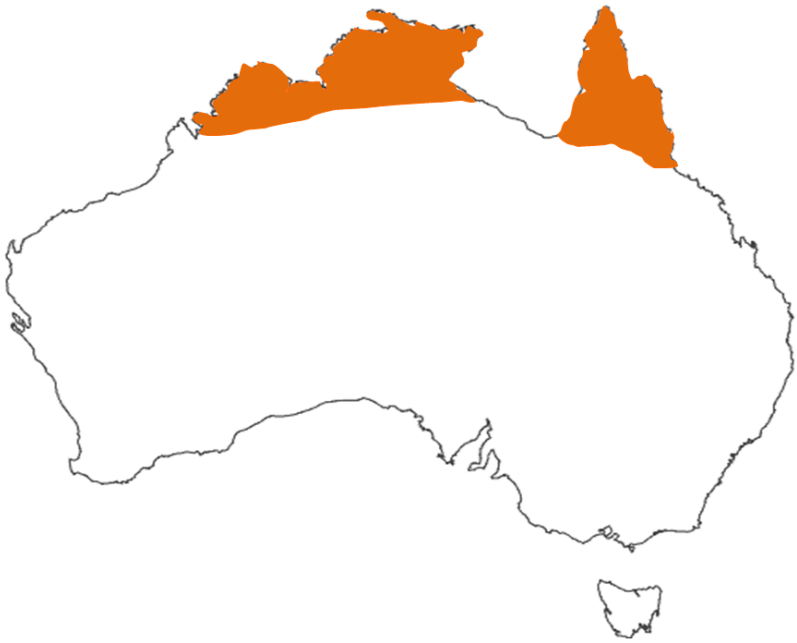

Figure 3: *(a) A photograph of Acanthophis praelongus (northern death adder) (kindly supplied by Peter Mirtschin) and (b) a map of Australia showing its distribution in orange (adapted from White et al., 1998).*

(a)

(b)

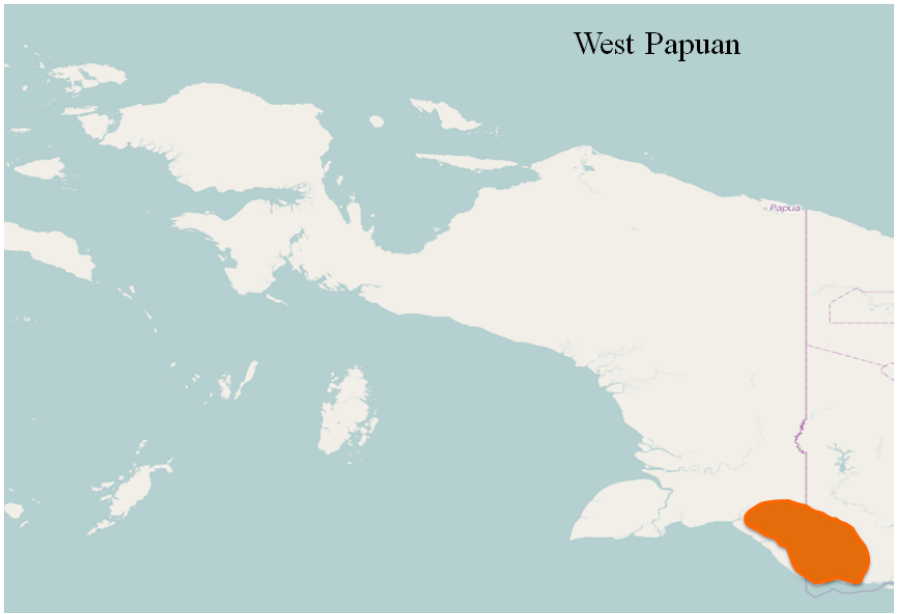

Figure 4: *(a) A photograph of Acanthophis rugosus (Irian Jayan death adder) (kindly supplied by David Williams) and (b) a map of West Papua New Guinea showing its distribution in orange (adapted from* http://www.avru.org/research/png_srp/implicated_spp/snakebite2.html).

3. Snake venom components and symptoms following envenoming

Venom has evolved for the immobilization and digestion of prey, as well as a defensive weapon. Variations in the composition of venom or the toxicity of snake venoms is dependent on the species, geographical location, habitat, season, sex and diet of the snake (Chippaux *et al.*, 1991; Currie, 2000; Menezes *et al.*, 2006; Queiroz *et al.*, 2008; White, 1991). In this literature review, venom components which induce toxic effects on important physiological systems are described.

3.1 Phospholipase A_2 (PLA_2)

Phospholipases are classified into 4 main groups (phospholipase A_1, A_2, C or D), based on their sites of hydrolysis at ester bonds of 3-*sn*-phosphoglycerides (Figure 5) (Fry, 1999). PLA_2 (EC 3.1.1.4) enzymes are the most common phospholipase found in animal venoms. They are water soluble hydrolytic enzymes which hydrolyze glycerophospholipids at the *sn*-2 acyl ester bond of membrane glycerol-3-phospholipids. Secreted PLA_2s ($sPLA_2$) have a molecular mass of 14-16 kDa and require calcium for enzymatic activity (Rizzo *et al.*, 2000). They have been identified in mammalian tissues such as the pancreas, synovial fluid and platelets (as inflammatory-type $sPLA_2$) as well as in venoms (Rizzo *et al.*, 2000). Secreted PLA_2 are categorized into three classes. Elapid or hydrophid venoms contain class I $sPLA_2$, while class II $sPLA_2$s are found in crotalid and viper venoms (Fry, 1999). Class III $sPLA_2$s are abundant in the venoms of beaded lizards (Fry, 1999) and bee venom (Arni and Ward, 1996).

Australasian elapid PLA_2s are basic and have 118 amino acids with 7 disulfide bonds (Fry, 1999). PLA_2 enzymes contribute to a number of pharmacological effects including neurotoxicity, myotoxicity, anticoagulation, smooth muscle relaxation/hypotension and hypersensitivity (Kini, 2003).

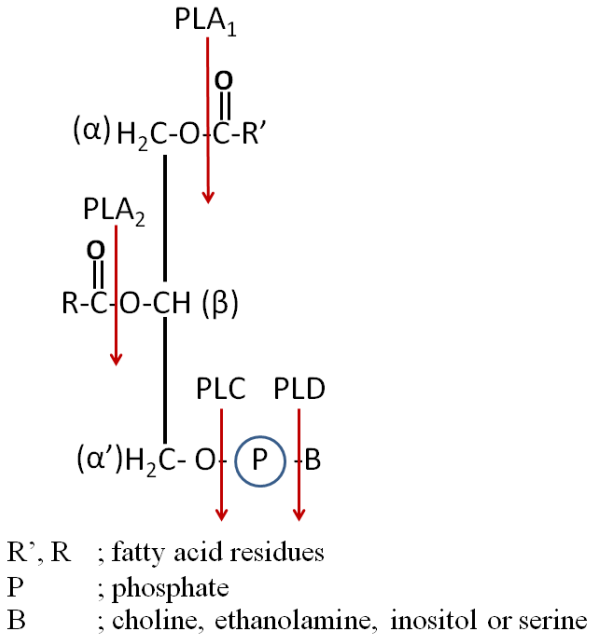

Figure 5: *The sites of hydrolysis (red arrows) of 3-sn-phosphoglycerides according to different phospholipase as type A_1, A_2, C or D (Harris, 1991).*

The pharmacological effects of PLA_2 may be produced either as a consequence of enzymatic activity of PLA_2 or as a consequence of binding activity of PLA_2 enzyme without catalytic activation (Kini, 2003). Thus, interaction between 'specific target binding sites' (membrane lipids or glycoproteins) on the surface of cells and 'pharmacological sites' on the PLA_2 molecule (independent of the catalytic site) can result in activation of the target site with pharmacological outcomes (Kini and Evans, 1989). A range of specific protein binding targets for snake PLA_2 toxins have been investigated. These include voltage-gated K^+ channel (K_V) for β-bungarotoxin (Petersen *et al.*, 1986), crotoxin acceptor protein from *Torpedo* (Faure *et al.*, 2003), neuronal pentraxin for taipoxin (Kirkpatrick *et al.*, 2000) and M-type PLA_2 receptor from skeletal muscle for OS1 and OS2 (Lambeau *et al.*, 1990).

For the catalytic-dependent pharmacological effects, hydrolytic activity of the PLA_2 enzyme at the phospholipid membrane induces the release of lysophospholipids (LysoPL) and fatty acids (FAs). These products generate pharmacological effects within cells, including membrane

damage, resulting in disruption of membrane-bound protein and functional disturbances (Kini, 2003).

Histidine-48 (His-48) plays an important role in phospholipid hydrolysis activity and is located close to the active catalytic site of PLA_2. The catalytic activity of PLA_2 can be abolished by selective alkylation of His-48 using 4-bromophenacyl bromide (4-BPB) (Volwerk *et al.*, 1974) or *p*-nitrobenzenesulfonate (Yang and King, 1980). The enzymatic activity of PLA_2 enzymes also requires Ca^{2+}. Therefore, the catalytic activity of PLA_2 can be inhibited by calcium chelators (e.g. EGTA, EDTA), removal of Ca^{2+} or replacement of Ca^{2+} by other ions such as Ba^{2+} and Sr^{2+} (Hodgson *et al.*, 2007). However, Ca^{2+} chelators or ion substitutions do not affect the non-enzymatic dependent mechanisms (Kini, 2003).

3.2 Effects of snake toxins on ion channel activities

Ion channel activation by snake neurotoxins has been investigated using electrophysiological techniques with a number of studies indicating the involvement of these toxins in the modulation of a range of ion channels.

3.2.1 Sodium (Na^+) channels

Voltage-gated Na^+ channels (Na_Vs) play a major role in the upstroke of the action potential in excitable cells e.g. neurons and muscle cells (Brandt *et al.*, 1976; Hiriart and Matteson, 1988; Tse and Hille, 1993; Yang *et al.*, 1991). Na_Vs consist of a single long polypeptide chain with four distinct domains (I-IV), each of which contains six transmembrane α-helices (S_1-S_6) (Shimomura *et al.*, 2011). Na_Vs are mostly closed at resting membrane potentials more negative than -65 mV. The large electrochemical gradient for Na^+ leads to rapid Na^+ entry during the opening of Na_Vs. Tetrodotoxin (TTX), a toxin from puffer fish, and saxitoxin (STX), from dinoflagellate of genus *Gonyaulax*, are natural blockers affecting the function of Na_Vs (Huang *et al.*, 2012).

Based on data from nomenclature studies (Goldin, 2001; Goldin *et al.*, 2000) and amino acid sequencing of transmembrane and extracellular domains (Catterall *et al.*, 2005), nine subtypes

of voltage-gated Na^+ channels have been identified i.e. $Na_V1.1$-$Na_V1.9$. Sensitivity to TTX is used as a means of classifying voltage-gated Na^+ channels. $Na_V1.1$-$Na_V1.4$, $Na_V1.6$ and $Na_V1.7$ are TTX-sensitive (TTX-S) as they are blocked by low concentrations of TTX (0.1-1 nM). $Na_V1.5$, $Na_V1.8$ and $Na_V1.9$ are classified as TTX-resistant (TTX-R) as they are blocked by TTX only at concentration above 10 μM (Akopian *et al.*, 1996; Cummins *et al.*, 1999; Roy and Narahashi, 1992; Satin *et al.*, 1992). $Na_V1.5$ is wildly distributed in mammalian heart (Marionneau *et al.*, 2012). $Na_V1.5$ (Renganathan *et al.*, 2002), $Na_V1.8$ and $Na_V1.9$ are abundant in dorsal root ganglia neurons (Akopian *et al.*, 1996; Dib-Hajj *et al.*, 1998a; Dib-Hajj *et al.*, 1998b). $Na_V1.4$ and $Na_V1.5$ are also found in skeletal muscle cells (Prasarnpun *et al.*, 2005) while $Na_V1.1$, $Na_V1.2$ and $Na_V1.6$ are predominantly found in CNS neurons (Catterall *et al.*, 2005).

A number of studies have examined the action of snake toxins on Na_Vs. For example, a secondary upregulation of $Na_V1.5$ mRNA in β-bungarotoxin-inoculated rat soleus muscle fibers (Prasarnpun *et al.*, 2005) has been postulated to be due to denervation and inactivity of skeletal muscle (Kallen *et al.*, 1990; Pasino *et al.*, 1996). Crotamine (a myotoxin from rattlesnake, *Crotalus durissus terrificus* venom) activates Na^+ channels causing an influx of Na^+ in skeletal muscle resulting in depolarization (Oguiura *et al.*, 2005).

3.2.2 Calcium (Ca^{2+}) channels

Ca^{2+} entry is involved in numerous physiological mechanisms e.g. initiation and modulation of muscle contraction, signal transduction, neurotransmitter release and hormone secretion (Berridge, 2005; Carafoli, 2005). Ca^{2+} regulates these functions either by acting directly on specific proteins such as troponin C on actin filament in excitation-contraction coupling (Mayrleitner *et al.*, 1994) or activation of calmodulin to modulate signal transduction in second messenger pathways (Berridge *et al.*, 2003; Berridge *et al.*, 2000a; Berridge *et al.*, 2000b).

Generally, cells maintain a Ca^{2+} concentration gradient of ~10,000:1 between the extracellular and intracellular compartments. Voltage-operated calcium channels (VOCCs) are a major pathway for Ca^{2+} entry across the plasma membrane. VOCCs are composed of four non-

covalently associated polypeptides, α_1, $\alpha_2\delta_1$, β_{1a} and γ_1 (Wang *et al.*, 2004). The α1 subunit is the Ca^{2+} pore forming subunit, similar in structure to the 4x6 loop arrangement of Na_Vs. The other subunits play a role in regulating channel gating (Leung *et al.*, 1987). VOCCs are activated by depolarization. A vast number of factors affect the function of VOCCs including stress, diseases, aging, chemical agents or toxins. These stimuli may damage healthy cells or neurons by permitting Ca^{2+} overload leading to apoptosis and cell degeneration.

Based on pharmacological, physiological properties and sequence analysis of α1 subunit genes (Ertel *et al.*, 2000), five families of VOCCs have been categorized (long-lasting, L; neuronal, N; Purkinje, P/Q; transient, T; residual (resistant), R).

L-type Ca^{2+} channels

The long-lasting Ca^{2+}channels are inhibited by dihydropyridine (DHP) derivatives (e.g. nifedipine, amlodipine) including the snake toxin, calciseptine (Kochegarov, 2003). L-type Ca^{2+} channels are classified into 4 subtypes (Ca_V1.1-1.4) based on the α1 subunit. Ca_V1.1 in skeletal muscle and Ca_V1.2 in cardiac cells are responsible for the excitation-contraction coupling (Wang *et al.*, 2004), while Ca_V1.3 and Ca_V1.4 are located in neurons and retina, respectively (Satin *et al.*, 2011).

N-type Ca^{2+} channels

N-type Ca^{2+} channels are not blocked by DHPs (Nowycky *et al.*, 1985). They are sensitive to ω-conotoxins GVIA and MVIIC from *Conus geographus* (cone shell mollusk) (Lewis *et al.*, 2012). These VOCCs are abundant in brainstem and responsible for neurotransmitter release (Mendelowitz *et al.*, 1995).

P/Q-type Ca^{2+} channels

P and Q components of the current through these channels are difficult to distinguish therefore, the term ‘P/Q-type’ current refers to both. These channels were discovered in Purkinje

cells (hence the 'P') and are insensitive to either DHPs or ω-conotoxin. In α1A gene knockout mice, there is an absence of both P-type and Q-type currents (Randall and Tsien, 1995) suggesting that these currents share the same gene. P/Q type Ca^{2+} channels are found in central or peripheral pre-synaptic membranes (Luisi *et al.*, 2009) as well as in nodose sensory neurons and arterial baroreceptors (Tatalovic *et al.*, 2012). P-type current is sensitive to low concentrations of ω-agatoxin IVA, a peptide from the venom of American funnel web spider, *Agelenopsis aperta*, while Q-type current is blocked by high concentration of ω-agatoxin IVA (Kochegarov, 2003).

T-type Ca^{2+} channels

T-type or low-voltage-activated (LVA) current is inhibited by very low concentrations of mibefradil and kurtoxin, a peptide toxin from the South African scorpion, *Parabuthus transvaalicus*. These channels are abundant in brain, kidney, liver, heart and CNS (Kochegarov, 2003).

R-type Ca^{2+} channels

R-type Ca^{2+} channels are inhibited by SNX-482, from the venom of the African tarantula, *Hysterocrates gigas* (Kochegarov, 2003). These channels are abundant in major neuronal systems and resistant to DHPs, ω-conotoxin GVIA and ω-agatoxin IVA.

Ligand-gated Ca^{2+} channels

Cytoplasmic Ca^{2+} availability can also be increased by pathways other than influx through VOCCs. These pathways include ligand-operated Ca^{2+} channels (LOCCs) or ionotropic receptors, which involve modulation of Ca^{2+} entry through NMDA (*N*-methyl-D-aspartate) receptors in glutamatergic transmission (Leist and Nicotera, 1998) or nicotinic acetylcholine receptors (nAChRs). Transient receptor potential (TRP) channels also have a significant Ca^{2+} permeability that permit Ca^{2+} influx (Verkhratsky, 2007). TRP channels belong to a super-family of cationic non-voltage gated channels found in many eukaryotic cells (Pedersen *et al.*, 2005). TRP channels

may generate physiological effects through G-protein coupled receptor pathways which are sensitive to environmental conditions such as temperature, diacylglycerols, mechanical force and hypotonic stress (Gosling *et al.*, 2005). Pathological activation of TRP channels may cause uncontrolled Ca^{2+} overload (Yoon *et al.*, 2000).

It has been shown that neurotoxicity of snake pre-synaptic PLA_2 neurotoxins (e.g. textilotoxin, taipoxin) is induced through an increase in intracellular calcium ($[Ca^{2+}]_i$) in isolated rat neuronal cells (Tedesco *et al.*, 2009). This may result in the depletion of neurotransmitters through excessive Ca^{2+} influx which is observed in neurons treated by a mixture of phospholipid hydrolysis products (i.e. LysoPL and FA) promoting a large influx of $[Ca^{2+}]_i$ (Rigoni *et al.*, 2007).

3.2.3 Potassium (K^+) channels

K^+ channels were identified by sequencing the *Caenorhabditis elegans* genome, with almost one hundred K^+ channels present in this organism (Bargmann, 1998). K^+ channels are critical for determining the negative value of the resting membrane potential in all cells. They also play a role in the regulation of neuronal or cardiac electrical transmission, release of neurotransmitters or hormones (e.g. insulin), skeletal muscle or cardiac contractions and modulation of signal transduction (Kaczorowski and Garcia, 1999). The structure of K_V comprises six transmembrane segments (S_1-S_6) with a pore-forming region located between S_5 and S_6 (Kaczorowski and Garcia, 1999).

K_V channels have been characterized as Shaker (K_V1), Shab (K_V2), Shaw (K_V3) and Shal (K_V4) in the homologues of the *Drosophila* potassium channel genome (Robertson, 1997). K_V channels can be inhibited by charybdotoxin (ChTX; from *Leiurus quinquestriatus hebraeus* venom; blocks $K_V1.2$ and 1.3; Miller, 1995), noxiustoxin (NxTX; from scorpion venom, *Centruroides noxius*; blocks $K_V1.2$ and 1.3; Leonard *et al.*, 1992), agitoxin (AgTX; from *Androctonus mauretanicus*; blocks $K_V1.3$; Garcia *et al.*, 1994) and dendrotoxin (DTX; from African mamba (*Dendroaspis angusticeps*) venom; blocks $K_V1.1$, 1.3 and 1.6; Penner *et al*., 1986).

Snake pre-synaptic neurotoxins (e.g. dendrotoxin, β-bungarotoxin) inhibit the K_V channel, allowing VOCCs to remain open for longer, prolonging a Ca^{2+} influx which may induce an enhanced release of neurotransmitter (Penner and Dreyer, 1986; Penner *et al.*, 1986; Petersen *et al.*, 1986). However, one report failed to confirm an inhibitory action of snake pre-synaptic neurotoxins on cloned K_V channels in B82, L929 mouse fibroblasts or mouse erythroleukemia cells (Fathi *et al.*, 2001). This result may be due to an absence of binding sites on the α-subunit of the five K_V channels used in the study and/or may reflect the absence of accessory K_V subunits in the cell lines used that are essential for toxin channel interaction.

3.2.4 Chloride (Cl^-) Channels

Chloride (Cl^-) is the most abundant anion in biological systems. It has a significant role in inducing membrane hyperpolarization, in inhibitory postsynaptic potentials (IPSP) and in the regulation of intracellular pH and cell volume (Edwards and Kahl, 2010). Cl^- channels are classified into 4 different categories, based on their functional and biophysical properties. Those are (1) cystic fibrosis chloride channel, (2) swelling active chloride channels, (3) calcium activated chloride channels and (4) ligand gated chloride channels or γ-aminobutyric acid (GABA)/glycine receptors (Jentsch *et al.*, 2002). Oenanthotoxin, a polyacetylenic alcohol extracted from a plant of genus *Oenanthe*, has been reported to block GABAergic currents in rat hippocampal neurons suggesting an inhibitory effect on chloride channels (Wyrembek *et al.*, 2010).

3.3 Neurotoxicity

Neurotoxicity is the most common symptom following envenoming by Australasian elapids. This is due to inhibitory effects at the skeletal neuromuscular junction and is an important mechanism of immobilizing the prey.

Snake neurotoxins interrupt signal transduction at the neuromuscular junction either presynaptically, at the motor nerve terminal or postsynaptically, at nAChRs on the plasma membrane of skeletal muscle cells. This results in a disturbance of transmitter release or disruption

of binding activity between ACh and nAChRs, respectively. Australasian elapid venoms contain potent neurotoxins that cause extraocular muscle weakness (ptosis), flaccid paralysis and respiratory failure due to respiratory muscle weakness in envenomed humans.

Signal transduction at the neuromuscular junction

The presynaptic and postsynaptic membranes are separated by a synaptic cleft. The presynaptic nerve terminal contains a large number of active zones and synaptic vesicles packed with neurotransmitters (Figure 6a). Acetylcholine (ACh) is the neurotransmitter at the neuromuscular junction (NMJ) and synthesized in the cytosol of the presynaptic nerve terminal by choline acetyltransferase (ChAT) (Palouzier-Paulignan *et al.*, 1991) (Figure 6a). Choline is the precursor for ACh synthesis and is taken up into cholinergic nerve terminals by a specific transporter (Black and Rylett, 2012). Synthesized ACh is transported from the cytoplasm to synaptic vesicles by a vesicle-associated transporter which can be interrupted by some snake pre-synaptic neurotoxins i.e. taipoxin and paradoxin (Treppmann *et al.*, 2011).

Release of vesicles of ACh is achieved by an action potential invading the axon terminal, inducing the opening of VOCCs. The increase in $[Ca^{2+}]_i$ causes the fusion of the synaptic vesicle membrane with the presynaptic membrane and the formation of a pore, permitting the release of transmitter into the synapse. This process is called exocytosis (Schneggenburger *et al.*, 2012), and occurs when vesicles are already docked at active zones (Figure 6). Docking of synaptic vesicles is initiated by interaction of SNAREs (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors) located on the terminal membrane and the vesicle membrane (step 1, Figure 6b). These proteins include a vesicle protein (synaptobrevin/VAMP), the Ca^{2+} sensor of membrane fusion (synaptotagmin), synaptophysin or plasma membrane protein complexes of syntaxin-1 and SNAP-25 (Becher *et al.*, 1999; Rizo and Sudhof, 2012).

It has been reported that snake pre-synaptic neurotoxins (e.g. β-bungarotoxin or taipoxin) induce disassociation of vesicular proteins (syntaxin, synaptophysin, synaptobrevin) (Bonanomi *et al.*, 2005; Prasarnpun *et al.*, 2005). In response to Ca^{2+} entry into the presynaptic membrane,

SNARE proteins generate membrane complexes and fusion resulting in neurotransmitter release (step 3, Figure 6b) (Sutton and Sprang, 1998). Following transmitter release, the vesicle is retrieved by endocytosis (step 4, Figure 6b) which is triggered by Ca^{2+} influx through VOCCs. Synaptic endocytosis is necessary for the re-uptake of secretory vesicles in synaptic transmission pathway (Keating *et al.*, 2006). In the CNS, the major pathway is clathrin-mediated endocytosis, which involves the building up of vesicle by coated membrane clathrin (Yamashita, 2012).

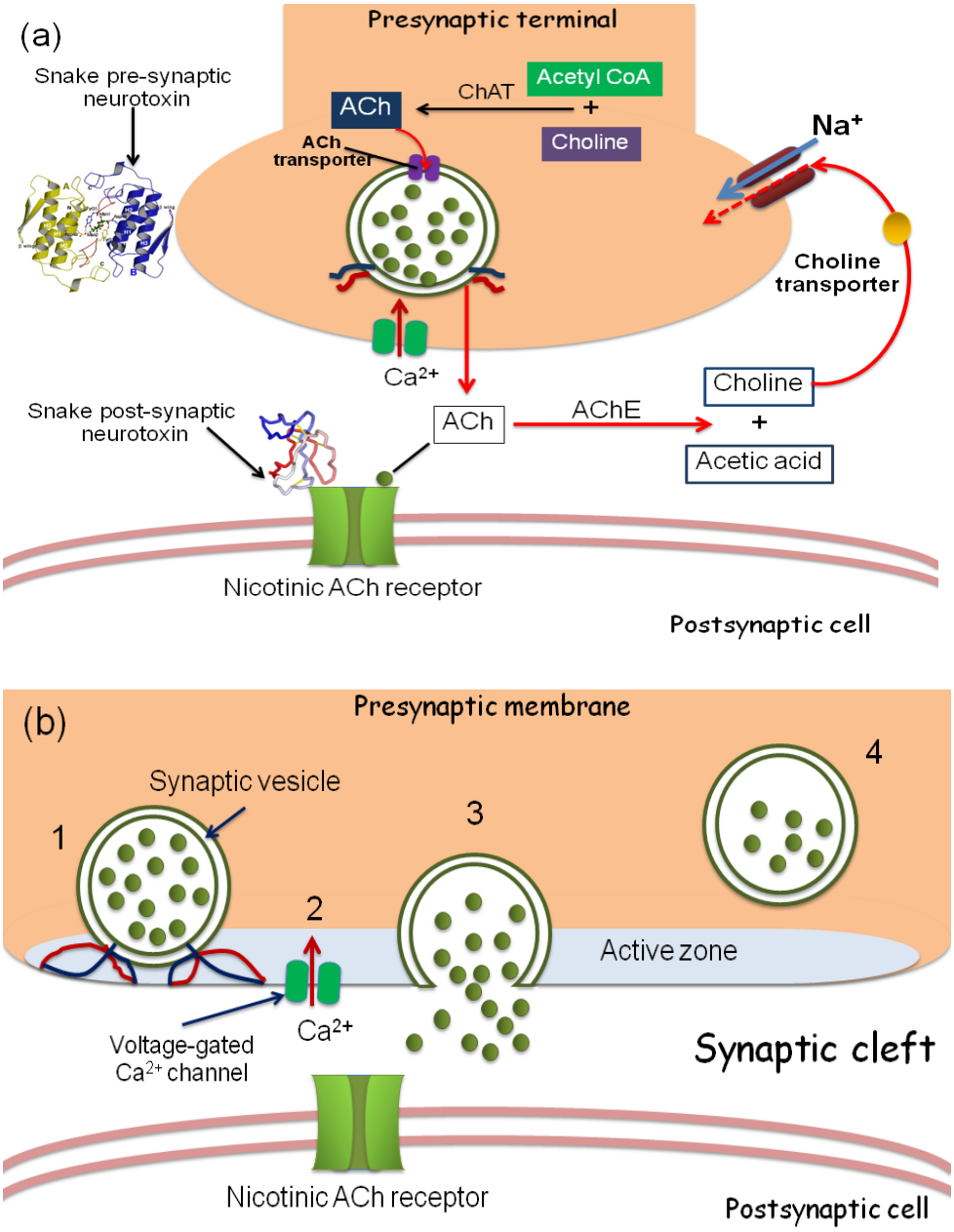

Figure 6: *Schematic diagrams showing (a) target sites of snake pre-synaptic or post-synaptic neurotoxins including the cycle of ACh synthesis and (b) mechanism of ACh release by exocytosis (Bear et al., 2007).*

The muscle type nAChR displays a pentameric structure made up of polypeptide subunits (e.g. $(\alpha1)_2\beta1\delta\varepsilon$ in adults, or $(\alpha1)_2\beta1\delta\gamma$ in the fetus) (Liu and Dilger, 2009). The ACh binding sites on nAChR are located at the interface between subunits α-δ and α-ε (Akk and Auerbach, 1996). As a consequence of ACh binding, the nAChR becomes an ionophore (an ion channel) allowing Na^+ influx across the muscle cell membrane, resulting in membrane depolarization and muscle contraction. Acetylcholinesterase (AChE) is responsible for ACh degradation to choline and acetate for subsequent reuptake into the nerve terminal, and resynthesis into ACh (Dvir *et al.*, 2010).

3.3.1 Snake pre-synaptic neurotoxins (β-neurotoxins)

Pre-synaptic neurotoxins or β-neurotoxins have been purified from the venoms of Elapid (e.g. textilotoxin from *P. textilis* venom, β-bungarotoxin from the venom of *Bungarus multicinctus*), Crotalidae (e.g. crotoxin from South American rattlesnake, *Crotalus durissus terrificus*) and Viperidae (e.g. Ammodytoxin A from *Vipera ammodytes*) snakes. Most snake pre-synaptic neurotoxins display PLA_2 activity which interrupts neurotransmitter release, synthesis, storage or turnover on the synaptic nerve terminal (Rossetto *et al.*, 2006). Nevertheless, a direct relationship between PLA_2 activity and β-neurotoxin-induced neurotoxicity is not proven (Hodgson *et al.*, 2007). The effects of pre-synaptic neurotoxins is difficult to neutralize by administration of antivenom or washing out with fresh medium unless performed within a short time period after envenoming (Montecucco and Rossetto, 2000).

Snake pre-synaptic neurotoxins (Table 1.1) display a molecular mass between 14-70 kDa and are comprised of different subunits (Fohlman *et al.*, 1979; Fohlman *et al.*, 1976). β-Bungarotoxin contains two subunits, A and B, bound together with covalently cross-linked disulphide bridge (Rowan, 2001), while taipoxin, paradoxin and cannitoxin contain three non-covalent cross-linked, α-, β- and γ-subunits (Kuruppu *et al.*, 2008). The α-subunit is the only catalytic subunit that displays the PLA_2 activity. The γ-subunit possesses acidic properties and appears to be heterogeneously glycosylated (Kuruppu *et al.*, 2008). The β- and γ-subunits work

together as chaperones to induce toxicity (Fohlman *et al.*, 1979). Thus, the activity of each subunit is significantly reduced upon dissociation from the other subunits (Fohlman *et al.*, 1976).

Table 1.1 *Alignment of certain elapid pre-synaptic neurotoxin subunits*

Species	Pre-synaptic neurotoxin subunit/ molecular mass (Da)	Partial amino acid sequences
A. antarcticus	P-EPTX-Aa1a; α-subunit (13,809)[a]	NLLQFGFMIR CANKRRRPVW PYEESGC
O. scutellatus	Cannitoxin; α-subunit (13,824)[b]	NLLQFGYMIR CANGRSRPVW - - - - - - -
O. s. scutellatus	Taipoxin; α-subunit (13,829)[c]	NLLQFGFMIR CANRRSRPVW HYMDYGC
A. antarcticus	P-EPTX-Aa1a; β-subunit (13,516)[a]	DLFQFGKMIE CANKGSRPSL DYMNY
O. scutellatus	Cannitoxin; β_2-subunit (13,242)[b]	NLVQFGFMIE CAIRNRQPAL DFMNY
O. s. scutellatus	Taipoxin; β-subunit (13,236)[c]	NLIQFGKMIE CAIRNRRPAL DFMNY
A. antarcticus	P-EPTX-Aa1a; γ-subunit (17,632)[a]	- SIPLPSLNFEQ FGNMIQCTIP - - - - - - - -
O. scutellatus	Cannitoxin; γ-subunit (17,762)[b]	S EIPQPSLDFEQ FSNMIQCTIP - - - - - - - -
O. s. scutellatus	Taipoxin; γ-subunit (18,354)[c]	S ELPQPSIDFEQ FSNMIQCTIP - - - - - - - -

[a](Blacklow *et al.*, 2010a) [b](Kuruppu *et al.*, 2005b) [c](Fohlman *et al.*, 1977)

The effect of snake pre-synaptic neurotoxins on evoked neuronal transduction in skeletal muscle preparations is triphasic, characterized by an initial inhibition of twitches and endplate potential (epp) amplitude (Phase 1), followed by a prolonged facilitatory phase (an increase in transmitter release, Phase 2), and a final decline in twitch amplitude until complete failure occurs (Phase 3) (Rowan, 2001). This triphasic effect is particularly evident in low Ca^{2+} or Sr^{2+} substituted solution as well as in high Mg^{2+} solution (Caratsch *et al.*, 1985; Chang and Su, 1982). Phase 1 appears to be independent of PLA_2 activity, and has been suggested to be due to the binding of the

toxin to the presynaptic membrane. However, the mechanism mediating the decrease in epp amplitude has not been determined (Rowan, 2001). Phases 2 and 3 require PLA_2 activity to induce toxicity (Kuruppu *et al.*, 2008). The facilitation observed in phase 2 might be induced by an inhibitory effect on K_V channels which causes presynaptic depolarization and facilitates Ca^{2+} influx through VOCCs, resulting in enhanced transmitter release. Phase 3 is characterized by a progressive decline in the epp amplitude and is associated with a failure of endocytotic retrieval of vesicular membrane, loss of docking sites in the presynaptic membrane, and damage of mitochondrial membranes (Landon *et al.*, 1980; Rowan, 2001).

It has been suggested that the effects of pre-synaptic PLA_2 neurotoxins are due to the toxins promoting the fusion of the synaptic vesicle membrane to the terminal membrane and the inhibition of vesicle recycling (Caccin *et al.*, 2006). Monteccucco and Rossetto (2000) suggested a model where the pre-synaptic neurotoxins bind to the external surface of the membrane allowing access to the cytoplasm. The toxin is then endocytosed to the synaptic vesicle causing hydrolysis of the inner phospholipid membrane of the synaptic vesicle. The phospholipid hydrolysis products (i.e. LysoPL and FA) also exhibit pre-synaptic neurotoxicity in similar manifestations which can be characterized by:

(1) the enlargement of axon terminals

(2) the appearance of large vesicles, vacuoles and damaged mitochondria

(3) the presence of membrane fusion of ready-releasable synaptic vesicles

(4) transient membrane pores which lead to the entry of external Ca^{2+}

(5) depletion of neurotransmitter from synaptic vesicles (Rigoni *et al.*, 2007).

Recent studies have shown that the anti-trypanosomiasis agent and antagonist of P2 purinoceptors, suramin, inhibits the early biphasic effects of pre-synaptic neurotoxins (i.e. β-bungarotoxin, taipoxin, ammodytoxin A) and significantly prolongs the time taken for complete inhibition of twitch contractions (Fathi *et al.*, 2011). Suramin has been reported to antagonize the inhibitory action of non-depolarizing agents (e.g. tubocurarine, pancuronium) but not depolarizing agents (e.g. suxamethonium) (Henning *et al.*, 1992). Suramin also inhibits the action of Ca^{2+}

channel blockers i.e. ω-conotoxin MVIIC or ω-agatoxin IVA (Lin *et al.*, 2000). It has been suggested that suramin interacts directly with the pre-synaptic neurotoxin to prevent neurotoxicity (Fathi *et al.*, 2011). The blocking activity (Phase 3) of toxin is reduced in high Mg^{2+} suggesting phospholipase dependence in this phase (Rowan, 2001). PLA_2 activity of pre-synaptic neurotoxins can be attenuated by selective alkylation of His48 using 4-BPB (Blacklow *et al.*, 2010a; Kuruppu *et al.*, 2005b).

3.3.2 Snake post-synaptic neurotoxins (α-neurotoxins)

Snake post-synaptic neurotoxins or α-neurotoxins display a similarity of action to d-tubocurarine, a competitive nicotinic receptor antagonist (Endo and Tamiya, 1991). Thus, they are also known as "curaremimetic toxins". Post-synaptic neurotoxins bind to nAChR at the NMJ blocking the binding of ACh (Figure 6a), and preventing muscle contraction. The pharmacological activity of some post-synaptic neurotoxins occurs quite rapidly in comparison to the effects of pre-synaptic neurotoxins.

Post-synaptic neurotoxins are classified into short-chain (Type I) and long-chain (Type II) neurotoxins based on their amino acid sequences and phylogenetic groups (Dufton and Harvey, 1989). Short chain neurotoxins have 60-62 amino acid residues and four disulfide bridges whereas long-chain neurotoxins contain 66-74 amino acid residues with five disulfide bridges (Endo and Tamiya, 1991).

In *in vitro* experiments using skeletal muscle preparations, addition of antivenom at the time point when the amplitude of the twitches is reduced by 50% (i.e. t_{50}) can often reverse the inhibitory effect of post-synaptic neurotoxins (Kuruppu *et al.*, 2005c). A number of post-synaptic neurotoxins have been purified and characterized from Australasian and other elapid venoms including acantoxin IVa from *A. laevis* venom (Wickramaratna *et al.*, 2004), oxylepitoxin from *O. microlepidotus* venom (Clarke *et al.*, 2006), hostoxin-1 from *Hoplocephalus stephensi* venom (Tan *et al.*, 2006), α-Elapitoxin-Ppr1 and α-Elapitoxin-Pc1 from *Pseudechis porphyriacus* and *Pseudechis colletti* (Hart *et al.*, 2013b), respectively.

3.4 Myotoxic activity

Myotoxicity is generated by small molecules which can induce direct cytotoxicity in skeletal muscle causing the release of myoglobin and creatine kinase (CK). Snake myotoxins have been classified into three different types (Lomonte and Rangel, 2012). They are (1) myotoxin 'a' and crotamine or 'small myotoxins' from rattlesnake venoms (Oguiura *et al.*, 2005), (2) "Cytolysins" or "cardiotoxins" , polypeptides found in cobra venom belonging to three finger toxin (3FTx) family (Kini and Doley, 2010) and (3) PLA_2s which appear to be the most abundant myotoxic compounds in snake venom.

As mentioned above, snake PLA_2 enzymes are divided into 2 classes, (1) class I; Elapid or hydrophid PLA_2 and class II; Viperid snake PLA_2. Within class II, myotoxins have been subdivided into catalytically-active, Asp49 PLA_2s and catalytically-inactive PLA_2s with the presence of Lys, Ser or Arg at position 49 (Lomonte *et al.*, 2003). Lys49 PLA_2 homologues appear to be frequently recognized catalytically-inactive PLA_2s which induce myotoxicity and cytotoxicity by a mechanism which does not involve phospholipid hydrolysis (Lomonte and Rangel, 2012).

The characteristics of snake venom-induced skeletal muscle cell damage are hypercontraction of myofilaments, disruption of the plasma membrane, release of CK and necrosis (Melo *et al.*, 2004). PLA_2 myotoxins evoke their myotoxicity rapidly at the plasma membrane of the muscle cell (approximately 3-4 min) causing membrane depolarization (Melo *et al.*, 2004). The mechanism behind myotoxin-induced muscle degeneration can be attributed to the binding of the myotoxin to specific plasma membrane receptors. Binding causes phospholipid membrane hydrolysis resulting in the generation of ionic pores for cations such as Na^+ and Ca^{2+} (Villalobos *et al.*, 2007). Cationic influx depolarizes the cell, opening VOCCs, which causes Ca^{2+} release from the sarcoplasmic reticulum (SR) and local hypercontraction of skeletal muscle cells (Dixon and Harris, 1996).

In Australia and Papua New Guinea, envenoming by the Mulga snake (*Pseudechis australis*), the taipan (genus *Oxyuranus*), tiger snake (genus *Notechis*) and the death adder (genus

Acanthophis) has been reported to cause myotoxicity. In addition, snake pre-synaptic PLA_2 neurotoxins (e.g. taipoxin (Harris and Maltin, 1982), notexin and crotoxin (Melo *et al.*, 2004)) also display myotoxicity in *in vitro* and *in vivo* studies. Myotoxic activity of snake venoms/toxins can be attenuated by prior incubation with 4-BPB (Kuruppu *et al.*, 2005a) or suramin (de Oliveira *et al.*, 2003; Murakami *et al.*, 2005).

3.5 Renal damage

Renal damage (i.e. nephrotoxicity) can result following Australasian elapid envenoming, and involves mechanisms related to myotoxic activity and elevated CK levels. Nephrotoxicity can be attributed to several mechanisms: (1) impairment of glomerular perfusion as a result of intravascular coagulation (Burdmann *et al.*, 1993); (2) a decrease in renal vascular resistance, glomerular filtration rate and urinary flow (Evangelista *et al.*, 2010) and (3) obstruction by released myoglobin from damaged smooth muscle in renal tubules which can lead to renal ischemia and acute renal failure (Ponraj and Gopalakrishnakone, 1995). Kidneys of animals treated with the venoms of coral snakes displayed extensive necrosis of tubular epithelial cells, rupture of basal membrane and tubule thickening. These lesions resulted from glomerular damage by myoglobin deposits (de Roodt *et al.*, 2012).

3.6 Cell death and cytotoxic activity

The terms cytotoxin, cardiotoxin, direct lytic factor or membrane-disruptive polypeptide have been used to describe a snake toxin causing cellular degeneration. Effects of snake cytotoxins include membrane depolarization and Ca^{2+} release from SR (Fletcher *et al.*, 1993), muscle contraction (Fletcher and Lizzo, 1987), hemolysis (Jiang *et al.*, 1989) and cytolysis. Numerous venoms and toxins from elapids (e.g. cobras (*Naja* spp.), kraits (*Bungarus* spp.)) and vipers (e.g. *Bothrops asper*, *Bothrops atrox*) induce marked cytotoxicity which can be due to either apoptosis or necrosis (Mora *et al.*, 2005).

Apoptosis (programmed cell death) and necrosis are two major modes of cell death. Necrosis is associated with $[Ca^{2+}]_i$ overload while apoptotic cell death is triggered by cellular Ca^{2+} signaling. Pathologically high Ca^{2+} levels are induced by two different mechanisms, (1) an overstimulation of the cell membrane leading to unregulated Ca^{2+} influx (e.g. hyper-release of glutamate, anoxia, ischemia) and/or (2) failure of Ca^{2+} homeostasis (e.g. exogenous toxins or physiological factors induce an increase in $[Ca^{2+}]_i$). In fact, cell degeneration, either apoptotic or necrotic, is related to increased Ca^{2+} flux through VOCCs (Barone *et al.*, 2004).

The mechanisms mediating β-bungarotoxin-induced cytotoxicity have been elucidated in mice neurons. β-bungarotoxin induces a rise in $[Ca^{2+}]_i$, leading to the activation of nitric oxide synthase (NOS) which promotes the production of nitrogenous species and nitric oxide (NO) (Tseng and Lin-Shiau, 2003a). Neuronal cell death was blocked by BAPTA-AM and EGTA (Ca^{2+} chelators), MK801 (NMDA receptor antagonist) and diltiazem (L-type Ca^{2+} channels blocker) suggesting that the elevation in $[Ca^{2+}]_i$ and neurotoxicity result from Ca^{2+} influx through NMDA receptors as well as via L-type VOCCs (Tseng and Lin-Shiau, 2003b).

Determination of toxin-induced cytotoxicity by calcium imaging

Snake toxin-induced cytotoxicity can be studied by Ca^{2+} imaging techniques. β-bungarotoxin, taipoxin, or textilotoxin generate an increase in $[Ca^{2+}]_i$ which is detected in neurons loaded with a fluorescent dye (e.g. fura-2, fluo-3, fluo-4) (Rigoni *et al.*, 2007; Tedesco *et al.*, 2009). Fluorescent dyes (Fluo-4) are administered to the cell as acetoxymethyl (AM) ester which is permeable to the cell membrane. Intracellular esterases hydrolyze the AM, leaving fluo-4^{5-} free in the cell (Figure 7). Fluo-4^{5-} is impermeable to the cell membrane and presents a negatively charged form which is capable of binding Ca^{2+}. Fluo-4^{5-} contains a BAPTA-like Ca^{2+} chelator site which bind to Ca^{2+} therefore measurement of Ca^{2+} can be performed (Hansen and Brauner-Osborne, 2009).

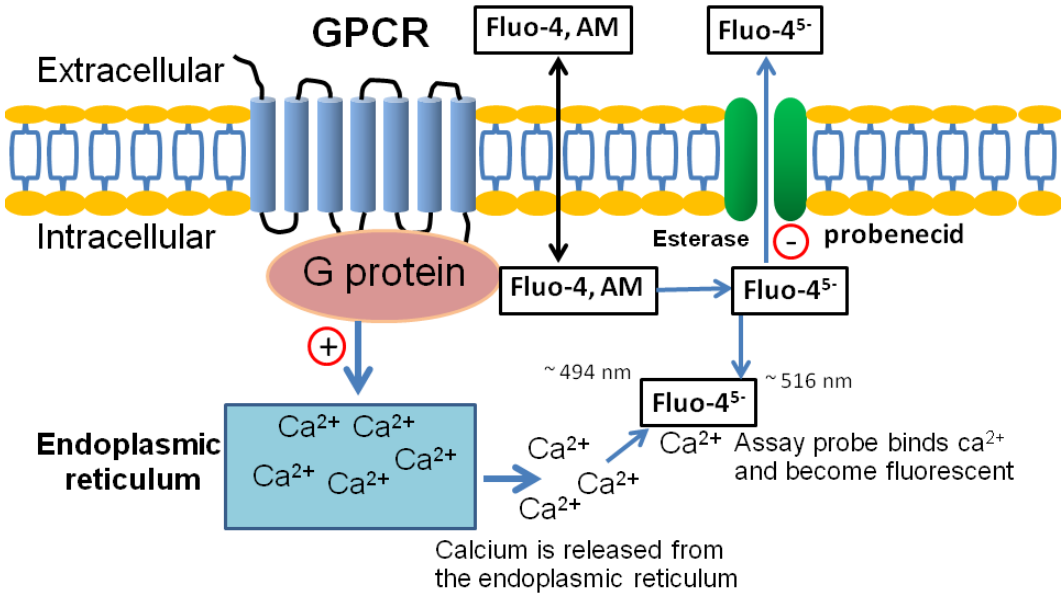

Figure 7: *Schematic diagram displaying the intracellular mechanisms of a calcium assay in mammalian cells following the administration of the fluorescent calcium binding dye, fluo-4. Modified from Hansen and Brauner-Osborne (2009).*

3.7 Haemostatic abnormalities

Haemostatic disturbances following Australian elapid envenoming such as coagulopathy, anticoagulation and thrombosis can cause significant mortality leading to intracranial hemorrhage and complete defibrination (White, 2005) or 'venom-induced consumption coagulopathy' (VICC) (Isbister *et al.*, 2006b). The characteristics of VICC include low or undetectable fibrinogen levels, depletion of cofactors V and VIII, and high concentrations of fibrin degradation products (Lalloo *et al.*, 1995b). These haemostatic abnormalities have been suggested to be responsible for the significant life-threatening events such as severe depression of cardiac output and sudden cardiovascular collapse following envenoming by some Australasian elapids (Tibballs, 1998; Tibballs *et al.*, 1989; Tibballs *et al.*, 1992).

3.7.1 Blood coagulation

Intrinsic and extrinsic pathways are involved in fibrin formation. The extrinsic, or *in vivo*, pathway is initiated by tissue factor (TF) and factor VII (stable factor). Vascular tissue damage

activates TF-VIIa complex formation in the presence of phospholipid (PL) and Ca^{2+} (Figure 8). The TF-VIIa complex and factor IXa (plasma thromboplastin component) activate factor X to Xa leading to the formation of prothrombinase complex (factor Xa, Ca^{2+}, PL and factor Va) which converts prothrombin (factor II) to thrombin (factor IIa) (Davie, 2003).

To stabilize clotting, thrombin induces the activation of factor XIII (fibrin-stabilizing factor) to XIIIa. Factor XIIIa and Ca^{2+} develop cross linked fibrin (stabilized form) by using intermolecular covalent bonds in a stabilizing process (Ichinose, 2001). Thrombin from the *in vivo* pathway also activates primarily factors V (proaccelerin), VIII (antihemophilic factor), and XI (plasma thromboplastin antecedent). Furthermore, the intrinsic pathway or contact pathway is also activated in order to promote further coagulation. Factor XII plays a role as an initiator in this pathway and participates in the *in vivo* pathway during the activation of factor X, as indicated above (Muller and Renne, 2008). The vascular endothelium also participates in the prevention of intravascular platelet activation and coagulation. Endothelial cells synthesize and release tissue plasminogen activator (TPA) which converts plasminogen to plasmin. Plasmin is a fibrin-specific protease which has a significant role in fibrin digestion (McMichael, 2012). Indeed, vascular endothelial cells also release vasoactive mediators such as NO and prostacyclin (PGI_2) that are potent inhibitors of platelet aggregation (Moncada *et al.*, 1988; Shankarraman *et al.*, 2012).

Prothrombin can be activated by exogenous prothrombin activators, present in the venoms of some snakes, leading to severe hematological disturbances (Rao and Kini, 2002). Venoms of snakes from the genus *Pseudonaja*, *Notechis* and *Oxyuranus* contain prothrombin activators, while New Guinea small eyed snake (*Micropechis ikaheka*), death adder and Australian mulga snake (*Pseudechis* spp.) venoms induce anticoagulation (White, 2005).

Snake prothrombin activators are classified into 4 categories based on their functional properties and cofactor requirements (Rao and Kini, 2002; Rosing and Tans, 1992).

Group A (e.g. ecarin) (Morita *et al.*, 1976) and B (e.g. carinactivase) (Yamada *et al.*, 1996) prothrombin activators are metalloproteinases found in viperid or crotalid venoms. The activity of group A prothrombin activators is independent of Ca^{2+} and cofactors (e.g. PL or FVa), while group

B prothrombin activators require Ca^{2+} but not cofactors for their activity (Rao and Kini, 2002; Rosing and Tans, 1992).

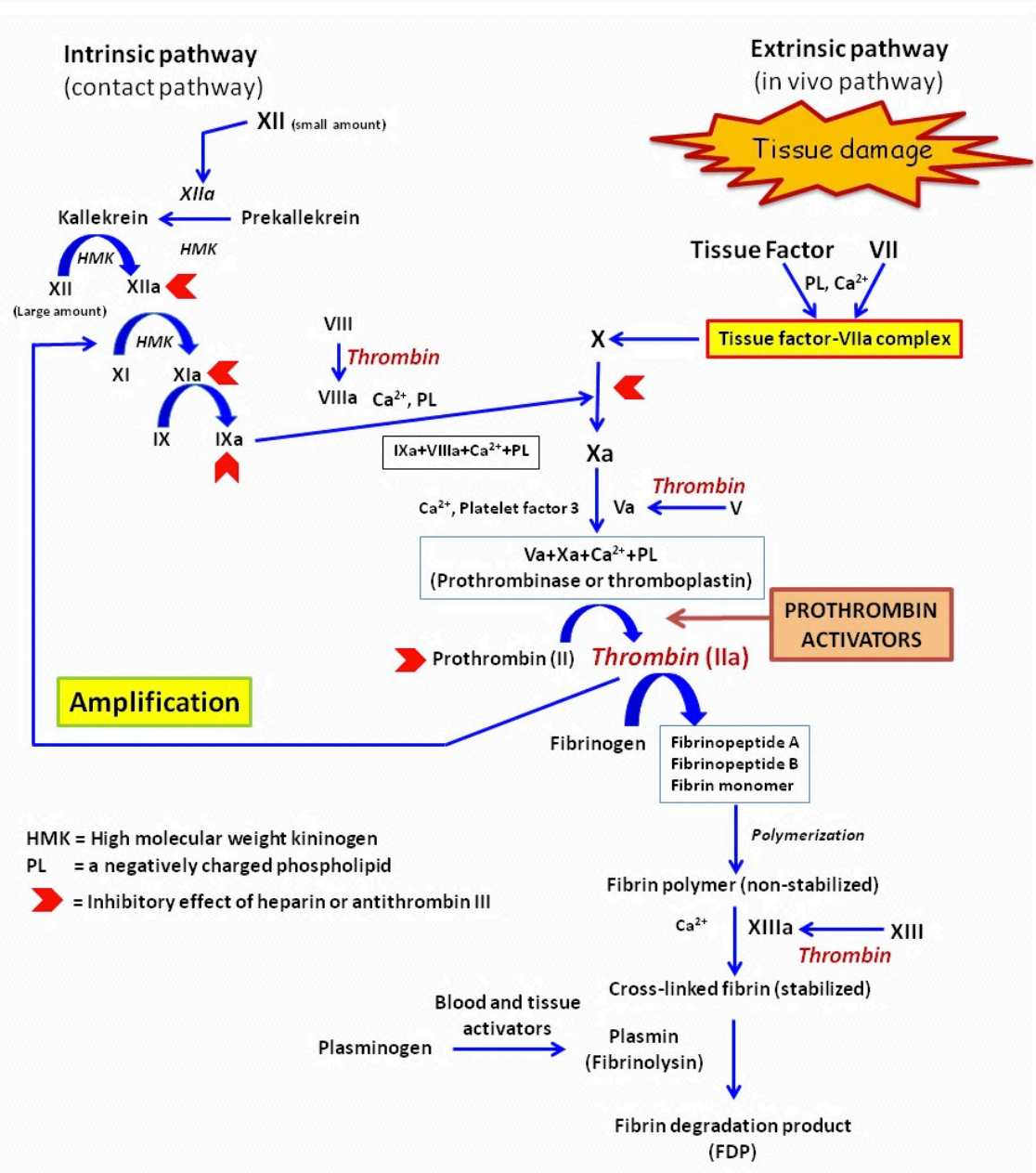

Figure 8: *Schematic diagram showing mechanism of blood coagulation. Modified from McMichael (2012).*

Group C and D prothrombin activators are serine proteinases (Rao *et al.*, 2003), which promote the conversion of prothrombin to thrombin rapidly upon reaching the systemic circulation. Group C activators contain large protein complexes (approximately 250 kDa) with multiple subunits (e.g. oscutarin, isolated from *O. scutellatus* venom, pseutarin C, isolated from *P. textilis*

venom) (Rao and Kini, 2002). Group C activators display functional and structural similarities to mammalian factor Xa-Va complex (Rao and Kini, 2002). Group D activators have been purified from venoms of *Notechis* sp. (e.g. notecarin) (Tans *et al.*, 1985) and *Tropidechis carinatus* (e.g. trocarin) (Joseph *et al.*, 1999).

Russell's viper (*Daboia russelii*) venom is a potent procoagulant venom containing factor V and X activators (Schiffman *et al.*, 1969) and metalloproteinase (Mukherjee, 2008) resulting in consumptive coagulopathy and bleeding (Ariaratnam *et al.*, 2009). The clotting activity of prothrombin activators is inhibited by reversible serine proteinase inhibitors e.g. aprotinin, and reducing agents such as hirudin, heparin and EDTA (Stocker *et al.*, 1994).

3.7.2 Hypotension and sudden cardiovascular collapse

The venom of the Gaboon viper (*Bitis gabonica*) has been reported to induce vasodilation, resulting in a fall in peripheral resistance, followed by a progressive reduction in stroke volume due to a cardiotoxic component (Adams *et al.*, 1981). In an isolated heart experiment, venom of *Bitis nasicornis* increased left ventricular pressure (LVP), pacemaker activity and heart rate (Alloatti *et al.*, 1991), suggesting changes in $[Ca^{2+}]_i$ movement and disruption of ion conductance during the sino-atrial node action potential.

Sudden cardiovascular collapse following envenoming by Australasian elapids has been suggested to be due to coronary occlusion induced by some snake prothrombin activators (Tibballs, 1998; Tibballs *et al.*, 1989; Tibballs *et al.*, 1992). However, this is controversial since thrombus formation at multiple sites would be expected to result from prothrombin activator activity. It has been suggested that the hypotensive or anaphylactic effects observed following snake envenoming may involve venom-induced release of depletable vasoactive compounds (e.g. bradykinin, histamine) (Myint *et al.*, 1985).

3.7.3 Mediators and venom components affecting vasculature

Histamine is released from mast cells during anaphylactic hypersensitivity and occurs mostly in the lung, the skin and, in particular high concentrations, in the gastrointestinal tract. At the cellular level, histamine is largely found in mast cells and basophils and is also inhibited by heparin (Carr, 1979). Four histamine receptors have been classified i.e. $H_{1\text{-}4}$. H_1 receptor is responsible for vasodilation and regulation of general smooth muscle contraction, except in blood vessels. Mepyramine is an antagonist for H_1 receptor (Carlson *et al.*, 2000). The H_2 receptor has a major role in cardiac stimulation and gastric secretion. The H_2 receptor antagonist, ranitidine, has been reported to attenuate tachycardia following the administration of *Bitis nasicornis* venom (Alloatti *et al.*, 1991). The H_3 receptor is involved in inhibition of transmitter release from presynaptic neurons in brain and myenteric plexus, while the H_4 receptor is implicated in the production of inflammatory and allergic mediators on eosinophil and neutrophils (Coruzzi *et al.*, 2007).

It has been reported that metalloproteinase from *Vibrio vulnificus* venom (Miyoshi *et al.*, 2003), as well as venom and PLA_2 from the cobra (*Naja* spp.), mediate degranulation of rodent mast cells and promote histamine release (Damerau *et al.*, 1975; Dutta and Narayanan, 1952; Wang and Teng, 1989). Atrahagin, a metalloproteinase from Chinese cobra (*Naja atra*) venom has also been shown to cause histamine release from human mast cells (Wei *et al.*, 2006).

Formation of ***bradykinin*** is initiated by prekallikrein, an inactive precursor of the proteolytic enzyme 'kallikrein' in plasma. Bradykinin causes vasodilation and increases vascular permeability due to PGI_2 and NO release. There are two major types of bradykinin receptors, B1 and B2 receptors which are blocked by [Leu^8]-bradykinin $_{1\text{-}8}$ (Seabrook *et al.*, 1995) and Icatibant (HOE140, pA_2,9) (Cole and Lundquist, 2013), respectively.

Serotonin (5-hydroxytryptamine or 5-HT) is a monoamine neurotransmitter in the wall of the intestine and in the CNS (Trakhtenberg and Goldberg, 2012). In the vasculature, 5-HT activates $5\text{-}HT_{2A}$-receptors to induce vasoconstriction (Morecroft *et al.*, 1999). 5-HT also mediates the release of NO from endothelial cells and induces vasodilation via activation of $5\text{-}HT_1$ and $5\text{-}HT_{2B}$

receptors (Glusa and Pertz, 2000; Glusa and Roos, 1996). Venom of the imperial cone snail, *Conus imperialis* contains substantial level of serotonin which may produce prolonged skeletal muscle paralysis (McIntosh *et al.*, 1993). The venom of *Vipera lebetina* induces vasodilation and inflammation in mouse hind paw which might be due to 5-HT release (Sebia-Amrane and Laraba-Djebari, 2013).

Natriuretic peptides (NPs) regulate electrolyte balance and vascular smooth muscle tone. 'The Natriuretic peptide' was named due to its effects on sodium excretion in the urine via an effect on the kidney and increased production of urine (Vink *et al.*, 2012). Mammalian NPs have been classified into three different types: the A-type or atrial natriuretic peptide (ANP), the B-type or brain natriuretic peptide (BNP), and the C-type natriuretic peptide (CNP) (Vink *et al.*, 2012). All NPs contain a 17-residual ring structure and induce pharmacological effects by activating membrane-bound receptors (Vink *et al.*, 2012). Physiological effects of ANP and BNP include natriuresis, vasodilation, hypotension and inhibition of renin-angiotensin and aldosterone cascades resulting in a decrease in the mechanical load on the heart (Koh and Kini, 2012). NPs also facilitate the vagal reflex to slow heart rate in response to a rapid increase in blood pressure (Ackermann *et al.*, 1988; Thomas *et al.*, 2002). Several NPs have been purified from snake venoms. Dendroaspis natriuretic peptide (DNP) (isolated from *Dendroaspis angusticeps* venom) was the first reptilian NP (Schweitz *et al.*, 1992). DNP gives rise to an increase in urinary and plasma cGMP (Johns *et al.*, 2007). PNP isolated from the venom of Iranian viper (*Pseudocerastes persicus*), induces an increase in urine flow, sodium excretion and reduction in blood pressure (Amininasab *et al.*, 2004). Three natriuretic-like peptides (TNP-a-c) have been purified from inland taipan venom (*Oxyuranus microlepidotus*) (Fry *et al.*, 2005).

Bradykinin potentiating peptide(s) (BPPs) were first discovered in the venom of the South American pit viper (*Bothrops jararaca*). BPPs have inhibitory effects on angiotensin converting enzyme (ACE) and also potentiate the action of bradykinin (Ferreira *et al.*, 1970). These peptides promote inhibition of bradykinin breakdown, activation of local bradykinin release (Hecker *et al.*, 1994) and also inhibit the synthesis of angiotensin II. The ACE inhibitor captopril, an

antihypertensive medication, was developed base on the structure of snake BPPs (Camargo *et al.*, 2011). BPPs have been purified from viperid and crotalid venoms e.g. *Bothrops jararaca*, *Bothrops neuwiedi, Agkistrodon blomhoffi, Agkistrodon halys pallas*, *Agkistrodon piscivorus* and *Crotalus atrox*. Moreover, scorpion and spider venoms also contain bradykinin potentiating peptide activity (Sosnina *et al.*, 1990; Verano-Braga *et al.*, 2008).

L-type Ca^{2+}-channel blockers have been primarily isolated from mamba venoms. Calciseptine (de Weille *et al.*, 1991) and FS2 toxin (Strydom, 1977) were identified in black mamba (*Dendroaspis polylepis*) venom (Koh and Kini, 2012). The hypotensive effect of both toxins is more marked than that of nifedipine (Watanabe *et al.*, 1995). Calciseptine and FS2 toxins contain 60 amino acid residues and structural similarity to three-finger toxins (Joseph *et al.*, 2004).

Isolated PLA_2s from snake venoms generate hypotensive effects by either a direct action on vascular smooth muscle or by indirect mechanisms such as the release of vasoactive mediators e.g. prostaglandins (Ho and Lee, 1981), lipoxygenase metabolites and cGMP (Huang and Lee, 1985). The hypotensive effects of snake PLA_2 can be blocked by indomethacin (a non-selective cyclooxygenase inhibitor), nordihydroguaiaretic acid (NDGA, a lipoxygenase inhibitor) and methylene blue (cGMP inhibitor) (Duan *et al.*, 2003). Isolated PLA_2s from *Vipera russelli* (Huang, 1984), *Notechis scutatus* (Francis *et al.*, 1993) and *Naja mossambica* (Cicala and Cirino, 1993) venoms have been shown to induce a hypotensive effect in anaesthetized animals.

3.8 Effects of snake venom on vascular smooth muscle

As mentioned above, snake venoms can have either a direct or indirect effects on vascular smooth muscle cells. As such, it is important to understand the key mediators responsible for maintaining vascular tone.

3.8.1 Smooth muscle contraction

Vasoconstrictors such as noradrenaline or angiotensin II evoke depolarization of the smooth muscle cells by increasing the membrane permeability to cations (Na^+ and Ca^{2+}) which

leads to an increase in the opening of VOCCs and further Ca^{2+} influx (Zamorano *et al.*, 1995). In smooth muscle cells, the SR is a major intracellular storage site for Ca^{2+}. Activation of receptors which mediate vasoconstriction (e.g. α-receptors, endothelin receptors) stimulates membrane-bound phospholipase C (PLC) to release inositol trisphosphate (IP3) into the cytoplasm (Figure 9). IP3 activates receptors on the SR, resulting in the release Ca^{2+} into the cytoplasm. A rise in $[Ca^{2+}]_i$ activates myosin-light-chain kinase (MLCK) and induces phosphorylation of myosin causing smooth muscle contraction (He *et al.*, 2011).

3.8.2 Smooth muscle relaxation

Smooth muscle relaxation is induced by mechanisms involving cAMP or cGMP activated protein kinases (PKA and PKG), activation of K^+ channels, activation of Ca^{2+} extrusion pumps (in the SR and plasma membranes) and/or suppression of the sensitivity of contractile apparatus to Ca^{2+}. A reduction in $[Ca^{2+}]_i$ is also facilitated by hyperpolarization following the opening of K^+ channels that subsequently inactivate the opening of VOCCs (Figure 9). Vascular endothelial cells produce a variety of vasodilator substance i.e. NO, PGI_2 and endothelium-derived hyperpolarizing factor (EDHF) (Busse *et al.*, 2002).

Nitric oxide

In large arteries (e.g. aorta, cerebral artery, basilar artery), NO is a major endothelial vasodilator. In smooth muscle cells, NO generates an increase in cGMP through guanylate cyclase (GC). Activation of cGMP-dependent protein kinase (PKG) inhibits vascular contraction via several mechanisms. Intracellular free Ca^{2+} is reduced by Ca^{2+} efflux from smooth muscle cells and uptake into the ER, causing smooth muscle relaxation (Somlyo and Somlyo, 1994). NO also mediates vascular relaxation by inducing hyperpolarization of smooth muscle cells (Tare *et al.*, 1990) through the opening of K^+ channels and BK_{ca} channels either directly or via second messengers. The production of NO is inhibited by NOS inhibitors (e.g. L-NAME; N^G-nitro-L-arginine methylester or L-NMNA; N^G-monomethyl-L-arginine).

Prostacyclin (PGI_2)

PGI_2 is an arachidonic acid (AA) metabolite and capable of inducing vasodilation through the activation of PGI_2 receptors (Zhou *et al.*, 2006). PGI_2-induced vasodilation is dependent upon the expression of specific PGI_2-receptors on the smooth muscle (Coleman *et al.*, 1994; Furchgott and Zawadzki, 1980). PGI_2 receptors are coupled to adenylate cyclase (AC), which elevates cAMP to activate cAMP-dependent protein kinase (PKA). PKA inhibits smooth muscle contraction by stimulation of Ca^{2+} pumps to increase Ca^{2+} extrusion from the cell and uptake of Ca^{2+} into the SR (Bukoski *et al.*, 1989), both mechanisms reduce free Ca^{2+} concentration in the smooth muscle cells. Furthermore, desensitization of the contractile apparatus to Ca^{2+} (by cAMP-kinase-mediated phosphorylation of MLCK) also contributes to PGI_2-induced vascular relaxation (Nishimura and van Breemen, 1989) (Figure 9).

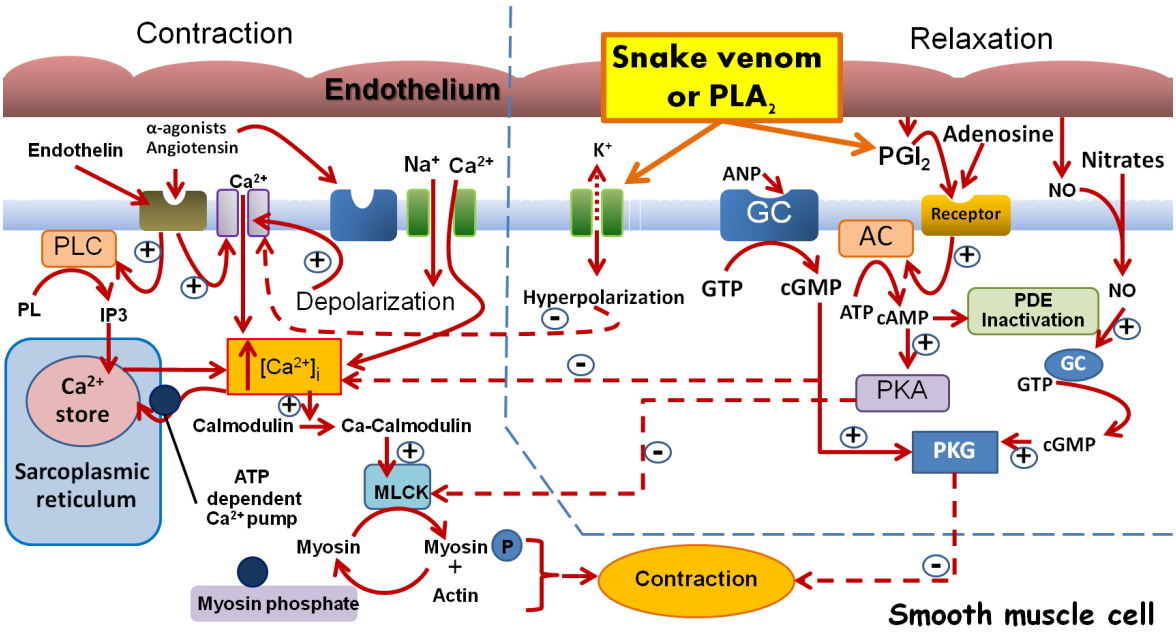

Figure 9: *Schematic diagram demonstrating regulation of vascular smooth muscle. Modified from Rang et al. (1999). (AC = adenylate cyclase; GC = guanylate cyclase; MLCK = myosin-light-chain kinase; cAMP = cyclic adenosine monophosphate; PKA = cAMP-dependent protein kinase; PKG = cGMP-dependent protein kinase; PLC = phospholipase C; NO = nitric oxide; PGI_2 = prostacyclin; ANP = atrial natriuretic peptide; ATP = adenosine triphosphate; PDE = phosphodiesterase).*

Endothelium-derived hyperpolarizing factor (EDHF)

EDHF is another important contributor to endothelium-dependent vasodilation in small arteries and arterioles. The relaxant effect of EDHF has been explored after inhibition of NO and prostaglandin synthesis (Sandow, 2004). EDHF induces relaxation by activation of K^+ channels on endothelial and/or vascular smooth muscle cells causing membrane hyperpolarization (Delaey *et al.*, 2007). The membrane hyperpolarization inhibits the opening of VOCCs (Nelson *et al.*, 1990) and decreases PLC activity (Itoh *et al.*, 1992). The relaxation induced by EDHF is blocked by apamin from bee venom or ChTX from scorpion venom (McGuire *et al.*, 2001; Sandow, 2004).

4. Australasian elapid antivenoms

Antivenoms are mainstay therapeutics for the treatment of systemically envenomed patients. In Australia, CSL Ltd (Melbourne, Victoria) is the only manufacturer of antivenoms. Antivenoms are produced by repetitive injection of venom into animals (usually horses or sheep). Therefore, they are polyclonal antibody mixtures with an affinity for the different antigenic components in the venom (Isbister, 2010). Antivenoms are classified as (1) monovalent antivenoms, which are prepared by the injection of venom from only one species of snake or (2) Polyvalent antivenoms which are produced by the injection of different snake venoms. Monovalent antivenoms have a low volume of specific antibodies for the snake species involved. Polyvalent antivenoms are more cost-effective to manufacture and are a better option for snakebite patients in order to minimize the problem of incorrect antivenom application due to diagnostic error (O'Leary and Isbister, 2009). However, polyvalent antivenom requires a larger volume and may increase the risk of adverse reactions (O'Leary and Isbister, 2009). Antivenom administration is necessary when the presence of systemic envenoming (e.g. coagulation, paralysis) is observed.

5. Purification of toxins from Australasian elapid venoms

High performance liquid chromatography (HPLC) is widely used for the purification of snake venoms components. Both single step and multiple step processes have been successfully employed to isolate and purify toxins, with the methodology required being dependent on the complexity of the structure of the toxin and the quantity of closely eluting components. Where the molecular weight of the toxin is known or can be predicted, isolation of the toxin can often be accomplished using size-exclusion HPLC, e.g. the purification of snake pre-synaptic neurotoxins (Blacklow *et al.*, 2010a; Kuruppu *et al.*, 2005b). The subunits of snake pre-synaptic neurotoxins are often held together by non covalent interactions which dissociate under ion exchange or reverse-phase HPLC resulting in loss or reduction of activity (Kuruppu *et al.*, 2008).

The purification of post-synaptic neurotoxins or PLA_2 myotoxins can be performed with multiple 'runs' under reverse phase HPLC, a purification technique based on the hydrophobic/hydrophilic properties of the venom components (Hart *et al.*, 2005; Kornhauser *et al.*, 2010). Highly predictable elution times of similar chemical compounds under reverse phase HPLC can be obtained by using the same solvents and consistent gradient conditions (Barber *et al.*, 2013).

A combination of gel filtration and hydroxyapatite chromatography was used to purify snake prothrombin activators (Rao and Kini, 2002). Hydroxyapatite chromatography is widely used for the purification of complex compounds such as recombinant proteins or DNA fragments (Dattolo *et al.*, 2010). This is a complicated methodology which separates proteins based on heterogeneous functional groups present on the crystal surface in the hydroxyapatite column (McCue *et al.*, 2007).

6. Project aims

The neurotoxic and cardiovascular effects following envenoming by some Australasian elapid snakes remain significant health issues given the potentially life-threatening effects. Moreover, a number of studies have shown that some snake PLA_2 enzymes also cause cytotoxicity.

Although numerous toxins responsible for snake venom-induced neurotoxicity have been isolated and characterized, those responsible for early cardiovascular collapse are largely unknown. In addition, the cellular mechanisms behind these important clinical effects have not been fully elucidated.

Therefore, the objectives of this project are to:

1. identify the pre-synaptic neurotoxins which play a significant role in the irreversible neurotoxicity induced by death adder (*Acanthophis* spp.) venoms
2. investigate the myotoxic and cytotoxic activities of death adder and taipan (*Oxyuranus* spp.) venoms and their isolated pre-synaptic neurotoxins
3. investigate the pathology of sudden cardiovascular collapse following taipan and brown snake (*Pseudonaja* spp.) envenoming
4. determine the mechanism behind the 'self protective' effect of elapid venoms (i.e. how incrementally increasing the dose of venom prevents sudden cardiovascular collapse)

This thesis will provide insight into the development of progressive neurotoxicity, cytotoxicity and cardiovascular collapse following envenoming by Australasian elapid snakes. The cellular mechanisms behind these effects, and the prevention of those toxicities will be investigated. The outcomes of the study will contribute to a better understanding of the mechanism behind significant outcomes and clinical approaches following envenoming by Australasian elapids.

CHAPTER 2

ISOLATION AND CHARACTERIZATION OF PRE-SYNAPTIC NEUROTOXINS FROM THE VENOMS OF NORTHERN AND IRIAN JAYAN DEATH ADDERS

Monash University
Declaration for Thesis Chapter 2

This chapter is made up of the following publication and additional work undertaken but not included in the manuscript.

Declaration by candidate

In the case of Chapter 2, the nature and extent of my contribution to the work was the following:

Nature of Contribution	Extent of Contribution (%)
I declare that experiments and writing of this manuscript were solely undertaken by the myself, taking into consideration the advice and recommendations of co-authors	85%

The following co-authors contributed to the work.

Name	Nature of Contribution
Dr Nicki Konstantakopoulos	Development of ideas, manuscript preparation
Professor Ian A Smith	Provision of expertise and facilities
Professor Wayne C Hodgson	Development of ideas, manuscript preparation

Candidate's signature: [redacted] Date: 07.06.2013

Janeyuth Chaisakul

Declaration by co-authors
The undersigned hereby certify that:
(1) the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors;
(2) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(3) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(4) there are no other author of the publication according to these criteria;
(5) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journal or other publications and (c) the head of the responsible academic unit; and
(6) the original data are stored at the following location (s) and will be held for at least five years from the date indicated below:

Location (s) Department of Pharmacology, Monash University

		Date
Dr Nicki Konstantakopoulos	[redacted]	06.06.2013
Professor Ian A Smith	[redacted]	04.06.2013
Professor Wayne C Hodgson	[redacted]	06.06.2013

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Isolation and characterisation of P-EPTX-Ap1a and P-EPTX-Ar1a: Pre-synaptic neurotoxins from the venom of the northern (*Acanthophis praelongus*) and Irian Jayan (*Acanthophis rugosus*) death adders

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ABSTRACT

The neurotoxicity observed following death adder envenoming has been thought to be solely due to the presence of potent post-synaptic neurotoxins. Clinically, these effects are often poorly reversed by death adder antivenom or anticholinesterase, particularly when patients present with established paralysis. This suggests that either the post-synaptic neurotoxins are irreversible/'pseudo' irreversible, or the venom contains pre-synaptic neurotoxins that do not respond to antivenom. To support the later hypothesis, a pre-synaptic neurotoxin (P-EPTX-Aa1a) has recently been isolated from the venom of *Acanthophis antarcticus*. We examined *Acanthophis praelongus* and *Acanthophis rugosus* venoms for the presence of pre-synaptic neurotoxins. P-EPTX-Ap1a (40,719 Da) and P-EPTX-Ar1a (40,879 Da) were isolated from *A. praelongus* and *A. rugosus* venoms, respectively. P-EPTX-Ap1a and P-EPTX-Ar1a are comprised of three different subunits, α, β1 and β2. The two toxins displayed similar levels of PLA_2 activity which was almost solely attributed to the α subunit in both toxins. P-EPTX-Ap1a (20–100 nM) and P-EPTX-Ar1a (20–100 nM) caused inhibition of indirect twitches of the skeletal muscle preparation without affecting contractile responses to nicotinic receptor agonists. Interestingly, only the α subunit of both toxins (300 nM) displayed neurotoxic activity. Inhibition of PLA_2 activity markedly reduced the effect of the toxins on muscle twitch height. These results confirm that P-EPTX-Ap1a and P-EPTX-Ar1a are pre-synaptic neurotoxins and represent the second and third such toxins to be isolated from death adder venom. The presence of pre-synaptic neurotoxins in *Acanthophis* sp. venoms indicates that treatment strategies for envenoming by these snakes needs to be reassessed given the likelihood of irreversible neurotoxicity.

1. Introduction

Death adders (*Acanthophis* genus) are found throughout Australia, the Torres Straight Islands, Papua New Guinea, Irian Jaya, and the Indonesian islands; Seram, Halmahera, Obi and Tanimbar. Although belonging to the Elapidae family, they resemble snakes of the Viperidae family in appearance and habit [1]. They are characterized by a somewhat flattened, almost rectangular head and thick muscular bodies ending in a thin rat-like tail. Death adder envenomings are a rare occurrence in Australia however these are still a significant health problem in Papua New Guinea [2–4]. Clinical symptoms of envenoming by *Acanthophis* sp. include paralysis of extra ocular muscles, abdominal pain, headache, drowsiness, enlargement of regional lymph nodes and death occurs through respiratory failure from paralysis of the voluntary muscles [1,5]. Myotoxicity is another important symptom of envenoming by some death adders. Two clinical studies reported myotoxic activity following the envenomings by *Acanthophis* sp. in Papua New Guinea and the northern death adder (*Acanthophis praelongus*) [5,6]. Patients developed renal failure and displayed elevated creatine kinase levels, indicating rhabdomyolysis and the presence of myotoxic activity in the venom [7].

The neurotoxicity observed following envenoming by *Acanthophis* sp. has been widely accepted to be due to the presence of post-synaptic neurotoxins, this has been supported by the fact that the neurotoxicity can be reversed by administration of antivenom or anticholinesterase [8]. However, there have been reports some envenomed patients have responded poorly to antivenom and

Abbreviations: ACh, acetylcholine; BSA, bovine serum albumin; CCh, carbachol; MALDI-TOF, matrix-assisted laser desorption ionization time-of-fight; PLA_2, phospholipase A_2; P-EPTX-Aa1a, P-elapitoxin-Aa1a; RP-HPLC, reverse-phase high-pressure liquid chromatography.

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neostigmine [9]. This suggests that either the α-neurotoxins may not be completely reversible, or that the venom contains pre-synaptic neurotoxins (i.e. β-neurotoxin) which, due to their mode of action, are unresponsive to anticholinesterase and antivenom.

Pre-synaptic neurotoxins act at the motor nerve terminal to either facilitate (e.g. dendrotoxin) or inhibit (e.g. β-bungarotoxin, taipoxin and paradoxin) the release of neurotransmitter resulting in dysfunction of transmission at the neuromuscular junction [10]. Numerous pre-synaptic neurotoxins have been isolated from the venom of the major families of venomous snakes (i.e. Crotalidae, Elapidae and Viperidae). Australian snake (i.e. Elapids) venoms contain PLA_2 enzymes, which differ in enzymatic activity and pharmacological effects. These include textilotoxin from the Australian brown snake (*Pseudonaja textilis*) which contains five subunits [11], taipoxin, from the Australian coastal taipan (*Oxyuranus scutellatus*) which contains three subunits [12] and notexin, from the Australian tiger snake (*Notechis scutatus scutatus*) which is a single chain PLA_2 [13].

Previously we showed that *Acanthophis rugosus* (Irian Jayan death adder) and *A. praelongus* venoms caused time-dependent inhibition of indirect twitches and blocked contractile response to exogenous acetylcholine and carbachol [14]. Thus, suggesting the presence of post-synaptic neurotoxins.

In terms of PLA_2 components, acanthin I and II, both potent inhibitors of platelet aggregation have been isolated from *A. antarcticus* venom [15]. In addition, acanthoxin A_1 and A_2, two PLA_2 isoforms with weak neurotoxic activity, have been isolated from *A. antarcticus* venom [16]. Three PLA_2 isoenzymes, praelongins 2bIII, 2cII and 2cIV, with antiplatelet activity have also been isolated from *A. praelongus* venom [17]. Acanmyotoxin-1, the first death adder PLA_2 myotoxin was isolated from *A. rugosus* [18]. Subsequently, acanmyotoxin-2 and acanmyotoxin-3 were isolated from the venom of *A.* sp. Seram [19].

Recently, P-EPTX-Aa1a [20], a pre-synaptic PLA_2 neurotoxin was isolated from the venom of the common death adder (*A. antarcticus*). P-EPTX-Aa1a, which displayed modest PLA_2 activity, inhibited nerve-evoked contractions in a skeletal muscle preparation without affecting responses to cholinergic agonists. This is the first pre-synaptic neurotoxin isolated from a death adder venom.

A subsequent study examining the HPLC profiles of 9 death adder venoms including 4 geographical variants of *A. antarcticus* venoms (i.e. New South Wales, Queensland, South Australia and Western Australia) identified a high molecular weight component, which corresponded to the elution time for P-EPTX-Aa1a, in seven of the nine venoms, including *A. praelongus* and *A. rugosus*. Interestingly, the venoms of *A. wellsi* and *A. pyrrhus* appeared to be devoid of this component [21]. The presence of a pre-synaptic toxin would explain the clinical outcomes of patients envenomed by some species of death adders who do not respond adequately to anticholinesterases or antivenom.

The aim of this study was to isolate and characterise the PLA_2 pre-synaptic neurotoxins from *A. praelongus* and *A. rugosus* venoms. It is important to understand the composition and mechanism of action of these venom components so that appropriate treatments can be administered to envenomed patients.

2. Materials and methods

2.1. Venom preparation

Freeze-dried venoms were gifted from Venom Supplies (Venom Supplies Pty Ltd., Tanunda, SA, Australia). Venom was dissolved in Milli-Q water and insoluble material was removed by centrifugation at 16,000 × *g* at 4 °C for 8 min.

2.2. Fractionation of venom

All chromatography separations were performed using a Shimadzu (Kyoto, Japan) high-performance liquid chromatography system (LC-10ATvp pump and SPD-10AVP detector).

2.2.1. Size exclusion chromatography

Venom (3 mg) was applied to a Superdex G-75 column (13 μm; 10 mm × 300 mm; GE Healthcare, Buckinghamshire, UK) equilibrated with ammonium acetate buffer (0.1 M; pH 6.8). The sample was eluted at a flow rate of 0.6 ml/min. The eluant was monitored at 280 nm. The fractions were collected and freeze-died prior to being screened for neurotoxicity as per Section 2.3.

2.2.2. Reverse phase-high performance liquid chromatography

The active freeze-dried, purified component (40 μg) obtained as described in Section 2.2.1 was reconstituted in Milli-Q water and applied to a Phenomenex (Torrance, CA, USA) Jupiter analytical C18 column (150 mm × 2 mm; 5 μm; 300 Å) after equilibrating with solvent A (0.1% trifluoroacetic acid (TFA)). The sample was eluted with the following gradient conditions of solvent B (90% acetonitrile in 0.09% TFA): 0–20% for 0–5 min (4% gradient), 20–60% for 5–45 min (1% gradient), and then 60–80% for 45–50 min (4% gradient) at a flow rate of 0.2 ml/min. The eluant was monitored at 280 and 214 nm and freeze-dried.

2.3. Chick biventer cervicis nerve–muscle preparation

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

Motor nerves within the biventer were stimulated every 10 s (0.2 ms duration) at supramaximal voltage using a Grass S88 stimulator. *d*-Tubocurarine (10 μM) was added and the subsequent abolition of twitches confirmed the selective stimulation of nerves. Responses to nerve stimulation were re-established by thorough washing. Contractile responses to acetylcholine (ACh; 1 mM for 30 s), carbachol (CCh; 20 μM for 60 s) and potassium chloride (KCl; 40 mM for 30 s) were obtained in the absence of stimulation. The preparations were then equilibrated for at least 30 min with continuous nerve stimulation (as described above) before the addition of toxin. In all experiments, toxin (20–100 nM), subunits (300 nM) or venom (10 μg/ml) was left in contact with the preparation until responses to nerve stimulation were abolished or for a maximum of 4 h if total twitch blockade did not occur. In separate experiments, $MgCl_2$ (5 mM) was added to suppress twitch contraction to approximately 30% for at least 30 min prior to the addition of toxin (100 nM). At the conclusion of the experiment, responses were measured via a Grass force displacement transducer (FT03) and recorded on a MacLab System.

Given the 'all or nothing' nature of the failure of neurotransmission which occurs in the skeletal muscle preparation in the presence of snake venoms/toxins, the time taken to reduce the amplitude of the indirect twitches by 90% (t_{90}) was calculated to provide a quantitative measure (i.e. potency) of the neurotoxins [22]. This parameter enables comparison of the potency of previously published post- and pre-synaptic neurotoxins [22,23]. Where indicated, the PLA_2 activity of the toxin was inhibited by alkylation with 4-bromophenacylbromide (4-BPB). Toxin (20 nM), made up in sodium cacodylate–HCl buffer (0.1 M; pH 6.0), and 4-BPB, made up in acetone, were added to produce a

final concentration of 1.8 mM. The mixture was incubated for 16 h at 30 °C and examined for neurotoxic activity as above.

2.4. Molecular mass determination of P-EPTX-Ap1a and P-EPTX-Ar1a

The molecular mass of the complex was determined by gel filtration on a Superdex G-75 column equilibrated with ammonium acetate buffer (0.1 M; pH 6.8). The column was calibrated with the series of known standard (6500–66,000 Da) using the molecular weight marker kit for gel filtration chromatography (MW-GF-70; Sigma–Aldrich, St. Louis, MO, USA). The eluant was monitored at 280 nM, and a flow rate of 0.6 ml/min was used. Void volume (V_o) of the column was determined by running blue dextran, and the elution volume (V_e) was calculated for each molecular weight marker before injecting the purified component (0.5 mg). The molecular weight of the toxin was determined from a plot of log (mol. wt.) versus V_e/V_o ratio.

2.5. Mass spectrometry

MALDI-TOF MS analysis was performed using an Applied Biosystem (Foster City, CA, USA) 4700 ToF ToF Proteomics Analyser. The instrument was operated in positive polarity in linear mode using sinapinic acid matrix (Agilent Technologies; Palo Alto, CA, USA) for low-resolution protein analysis. Matrix (1 μl) was spotted on the sample plate and allowed to air-dry; sample (1 μl) diluted in acetonitrile/water (1:1) containing 0.1% (v/v) formic acid was subsequently spotted on dried matrix and allowed to air-dry. Data from 500 laser shots (337-nm nitrogen laser) were collected, and the signal was averaged and processed with the instrument manufacturer's 4000 series Data Explorer software.

2.6. Determination of PLA_2 activity

PLA_2 activity of the toxin and toxin subunits was determined using a secretory PLA_2 colourmetric assay kit (Cayman Chemical; MI, USA) according to manufacturer's instructions. This assay uses the 1,2-dithio analog of diheptanoyl phosphatidylcholine, which serves as a substrate for PLA_2 enzymes. Free thiols generated following the hydrolysis of the thio ester bond at the *sn*-2 position by PLA_2 are detected using DTNB (5,5′-dithio-bis-(2-nitrobenzoic acid)). Colour changes were monitored at 405 nm in a fusion α microplate reader (PerkinElmer; MA, USA), sampling every minute for a 5 min period. PLA_2 activity was expressed as micromoles of phosphatidylcholine hydrolyzed per minute per milligram of enzyme. For some samples, the PLA_2 activity of venom was inhibited by alkylation with 4-BPB (as above).

2.7. Chemical and drugs

The following drugs were used: ACh, BSA, CCh, *d*-tubocurarine, 4-BPB, ammonium acetate, acetone and cacodylic acid (Sigma Chemical Co., MO, USA); KCl, NaCl, $NaHCO_3$ and glucose (Ajax Finechem, New South Wales, Australia); TFA (Auspep, Victoria, Australia); acetonitrile, KH_2PO_4 and $CaCl_2$ (Merck, Darmstadt, Germany).

2.8. Analysis of results and statistics

Statistical analysis was performed using the Prism 5.0 software package (GraphPad Software; San Diego, CA, USA). Twitch height and contractile responses to agonists were expressed as a percentage of the corresponding value prior to the administration of toxins. For twitch height, statistical difference was determined by a one-way analysis of variance (ANOVA) of the change in twitch height at the 240 min time point followed by Tukey's multiple comparison test. Statistical significance was indicated when $P < 0.05$.

3. Result

3.1. Isolation and purification of pre-synaptic neurotoxins from A. praelongus and A. rugosus venoms

A. praelongus and *A. rugosus* venoms were fractionated by size exclusion chromatography on a Superdex G-75 column. The venom profile, of both species, indicated six to seven major peaks which were individually collected (Fig. 1a and b). Screening in the chick biventer cervicis nerve–muscle preparation confirmed pre-synaptic neurotoxic activity in the first peak of each venom which was subsequently named: P-EPTX-Ap1a (*A. praelongus*) and P-EPTX-Ar1a (*A. rugosus*) based on the suggested nomenclature of King et al. [24]. The samples were freeze-dried and then reconstituted in Milli-Q water. To verify the purity and determine the homogeneity and location of the isolated toxins, P-EPTX-Ap1a and P-EPTX-Ar1a were re-administered under the same conditions on the Superdex G-75 column (Fig. 1c and d). P-EPTX-Ap1a (8.97% of whole venom) and P-EPTX-Ar1a (7.25% of whole venom) eluted as clean peaks with retention time of 17.98 and 17.97 min (Table 1), respectively.

3.2. Separation of toxin subunits using reverse phase-high performance liquid chromatography

P-EPTX-Ap1a and P-EPTX-Ar1a were broken up into individual subunit using RP-HPLC on a Phenomenex Jupiter analytical column. Each toxin produced three peaks that eluded at different retention times. These components were collected for molecular weight determination by MALDI-TOF (see below) and analysis of PLA_2 activity (Fig. 1e and f). The subunits were designated as α, β1 and β2 based on their PLA_2 activity and neurotoxic effects (Table 1).

3.3. Molecular mass determination

3.3.1. Size exclusion chromatography in nondenaturing media

The molecular mass of P-EPTX-Ap1a and P-EPTX-Ar1a was determined by size exclusion chromatography on a Superdex G-75 column calibrated with the following protein standards: BSA (66,000 Da), carbonic anhydrase (29,000 Da), cytochrome c (12,400 Da), and aprotinin (6500 Da). The V_o of the column was determined as 8.17 ml using blue dextran (2,000,000 Da) and the V_e of P-EPTX-Ap1a and P-EPTX-Ar1a were each calculated as

Table 1
PLA_2 activity, retention times of isolated fractions/toxin subunits and molecular weights from MALDI-TOF.

Toxin/subunit	PLA_2 activity (μmol/min/mg)	Retention time (min)	Molecular weight (Da)
P-EPTX-Ap1a	101.7 ± 9.9	17.98[a]	40,719
β_1-P-EPTX-Ap1a	9.5 ± 0.6	20.93[b]	13,449
α-P-EPTX-Ap1a	220.4 ± 19.2	30.76[b]	13,804
β_2-P-EPTX-Ap1a	6.7 ± 1.1	33.9[b]	13,466
P-EPTX-Ap1a + 4-BPB	2.5 ± 0.3[**]	N/A	N/A
P-EPTX-Ar1a	115.2 ± 4.1	17.97[a]	40,879
β_1-P-EPTX-Ar1a	2.6 ± 0.5	21.24[b]	13,475
β_2-P-EPTX-Ar1a	32.1 ± 1.2	24.23[b]	13,592
α-P-EPTX-Ar1a	174.3 ± 14.3	31.08[b]	13,812
P-EPTX-Ar1a + 4-BPB	13.1 ± 1.5[**]	N/A	N/A

[a] Data from size exclusion HPLC.
[b] Data from reverse phase HPLC.
[**] $P < 0.05$, significantly different compared with P-EPTX-Ap1a and P-EPTX-Ar1a in the absence of 4-BPB, one-way ANOVA.

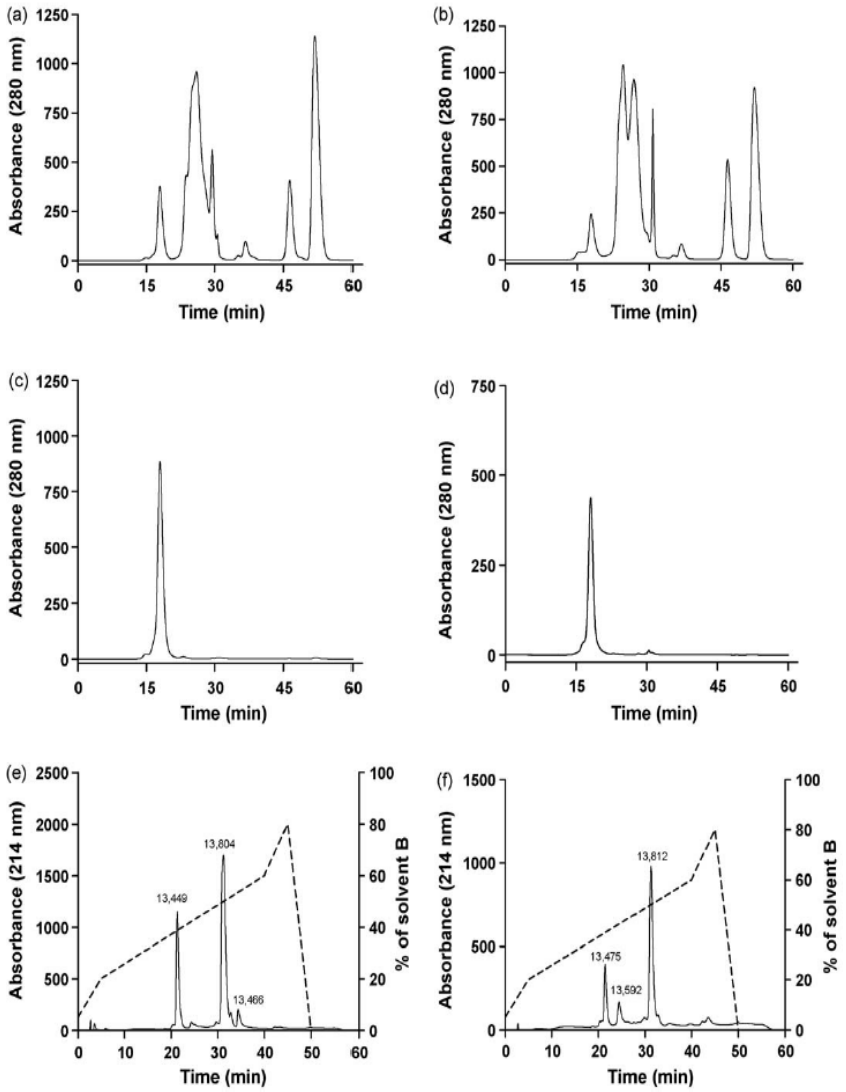

Fig. 1. Size exclusion chromatograph of (a) *A. praelongus* venom and (b) *A. rugosus*, (c) P-EPTX-Ap1a and (d) P-EPTX-Ar1a run on a Superdex G-75 column equilibrated with ammonium acetate (0.1 M; pH 6.8) at a flow rate of 0.6 ml/min. RP-HPLC chromatograph of (e) P-EPTX-Ap1a and (f) P-EPTX-Ar1a run on a Jupiter analytical C18 column equilibrated with solvent A (0.1% TFA) and eluted with the following gradient of solvent B (90% acetonitrile in 0.09% TFA) and solvent A: 0–20% for 0–5 min, 20–60% for 5–45 min and 60–80% for 45–50 min. Flow rate of 0.2 ml/min.

10.9 ml. The molecular mass of both toxins was determined from a plot of log mol. wt. versus corresponding V_e/V_o ratio (of the protein standards described above) to be approximately 41,000 Da (Fig. 2).

3.3.2. Mass spectrometry

The individual subunits of P-EPTX-Ap1a obtained in Section 3.2 were found to be of the following molecular mass by MALDI-TOF analysis: 13,449 Da (β_1), 13,804 Da (α) and 13,466 Da (β_2), while the subunits of P-EPTX-Ar1a were 13,475 Da (β_1), 13,592 Da (β_2) and 13,812 Da (α) (Table 1). The sum of the molecular masses of subunits resulted in a mass of 40,719 and 40,879 Da for P-EPTX-Ap1a and P-EPTX-Ar1a, respectively.

3.4. Chick biventer cervicis nerve–muscle preparation

3.4.1. Whole venom studies

Both *A. rugosus* and *A. praelongus* venoms (10 μg/ml; $n = 5$) abolished indirect twitches of the chick biventer cervicis nerve–muscle preparation (Fig. 3). The time taken to cause 90% inhibition (i.e. t_{90} values) was significantly less for the venom of *A. rugosus* (Table 2). Both venoms (10 μg/ml) abolished contractile responses to exogenous ACh (1 mM) and CCh (20 μM), but had no significant effect on responses to KCl (40 mM) (Fig. 3). Vehicle had no significant inhibitory effect on twitches or on the contractile responses to exogenous agonists ($n = 5$; one-way ANOVA, $P < 0.05$).

3.4.2. Isolated toxin studies

P-EPTX-Ar1a (20 and 100 nM) caused significant concentration-dependent decreases in twitch height compared to vehicle ($n = 5$–6; one-way ANOVA, $P < 0.05$; Fig. 4b). In contrast, P-EPTX-Ap1a (20 and 100 nM, $n = 5$) did not display a significant difference in the level of inhibition produced by the concentrations tested ($n = 5$; one-way ANOVA, $P < 0.05$; Fig. 4a). The time taken by P-EPTX-Ap1a (20 and 100 nM) to produce 90% inhibition of twitch height (i.e. t_{90}) was significantly shorter compared to P-EPTX-Ar1a at 20 nM (Table 2). Neither toxin had a significant inhibitory effect

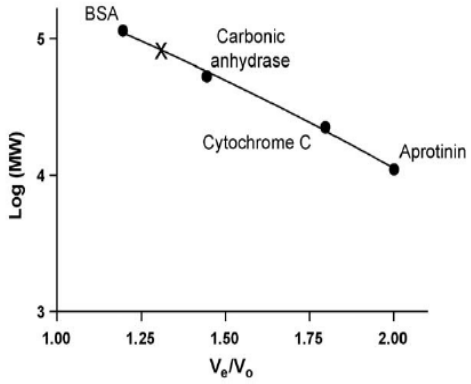

Fig. 2. Plot of log molecular weight versus V_e/V_o of a series of molecular weight standards run on a Superdex G 75 column equilibrated with ammonium acetate (0.1 M; pH 6.8) at a flow rate of 0.6 ml/min. V_o of the column was 8.17 ml. Cross indicates P-EPTX-Ap1a and P-EPTX-Ar1a.

on the tissue response to ACh, CCh, or KCl, indicating an action at the pre-synaptic nerve terminal (Fig. 4c).

The addition of Mg^{2+} (5 mM) to the physiological solution unmasked a triphasic effect of the toxins (100 nM) on twitch height. This was characterized by a transient inhibitory phase, followed by a transient facilitatory phase, prior to complete inhibition (Fig. 5).

The prior incubation of 4-BPB with either P-EPTX-Ar1a (20 nM) or P-EPTX-Ap1a (20 nM) prevented the complete inhibition of indirect twitches over the period of 4 h with 53 ± 12 and 30 ± 9% reduction in twitch response, respectively (Fig. 6). Both modified

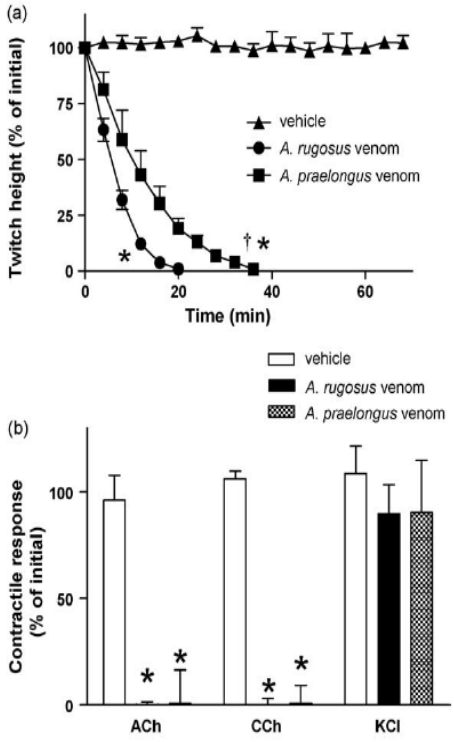

Fig. 3. Effect of *A. praelongus* (10 μg/ml; $n = 5$), *A. rugosus* venom (10 μg/ml; $n = 5$) or vehicle ($n = 5$) on (a) nerve-mediated twitches or (b) contractile responses to exogenous ACh, CCh and KCl in the chick isolated biventer cervicis nerve–muscle preparation. $^{\dagger}P < 0.05$, significantly different from *A. rugosus* venom 10 μg/ml, one-way ANOVA. $^{*}P < 0.05$, significantly different from vehicle, one-way ANOVA.

Table 2
Time to 90% inhibition (t_{90}) of twitch height as an indication of venom/toxin potency.

	t_{90} (min)
A. praelongus venom (10 μg/ml)	27.5 ± 1.9*
P-EPTX-Ap1a (20 nM)	96 ± 9.8**
P-EPTX-Ap1a (100 nM)	79.5 ± 8.0**
P-EPTX-Ap1a (20 nM) with 4-BPB	>240
P-EPTX-Ap1a (20 nM) with vehicle	100 ± 13.4
P-EPTX-Ap1a (100 nM) with Mg^{2+}	70.5 ± 1.9
A. rugosus venom (10 μg/ml)	12.4 ± 0.9
P-EPTX-Ar1a (20 nM)	132 ± 23.6
P-EPTX-Ar1a (100 nM)	79.6 ± 6.3**
P-EPTX-Ar1a (20 nM) with 4-BPB	>240
P-EPTX-Ar1a (20 nM) with vehicle	118.5 ± 14.3
P-EPTX-Ar1a (100 nM) with Mg^{2+}	69.5 ± 10.2

Values are mean ± s.e.m., $n = 4$–6.
* $P < 0.05$ compared to *A. rugosus venom* (10 μg/ml).
** $P < 0.05$ compared to P-EPTX-Ar1a (20 nM).

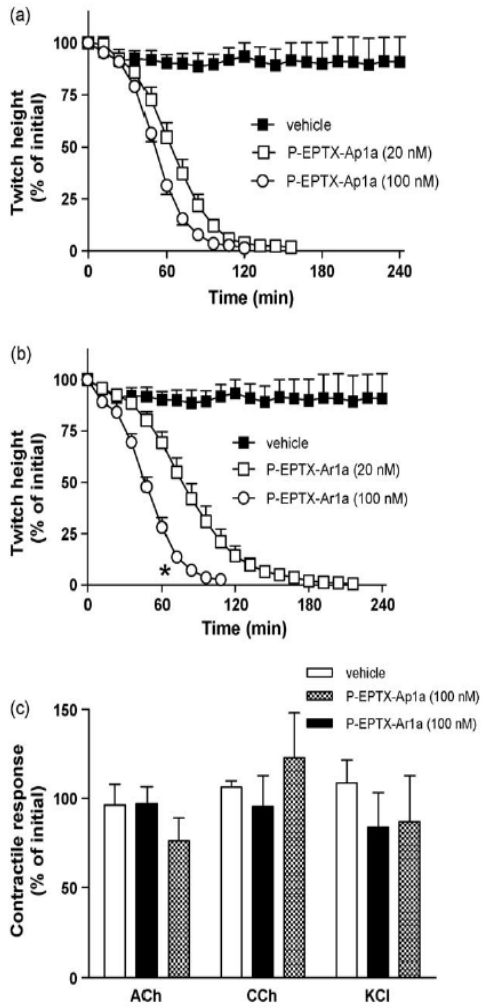

Fig. 4. The effect of (a) P-EPTX-Ap1a (20 and 100 nM, $n = 5$), (b) P-EPTX-Ar1a (20 and 100 nM, $n = 6$), or vehicle ($n = 5$) on nerve-mediated twitches in the chick isolated biventer cervicis nerve–muscle preparation. (c) Effect of P-EPTX-Ap1a (100 nM, $n = 5$), P-EPTX-Ar1a (100 nM, $n = 6$) or vehicle ($n = 5$) on contractile responses to exogenous ACh, CCh and KCl in the chick isolated biventer cervicis nerve–muscle preparation. $^{*}P < 0.05$, significantly different compared with toxin in the concentration of 20 nM, one-way ANOVA.

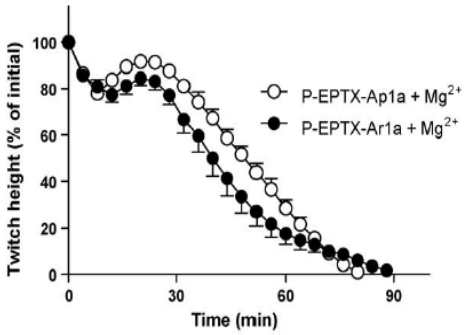

Fig. 5. The effect of P-EPTX-Ap1a or P-EPTX-Ar1a (100 nM), in the presence of Mg^{2+} (5 mM, $n = 4$), on nerve-mediated twitches in the chick isolated biventer cervicis nerve–muscle preparation.

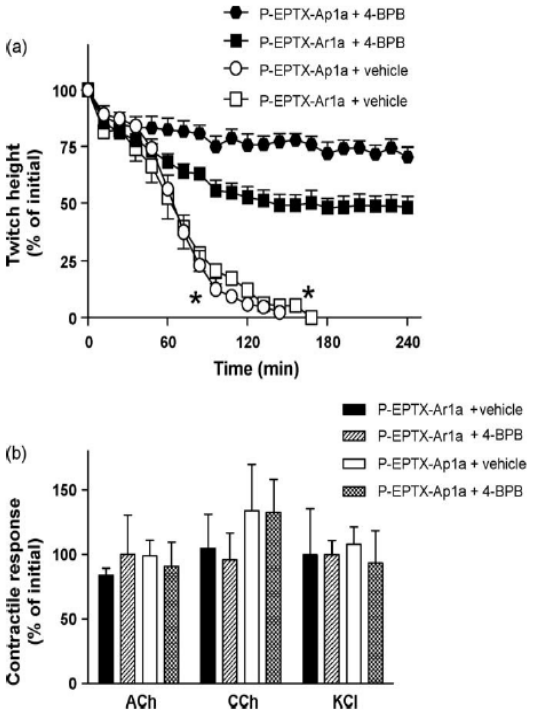

Fig. 6. The effect of P-EPTX-Ap1a or P-EPTX-Ar1a in the presence of 4-BPB (1.8 nM, $n = 6$) or vehicle ($n = 5$) on (a) nerve-mediated twitches or (b) contractile responses to exogenous ACh, CCh and KCl in the chick isolated biventer cervicis nerve–muscle preparation. $^{*}P < 0.05$, significantly different from corresponding toxin in the presence of 4-BPB, one-way ANOVA.

toxins did not affect the contractile responses to exogenous nicotinic receptor agonists.

3.4.3. Subunits of P-EPTX-Ar1a or P-EPTX-Ap1a

The β_1 and β_2 subunits (300 nM) of P-EPTX-Ar1a or P-EPTX-Ap1a had no significant inhibition on indirect twitches of chick biventer cervicis nerve–muscle preparation. However, the α subunits (300 nM) from both toxins significant inhibited twitch height compared with vehicle (Fig. 7).

3.5. Phospholipase A_2 activity

PLA_2 activity was detected in both P-EPTX-Ar1a and P-EPTX-Ap1a ($n = 4$). The positive control, bee venom showed a specific

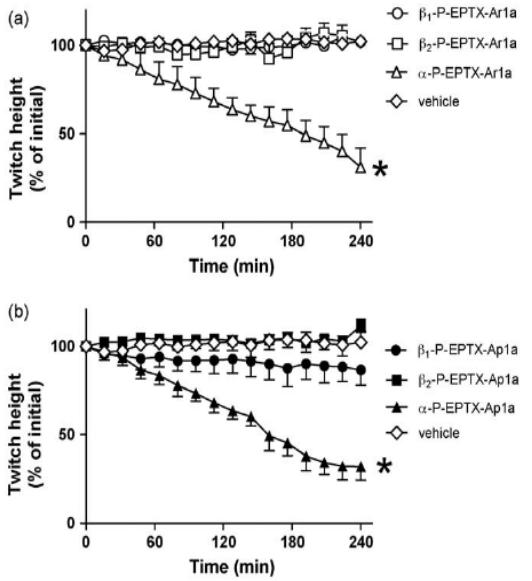

Fig. 7. Effect of α, $\beta 1$ and $\beta 2$ subunits of (a) P-EPTX-Ar1a or (b) P-EPTX-Ap1a (300 nM, $n = 3–4$) on nerve-mediated twitches. $^{*}P < 0.05$, significantly different from vehicle, one-way ANOVA.

activity of 309.8 ± 12.1 μmol/min/mg ($n = 4$). 4-BPB significantly inhibited the PLA_2 activity of P-EPTX-Ar1a and P-EPTX-Ap1a ($n = 4$). The α-subunit seems to be the only subunit with a significant level of activity in both toxins (Table 1).

4. Discussion

Whilst death adder neurotoxicity has been thought to be primarily due to the presence of reversible, competitive post-synaptic neurotoxins, clinical reports have indicated cases that responded poorly to treatment with antivenom and/or neostigmine [9]. This is surprising as it would be expected that, given their mode of action, these toxins would respond to this treatment protocol. However, a recent study has reported the characterisation and isolation of a pre-synaptic neurotoxin (i.e. P-EPTX-Aa1a) from the venom of the common death adder (*A. antarticus*) [20], a class of toxins that was previously thought to be absent from this genus. In addition, a recent study has indicated the likely presence of pre-synaptic neurotoxins in a number of species of death adder [21]. In the present study, we report the isolation and characterisation of pre-synaptic PLA_2 neurotoxins from the venoms of *A. praelongus* and *A. rugosus*.

Size exclusion chromatography, determined the molecular mass of P-EPTX-Ap1a and P-EPTX-Ar1a to be 41 kDa. This value corresponded well with the combined molecular masses of three subunits isolated from RP-HPLC determined by MALDI-TOF (i.e. 40,719 Da for P-EPTX-Ap1a and 40,879 Da for P-EPTX-Ar1a, respectively). In addition, the toxins display a similar molecular weight to that of P-EPTX-Aa1a, a heterotrimeric complex of 44,698 Da composed of three subunits, recently isolated from the venom of the common death adder [20]. It is well documented that elapid snake venom PLA_2 components usually have a molecular mass in the range of 12–14 kDa [17]. This was supported by MALDI-TOF analysis of the individual subunits for P-EPTX-Ap1a and P-EPTX-Ar1a which showed a range of mass from 13,449–13,812 Da. MALDI-TOF spectra did not indicate any areas of heterogenous glycosylation to aid identification of the γ subunit of both toxins. Thus, we labeled the two subunits with low PLA_2

activity as β1 and β2 subunit, respectively. The similar molecular mass of the α subunit of P-EPTX-Ap1a and P-EPTX-Ar1a is consistent with snake venom pre-synaptic PLA_2 neurotoxin components isolated from *A. antarcticus* [20] and *O. scutellatus canni* venoms [25].

Many elapid venom PLA_2 components are pre-synaptically neurotoxic [26]. Hence, P-EPTX-Ap1a and P-EPTX-Ar1a were examined for in vitro neurotoxicity using a skeletal muscle preparation. Both toxins inhibited indirect twitches but did not inhibit the contractile responses to exogenous ACh, CCh or KCl. This pharmacological profile indicates a pre-synaptic mode of action. Under conditions of reduced safety margin for neurotransmitter release, the reduction of indirect twitches by P-EPTX-Ap1a and P-EPTX-Ar1a was triphasic, characterized by an initial decrease and a transient increase followed by the complete inhibition of twitches. This triphasic effect is commonly observed with many other pre-synaptic neurotoxins, taipoxin, notexin, and β-bungarotoxin [27]. The initial two phases seem to be independent of PLA_2 activity [28], and are 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 [29].

P-EPTX-Ap1a, P-EPTX-Ar1a and their subunits were examined for PLA_2 activity. Studies from our laboratory have shown that the venom of *A. praelongus* possesses higher PLA_2 activity than that of *A. rugosus* [30]. In the current study, there was no significant difference between the PLA_2 activity of P-EPTX-Ar1a and P-EPTX-Ap1a indicating the difference observed between the whole venoms is likely to be due to the presence of other PLA_2 components. To test whether the PLA_2 activity of P-EPTX-Ap1a and P-EPTX-Ar1a was essential for their neurotoxic effects, we subjected the toxins to 4-BPB modification. Previous studies have shown that PLA_2 activity can be inhibited by selective alkylation of the His-48 residue using 4-BPB [31,32]. The complete block of indirect twitches of the chick preparation was significantly attenuated when P-EPTX-Ap1a and P-EPTX-Ar1a were incubated with 4-BPB prior to addition. However, a partial decrease in twitch height was observed for both alkylated toxins. This finding is in agreement with previous studies which have shown that chemical inactivation of the PLA_2 active site by 4-BPB inhibits the effects of pre-synaptic neurotoxins such as P-EPTX-Aa1a [20], taipoxin [33], β-bungarotoxin [29,32] and cannitoxin [25].

Pre-synaptic neurotoxins with PLA_2 activity can be classified based on their structures as being single, diametric, or multichain complexes [34]. Multichain neurotoxins consist of several different polypeptide chains held together by noncovalent interactions, with at least one of the subunits having toxic activity on its own [34]. Previous studies have shown that the activity of the toxic subunit is far less than the activity of native toxin [35]. Therefore, the subunits of P-EPTX-Ap1a and P-EPTX-Ar1a were examined for PLA_2 activity and neurotoxicity. The current study clearly showed that the α-subunit of both toxins were the only components to display marked activity as well as the lack of PLA_2 activity and neurotoxicity in the β1 and β2 subunits supporting previous studies of P-EPTX-Aa1a [20] and cannitoxin [25].

In conclusion, P-EPTX-Ap1a and P-EPTX-Ar1a are the first pre-synaptic PLA_2 neurotoxin complexes to be characterized from *A. praelongus* and *A. rugosus* venom, respectively, and the second and third pre-synaptic neurotoxins isolated from *Acanthophis* spp. venoms. Both toxins caused in vitro neurotoxicity in a skeletal muscle preparation which was markedly reduced by 4-BPB indicating that PLA_2 activity is essential for their neurotoxicity. Given the results of this study clinicians may need to be mindful of persistent neurotoxicity following death adder envenoming. Early intervention with antivenom may be important in severe death adder neurotoxicity.

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References

[1] Campbell CH. The death adder (*Acanthophis antarcticus*): the effect of the bite and its treatment. The Medical Journal of Australia 1966;2:922–5.
[2] Currie BJ. Snakebite in tropical Australia, Papua New Guinea and Irian Jaya. Emergency Medicine 2000;12:285–94.
[3] Lalloo DG, Trevett AJ, Saweri A, Naraqi S, Theakston RDG, Warrell DA. The epidemiology of snake bite in Central Province and National Capital District, Papua New Guinea. Transactions of the Royal Society of Tropical Medicine and Hygiene 1995;89:178–82.
[4] Sutherland SK. Treatment of snake bite in Australia and Papua New Guinea. Australian Family Physician 1976;5:272–88.
[5] Lalloo DG, Trevett AJ, Black J, Mapao J, Saweri A, Naraqi S, et al. Neurotoxicity, anticoagulant activity and evidence of rhabdomyolysis in patients bitten by death adders (*Acanthophis* sp.) in southern Papua New Guinea. QJM—Monthly Journal of the Association of Physicians 1996;89:25–35.
[6] Isbister GK, Halkidis L, O'Leary MA, Whitaker R, Cullen P, Mulcahy R, et al. Human anti-snake venom IgG antibodies in a previously bitten snake-handler, but no protection against local envenoming. Toxicon 2010;55:646–9.
[7] Sutherland SK. Recent advances in the care of snake-bite patients in Australia. Toxicon 1983;21:437.
[8] Flachsenberger W, Mirtschin P. Anticholinesterases as antidotes to envenomation of rats by the death adder (*Acanthophis antarcticus*). Toxicon 1994;32:35–9.
[9] Gunja N, Ling M, Dowsett R, Murray L, Isbister G. Antivenom and neostigmine failure in death adder envenoming. Clinical Toxicology 2007;45:389.
[10] Rossetto O, Rigoni M, Montecucco C. Different mechanism of blockade of neuroexocytosis by presynaptic neurotoxins. Toxicology Letters 2004;149:91–101.
[11] Su MJ, Coulter AR, Sutherland SK, Chang CC. The presynaptic neuromuscular blocking effect and phospholipase A_2 activity of textilotoxin, a potent toxin isolated from the venom of the Australian brown snake, *Pseudonaja textilis*. Toxicon 1983;21:143–51.
[12] Fohlman J, Lind P, Eaker D. Taipoxin an extremely potent presynaptic snake venom neurotoxin. Elucidation of the primary structure of the acidic carbohydrate-containing taipoxin-subunit, a prophospholipase homolog. FEBS Letters 1977;84:367–71.
[13] Westerlund B, Nordlund P, Uhlin U, Eaker D, Eklund H. The three-dimensional structure of notexin, a presynaptic neurotoxic phospholipase A_2 at 2.0 Å resolution. FEBS Letters 1992;301:159–64.
[14] Fry BG, Wickramaratna JC, Jones A, Alewood PF, Hodgson WC. Species and regional variations in the effectiveness of antivenom against the in vitro neurotoxicity of death adder (*Acanthophis*) venoms. Toxicology and Applied Pharmacology 2001;175:140–8.
[15] Chow G, Subburaju S, Kini RM. Purification, characterization, and amino acid sequence determination of Acanthins, potent inhibitors of platelet aggregation from *Acanthophis antarcticus* (Common death adder) venom. Archives of Biochemistry and Biophysics 1998;354:232–8.
[16] van der Weyden L, Hains P, Morris M, Broady K. Acanthoxin, a toxic phospholipase A_2 from the venom of the common death adder (*Acanthophis antarcticus*). Toxicon 1997;35:1315–25.
[17] Sim KL. Purification and preliminary characterisation of praelongin phospholipases, antiplatelet agents from the snake venom of *Acanthophis praelongus*. Biochimica et Biophysica Acta (BBA)—General Subjects 1998;1379:198–206.
[18] Wickramaratna JC, Fry BG, Aguilar MI, Kini RM, Hodgson WC. Isolation and pharmacological characterization of a phospholipase A_2 myotoxin from the venom of the Irian Jayan death adder (*Acanthophis rugosus*). British Journal of Pharmacology 2003;138:333–42.
[19] Hart AJ, Smith AI, Reeve S, Hodgson WC. Isolation and characterisation of acanmyotoxin-2 and acanmyotoxin-3, myotoxins from the venom of the death adder *Acanthophis* sp. Seram. Biochemical Pharmacology 2005;70:1807–13.
[20] Blacklow B, Escoubas P, Nicholson GM. Characterisation of the heterotrimeric presynaptic phospholipase A_2 neurotoxin complex from the venom of the common death adder (*Acanthophis antarcticus*). Biochemical Pharmacology, doi:10.1016/j.bcp.2010.03.030.
[21] Blacklow B, Konstantakopoulos N, Hodgson WC, Nicholson GM. Presence of presynaptic neurotoxin complexes in the venoms of Australo-Papuan death adders (*Acanthophis* spp.). Toxicon 2010;55:1171–80.
[22] Hodgson WC, Wickramaratna JC. In vitro neuromuscular activity of snake venoms. Clinical and Experimental Pharmacology and Physiology 2002;29:807–14.

[23] Kuruppu S, Smith AI, Isbister GK, Hodgson WC. Neurotoxins from Australo-Papuan elapids: a biochemical and pharmacological perspective. Critical Reviews in Toxicology 2008;38:73–86.
[24] King GF, Gentz MC, Escoubas P, Nicholson GM. A rational nomenclature for naming peptide toxins from spiders and other venomous animals. Toxicon 2008;52:264–76.
[25] Kuruppu S, Reeve S, Banerjee Y, Kini RM, Smith AI, Hodgson WC. Isolation and pharmacological characterization of cannitoxin, a presynaptic neurotoxin from the venom of the papuan taipan (*Oxyuranus scutellatus canni*). Journal of Pharmacology and Experimental Therapeutics 2005;315:1196–202.
[26] Harris JB, Grubb BD, Maltin CA, Dixon R. The neurotoxicity of the venom phospholipases A_2, notexin and taipoxin. Experimental Neurology 2000;161:517–26.
[27] Harris JB. Phospholipases in snake venoms and their effects on nerve and muscle. Pharmacology & Therapeutics 1985;31:79–102.
[28] Harvey AL, John RS, Ronald JB. Presynaptic effects of toxins. International Review of Neurobiology. Academic Press; 1990. p. 201–39.
[29] Chang CC, Lee JD, Eaker D, Fohlman J. The presynaptic neuromuscular blocking action of taipoxin. A comparison with [beta]-bungarotoxin and crotoxin. Toxicon 1977;15:571–6.
[30] Wickramaratna JC, Fry BG, Hodgson WC. Species-dependent variations in the vitro myotoxicity of death adder (*Acanthophis*) venoms. Toxicological Sciences 2003;74:352–60.
[31] Volwerk J, Pieterson W, de Hass G. Histidine at the active site of phospholipase A_2. Biochemistry 1974;13:1446–54.
[32] Abe T, Alema S, Miledi R. Isolation and characterization of presynaptically acting neurotoxins from the venom of Bungarus snakes. European Journal of Biochemistry 1977;80:1–12.
[33] Fohlman J, Eaker D, Dowdall MJ. Chemical modification of taipoxin and the consequences for phospholipase activity, pathophysiology, and inhibition of high-affinity choline uptake. European Journal of Biochemistry 1979;94:531–40.
[34] Yang C. Chemical modification and functional sites of phospholipase A2, in Venom Phospholipase A2 Enzymes. In: Kini RM, editor. Structure function and mechanism. Chiches, UK: John Wiley & Sons, Ltd.; 1997.
[35] Fohlman J, Eaker D, Karlsson E, Thesleff S. Taipoxin, an extremely potent presynaptic neurotoxin from the venom of the Australian snake taipan (*Oxyuranuss scutellatus*). Isolation, characterization, quarternary structure and pharmacological properties. European Journal of Biochemistry 1976;68:457–69.

1. Introduction

The present study raised interesting questions regarding the effectiveness of antivenom and increasing the frequency of electrical stimulation on the neurotoxicity induced by pre-synaptic neurotoxins. Therefore, addition experiments were carried out. This section includes additional work not included in the manuscript published in Biochemical Pharmacology.

2. Materials and Methods (continued)

2.1 Reverse-phase-HPLC

As per manuscript.

2.2 Mass spectrometry

As per manuscript.

2.3 Chick Biventer Cervicis Nerve-Muscle Preparation (continued)

In additional experiments, death adder antivenom (5 U/ml) was added 10 min prior to the addition of P-EPTX-Ap1a or P-EPTX-Ar1a. CSL death adder antivenom was left in contact with the tissue for 3 h.

In a separate experiment, twitches were evoked by stimulating the motor nerve at 1 Hz, 0.2 ms duration and a voltage greater than that required to evoke a maximal twitch.

2.4 Drug

Death adder antivenom (CSL Ltd., Melbourne, Australia) was used.

2.5 Analysis of result and statistics

The statistical significance of the effect of toxin was determined by a one-way ANOVA on the twitch height at the indicated time point. All ANOVAs were followed by a Tukey's multiple comparison test. A Student's unpaired *t*-test was used to compare the effect of treatment on different tissues. Statistical significance was indicated when $P < 0.05$.

3. Results

3.1 The presence of gamma subunit of isolated pre-synaptic neurotoxins

Under RP-HPLC, P-EPTX-Ap1a (100 µg) eluted as 4 peaks (Figure 1). Each subunit was collected individually for molecular mass determination using MALDI-TOF analysis. The subunits of P-EPTX-Ap1a were found to be of the following molecular masses: 13,406 Da (β1), 13,977 Da (α), 17,339 Da (γ) and 10,414 Da (β2) (Figures 2 a-d). The sum of the molecular masses of α, γ, β1 or β2 subunits resulted in mass of 43,226 Da. P-EPTX-Ar1a (100 µg) also eluted as 4 peaks. Data regarding the molecular masses of P-EPTX-Ar1a subunits are described as per Chapter 3.

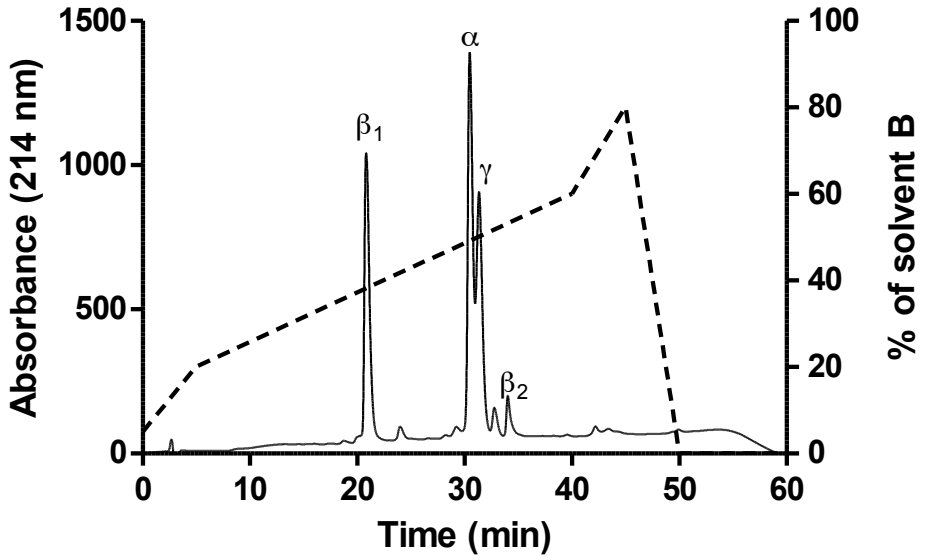

Figure 1: *RP-HPLC chromatograph of P-EPTX-Ap1a on a Phenomenex (Torrance, CA, USA) Jupiter analytical C18 column (150 mm x 2 mm; 5 µm; 300 A), equilibrated with solvent A [0.1% TFA]. The samples were eluted with the following gradient conditions of solvent B [90% ACN in 0.09% TFA]: 0-20% over 5 min, 20-60% for 5-40 min and then 60-80% for 40-45 min at a flow rate of 0.2 ml/min.*

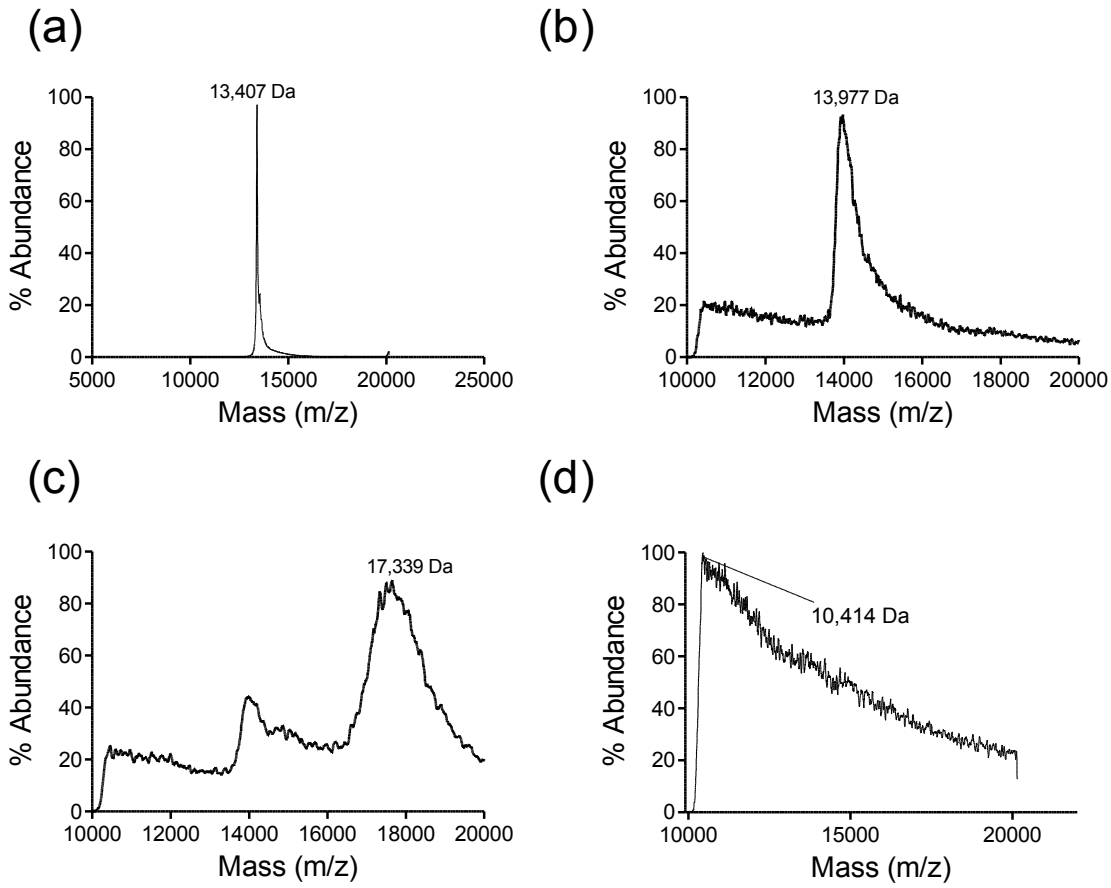

Figure 2: *MALDI-TOF mass spectrometry showing nominal masses of P-EPTX-Ap1a (a) β_1-subunit, (b) α-subunits (c) γ-subunits and (d) β_2-subunit.*

3.2 The inhibitory effect of death adder antivenom on P-EPTX-Ar1a and P-EPTX-Ap1a

The inhibition of twitch height induced by P-EPTX-Ar1a (100 nM) or P-EPTX-Ap1a (100 nM) was markedly attenuated by the prior addition of CSL death adder antivenom (5 units/ml, Figure 3) ($P < 0.05$, one-way ANOVA, $n = 4$-5).

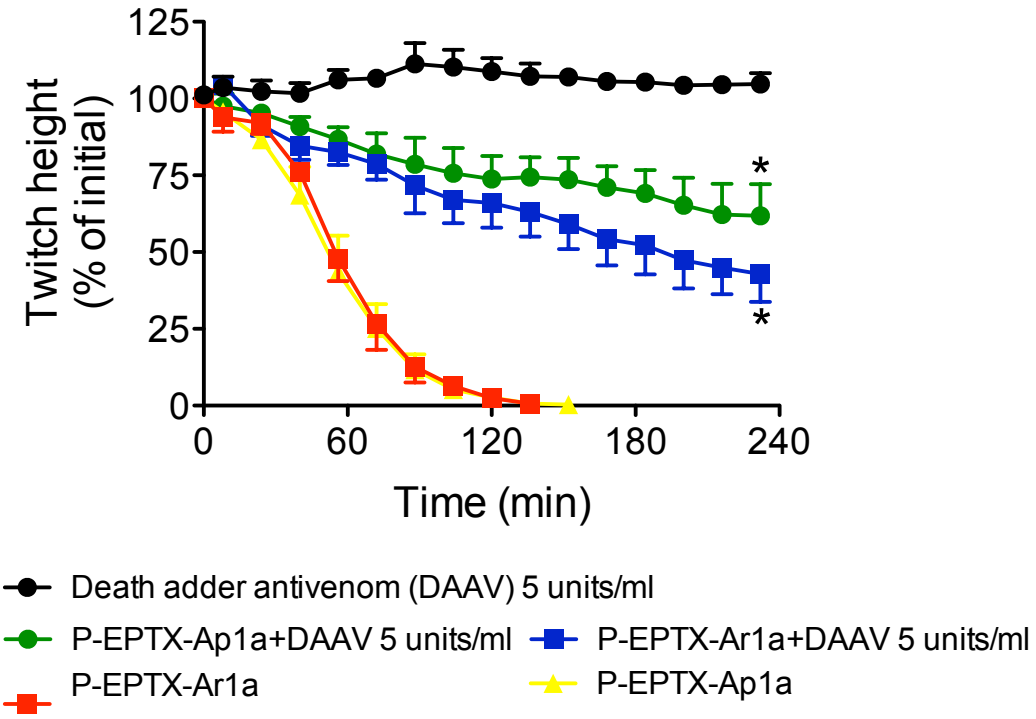

Figure 3: *The effect of P-EPTX-Ap1a (100 nM) or P-EPTX-Ar1a (100 nM) on twitch height of the CBCNM preparation in the presence or absence of CSL death adder antivenom (5 units/ml), *P < 0.05, one-way ANOVA compared to P-EPTX-Ap1a or P-EPTX-Ar1a in the absence of death adder antivenom (n = 4-5).*

3.3 Effect of 1 Hz indirect stimulation on twitches in the presence of isolated pre-synaptic neurotoxins

Time to 90% inhibition (t_{90}) was significantly ($P < 0.05$, one-way ANOVA, $n = 4$) decreased by increasing the stimulation frequency from 0.2 Hz to 1 Hz (Figures 4a and b) in the presence of P-EPTX-Ap1a (100 nM, 53 ± 5 min) or P-EPTX-Ar1a (100 nM, 43 ± 8 min).

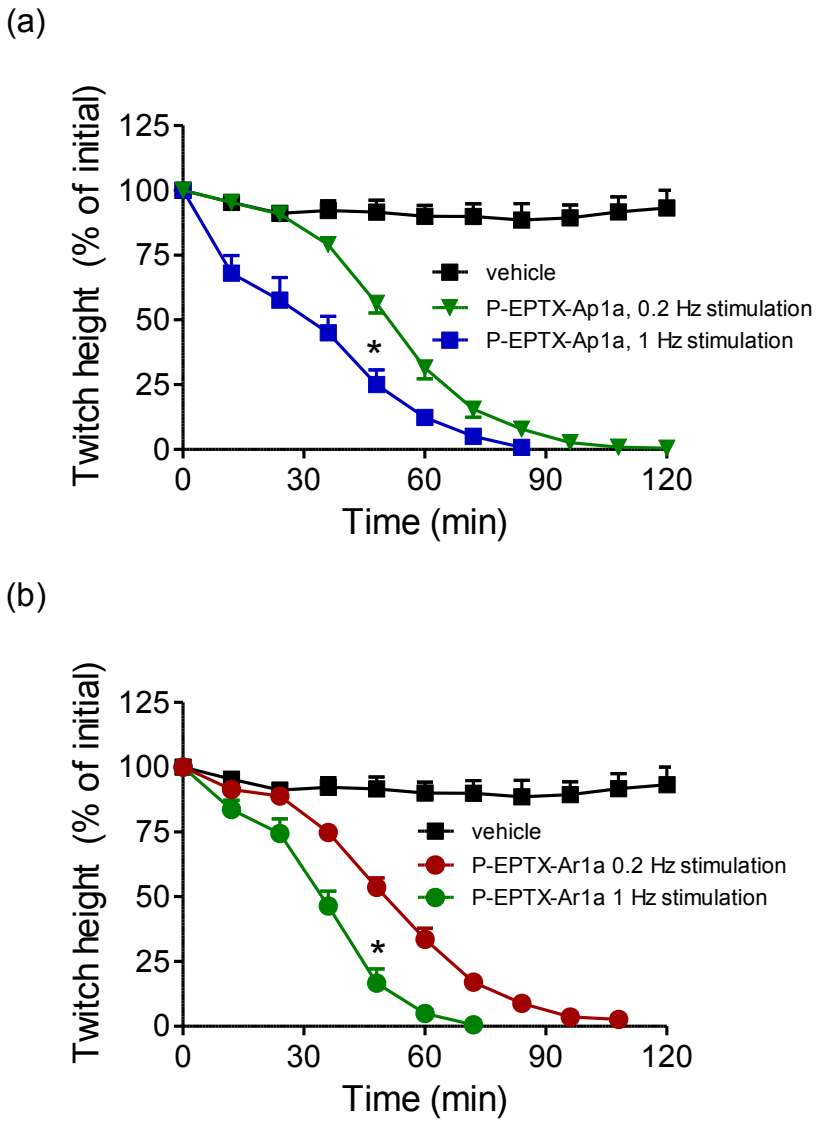

Figure 4: *The effect indirect stimulation at 1 Hz on the CBCNM preparation in the presence of (a) P-EPTX-Ap1a (100 nM) or (b) P-EPTX-Ar1a (100 nM). * $P < 0.05$, Student's unpaired t-test, compared with indirect stimulation at 0.2 Hz (n = 4).*

4. Discussion (continued)

Further discussion concerning the additional data is included in the General Discussion (Chapter 9).

CHAPTER 3

MYOTOXIC AND CYTOTOXIC ACTIVITIES OF VENOMS AND PRE-SYNAPTIC NEUROTOXINS FROM IRIAN JAYAN DEATH ADDER AND PAPUAN TAIPAN

Monash University
Declaration for Thesis Chapter 3

This chapter is made up of the following publication and additional work undertaken but not included in the manuscript.

Declaration by candidate

In the case of Chapter 3, the nature and extent of my contribution to the work was the following:

Nature of Contribution	Extent of Contribution (%)
I declare that experiments and writing of this manuscript were solely undertaken by the myself, taking into consideration the advice and recommendations of co-authors	90 %

The following co-authors contributed to the work.

Name	Nature of Contribution
Professor Helena C Parkington	Manuscript preparation
Associate Professor Geoffrey K Isbister	Development of ideas, manuscript preparation
Dr Nicki Konstantakopoulos	Development of ideas, manuscript preparation
Professor Wayne C Hodgson	Development of ideas, manuscript preparation

Candidate's signature: [redacted] Date: 07.06.2013

Janeyuth Chaisakul

Declaration by co-authors

The undersigned hereby certify that:

(1) the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors;
(2) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(3) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(4) there are no other author of the publication according to these criteria;
(5) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journal or other publications and (c) the head of the responsible academic unit; and
(6) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location (s) Department of Pharmacology, Monash University

		Date
Professor Helena C Parkington	[redacted]	06.06.2013
Associate Professor Geoffrey K Isbister	[redacted]	07.06.2013
Dr Nicki Konstantakopoulos	[redacted]	04.06.2013
Professor Wayne C Hodgson	[redacted]	07.06.2013

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Differential Myotoxic and Cytotoxic Activities of Pre-synaptic Neurotoxins from Papuan Taipan (*Oxyuranus scutellatus*) and Irian Jayan Death Adder (*Acanthophis rugosus*) Venoms

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Abstract: Pre-synaptic PLA_2 neurotoxins are important components of many Australasian elapid snake venoms. These toxins disrupt neurotransmitter release. Taipoxin, a pre-synaptic neurotoxin isolated from the venom of the coastal taipan (*Oxyuranus scutellatus*), causes necrosis and muscle degeneration. The present study examined the myotoxic and cytotoxic activities of venoms from the Papuan taipan (*O. scutellatus*) and Irian Jayan death adder (*Acanthophis rugosus*), and also tested their pre-synaptic neurotoxins: cannitoxin and P-EPTX-Ar1a. Based on size-exclusion chromatography analysis, cannitoxin represents 16% of *O. scutellatus* venom, while P-EPTX-Ar1a represents 6% of *A. rugosus* venom. In the chick biventer cervicis nerve-muscle preparation, *A. rugosus* venom displayed significantly higher myotoxic activity than *O. scutellatus* venom as indicated by inhibition of direct twitches, and an increase in baseline tension. Both cannitoxin and P-EPTX-Ar1a displayed marked myotoxic activity. *A. rugosus* venom (50–300 μg/ml) produced concentration-dependent inhibition of cell proliferation in a rat skeletal muscle cell line (L6), while 300 μg/ml of *O. scutellatus* venom was required to inhibit cell proliferation, following 24-hr incubation. P-EPTX-Ar1a had greater cytotoxicity than cannitoxin, inhibiting cell proliferation after 24-hr incubation in L6 cells. Lactate dehydrogenase levels were increased after 1-hr incubation with *A. rugosus* venom (100–250 μg/ml), *O. scutellatus* venom (200–250 μg/ml) and P-EPTX-Ar1a (1–2 μM), but not cannitoxin (1–2 μM), suggesting venoms/toxin generated cell necrosis. Thus, *A. rugosus* and *O. scutellatus* venoms possess different myotoxic and cytotoxic activities. The proportion of pre-synaptic neurotoxin in the venoms and PLA_2 activity of the whole venoms are unlikely to be responsible for these activities.

While snake envenoming is an infrequent occurrence in Australia, it remains an important cause of morbidity and mortality in Papua New Guinea and Irian Jaya [1,2]. Myotoxicity is a critical outcome of systemic toxicity produced by many Australasian elapids, including rarely with death adders (genus *Acanthophis*) and taipans (genus *Oxyuranus*). It has been suggested that phospholipase A_2 (PLA_2) is the major venom component responsible for myotoxicity [3–5]. Indeed, many snake venoms and isolated PLA_2 toxins display myotoxicity and cytotoxicity (e.g. Lys-49 phospholipase A_2 of *Bothrops asper* snake venom) [6–10] which induce membrane disruption and Ca^{2+} influx.

Although the Papuan taipan has been regarded as a separate subspecies to the Australian populations, recent taxonomic studies have indicated no significant differentiation between the two populations [11,12]. The clinical features of systemic taipan envenoming include venom-induced consumption coagulopathy, renal toxicity, cardiac disturbances and a descending neuromuscular paralysis [13–15]. The venom of the Australian coastal taipan has myotoxic activity causing necrosis and degeneration of mammalian muscle [16]. In contrast, Papuan taipan venom failed to inhibit direct muscle stimulation of the chick biventer cervicis nerve-muscle preparation (CBCNM) [17], despite myotoxicity with elevated creatine kinase being reported after Papuan taipan envenoming [18].

Death adder (*Acanthophis* spp.) venoms were thought to be devoid of myotoxic activity [19,20] prior to a clinical report of myotoxicity following death adder envenoming in Papua New Guinea. One patient developed renal failure and significantly elevated creatine kinase levels [21]. This report resulted in our previous examination of a range of death adder venoms for myotoxic activity with *A. laevis* (previously known as *A.* sp. Seram), *A. praelongus*, *A. rugosus* and *A. wellsi* all displaying *in vitro* myotoxicity [22]. Subsequently, myotoxins were isolated from *A. rugosus* (Irian Jayan death adder) [23] and *A. laevis* [24] venoms. Death adder venoms also displayed weak cytotoxic effects on skeletal muscle (L6) and aortic smooth muscle (A7r5) cell lines [25]. In addition, there has been a recent report of myotoxicity following confirmed envenoming by the northern death adder (*A. praelongus*) [26].

Pre-synaptic PLA_2 neurotoxins are key components of many elapid venoms and cause neuromuscular blockade by inhibiting transmitter release at the motor nerve terminal [27]. Some pre-synaptic PLA_2 neurotoxins (e.g. taipoxin [16], notexin and notechis II [28,29]) also display marked myotoxic activity. The alpha and gamma subunits of taipoxin induced cytotoxic effects in a rat adrenal pheochromocytoma (PC12) cell line, while the beta subunits were found to have mitogenic activity [30]. Crotoxin, a pre-synaptic neurotoxin and myotoxic PLA_2 from *Crotalus durissus terrificus*, induced plasma membrane

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disruption and depolarization [31]. We have previously isolated pre-synaptic PLA_2 neurotoxins from Papuan taipan (*O. scutellatus*; cannitoxin [32]) and Irian Jayan death adder (*A. rugosus*; P-EPTX-Ar1a [33]) venoms. However, the myotoxic and cytotoxic activities of these toxins have not been studied. Therefore, the aim of this study was to investigate the myotoxic and cytotoxic activities of pre-synaptic neurotoxins isolated from Papuan taipan and Irian Jayan death adder venoms as well as the possible contributing factors to myotoxic and cytotoxic activities. This work will provide insight into the mechanism behind the myotoxic activities of both the venoms.

Materials and Methods

Venom preparation and storage. *Oxyuranus scutellatus* (Merauke, Irian Jaya, Indonesia) and *A. rugosus* (Merauke, Irian Jaya, Indonesia) venoms were purchased from Venom Supplies (Tanunda, South Australia). Aliquots of freeze-dried venoms were reconstituted in distilled water. Stock solutions were filter-sterilized using a 0.22-μm membrane, and the protein content was determined via a BCA Protein Assay Kit (Thermo Fisher Scientific, Rockford, IL, USA), as per the manufacturer's instructions. Briefly, venoms/toxins (25 μl) were added in triplicate to a 96-well micro-titre plate. Bovine serum albumin (BSA) solutions, diluted from 0.025 to 1 mg/ml, were used as reference standard and distilled water was used as the blank. Absorbance was measured at 562 nm using a fusion α microplate reader (Perkin-Elmer, Waltham, MA, USA). Venom stock solutions were stored at 4°C until required.

Chromatography. All chromatography separations were performed using a Shimadzu (Kyoto, Japan) high-performance liquid chromatography system (LC-10ATvp pump and SPD-10AVP detector).

Size-exclusion chromatography. To isolate the pre-synaptic neurotoxins, size-exclusion chromatography was performed using fast protein liquid chromatography (FPLC). Venom (3 mg) was applied to a Superdex G-75 column (13 μm; 10 × 300 mm; GE Healthcare, Buckinghamshire, UK) equilibrated with ammonium acetate buffer (0.1 M; pH 6.8). The sample was eluted at a flow rate of 0.5 ml/min. The sample was monitored at 280 nm. The fractions were collected and pooled, frozen at −80°C and then freeze-dried to remove the solvent. The neurotoxic activity of pre-synaptic neurotoxins was confirmed using the CBCNM preparation as per our previous study [33].

RP-HPLC. Venom profiles for *O. scutellatus* (Papuan species) and *A. rugosus* venoms were obtained using RP-HPLC (reverse-phase-high-performance liquid chromatography). In addition, the pre-synaptic neurotoxins obtained from size-exclusion FPLC, as described previously, were dissociated into their subunits via RP-HPLC for further determination. Samples (100 μg) were reconstituted in Milli-Q water and applied to a Phenomenex (Torrance, CA, USA) Jupiter analytical C18 column (150 mm × 2 mm; 5 μm; 300 Å) after equilibrating with solvent A [0.1% trifluoroacetic acid (TFA)]. The sample was eluted with the following gradient conditions of solvent B [90% acetonitrile (ACN) in 0.09% TFA]: 0–20% over 5 min., 20–60% for 5–40 min. and then 60–80% for 40–45 min. at a flow rate of 0.2 ml/min. The eluent was monitored at 214 nm.

Mass spectrometry. MALDI-TOF mass spectrometry analysis was performed using an Applied Biosystems (Foster City, CA, USA) 4700 TOF TOF Proteomics Analyser [33]. The instrument was operated in positive polarity in linear mode using α-cyano-4-hydroxycinnamic acid (CHCA) matrix (Agilent Technologies, Palo Alto, CA, USA) for low-resolution protein analysis. Matrix (0.5 μl) was spotted on the sample plate and allowed to air-dry; sample (0.5 μl) diluted in ACN/water (1:1) containing 0.1% (v/v) formic acid was subsequently spotted on dried matrix and allowed to air-dry. Data from 2000 laser shots (337-nm nitrogen laser) were collected, and the signal was averaged and processed with the instrument manufacturer's 4000 series Data Explorer software.

N-terminal amino acid sequence determination. Purified peptides were loaded into the sequencing chamber of a Procise N-terminal amino acid sequencer (Applied Biosystems), and the amino acid sequence was determined (Edman degradation, phenylthiohydantoin derivatization chemistry, and separation of derivatized amino acids by RP-HPLC) using the manufacturer's recommended methods and reagents [32].

Chick biventer cervicis nerve-muscle preparation. All animal experiments used in this study were approved by the SOBS-B Monash University Animal Ethics Committee. Male chickens aged between 4 and 10 days were killed by CO_2 asphyxiation and both biventer cervicis nerve-muscles removed. These muscles were mounted under 1 g resting tension in 5-ml organ baths containing physiological salt solution (118.4 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, 25 mM $NaHCO_3$ and 11.1 mM glucose). The solution was maintained at 34°C and bubbled with carbogen (95% O_2 and 5% CO_2). Direct twitches were established by electrical stimulation (0.1 Hz, 2 ms, supramaximal voltage) using a Grass S88 stimulator with an electrode placed around the body of the muscle. To ensure selective stimulation of muscle, *d*-tubocurarine (10 μM) was added and left in the organ bath for the duration of the experiment. Snake venoms (10 and 30 μg/ml), isolated pre-synaptic neurotoxins (0.1 and 1 μM) or vehicle were then added and left in contact with the preparation for up to 3 hr. A significant contracture of skeletal muscle (i.e. a rise in baseline) and/or inhibition of direct twitches were considered signs of myotoxicity.

Cell culture. Rat skeletal muscle myoblast cell line, L6, was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). L6 cells were grown in 175-cm^2 flasks in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Foetal Calf Serum (FCS) and 1% penicillin/streptomycin (10% DMEM), incubated at 37°C with 5% CO_2 until 70% confluence. Cells were lifted using trypsin, and pelleted. The cell pellet was re-suspended in 30 ml culture medium to seed at 100 μl/well into four 96-well cell culture plates. Plates were incubated at 37°C in an atmosphere of 5% CO_2 and medium changed every second day until cells were grown to 90% confluence. Medium was then removed from wells and replaced with DMEM medium supplemented with 2% FCS and 1% penicillin/streptomycin (2% DMEM), to enable the differentiation of skeletal myoblast cells into skeletal myocytes. Plates were incubated at 37°C in an atmosphere of 5% CO_2. Medium was replaced every second day, for 1 week, until cell differentiation (appearance of long striated cells) was observed. Cells were passaged up to passage 12 before being discarded and a new vial of cells thawed.

Cell proliferation assay (MTS assay). Medium was removed from wells in L6 cell culture plates, and the wells were washed once with pre-warmed Dulbecco's phosphate-buffered saline (PBS). Venom stock solutions were diluted in 2% DMEM to a final concentration of 50–300 μg/ml. Isolated toxin stock solutions were diluted in 2% DMEM to a final concentration of 0.1–2 μM. Dilutions were added to wells in a cell culture plate in quadruplicate (100 μl/well). Culture medium control (cells and medium with no venom/toxin) and medium blanks (no cells) were also run in parallel. The plates were incubated at 37°C with 5% CO_2 for 1 hr or 24 hr. After incubation, the cell

culture plates were removed and washed with pre-warmed PBS three times. Fresh DMEM (50 µl/well) and 10 µl of 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) solution were added to each well. The plates were further incubated at 37°C with 5% CO_2 for 3 hr. Absorbance was measured at 492 nm utilizing the VersaMax microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Lactate dehydrogenase assay. The lactase dehydrogenase (LDH) assay (Promega, Melbourne, Australia) was performed as per the manufacturer's instructions. Briefly, L6 cells were exposed to multiple concentrations of venoms at 50–250 µg/ml for a period of 1 hr under 37°C in an atmosphere of 5% CO_2, followed by an equilibration at 22°C for 20 min. Three controls were performed: a no-cell control (blank), untreated cell control (negative control) and a maximum LDH release control (2 µl Lysis Solution positive control). Venoms and pre-synaptic neurotoxins were performed in the identical condition. CytoTox-ONE™ Reagent (Promega, Melbourne, Australia) (100 µl) was added to all wells and then incubated for a further 10 min. at 22° C. Stop solution (50 µl) was added to each well, and fluorescence was measured with an excitation wavelength of 560 nm and an emission wavelength of 590 nm. All solutions were plated in quadruplicate.

Determination of PLA_2 activity. PLA_2 activity of the venom/isolated toxin was determined using a secretory PLA_2 colorimetric assay kit (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's instructions. This assay uses the 1, 2-dithio analogue of diheptanoyl phosphatidylcholine, which serves as a substrate for PLA_2 enzymes. Free thiols generated following the hydrolysis of the thioester bond at the *sn*-2 position by PLA_2 are detected using DTNB [5, 5′-dithio-bis (2-nitrobenzoic acid)]. Colour changes were monitored at 405 nm in a fusion α microplate reader (Perkin-Elmer, Waltham, MA, USA), sampling every minute for a 5-min. period. PLA_2 activity was expressed as micromoles of phosphatidylcholine hydrolysed per minute per milligram of enzyme.

Drugs and chemicals. The following chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA): ammonium acetate, bovine serum albumin (BSA), *d*-tubocurarine, Dulbecco's modified Eagle's medium (DMEM) with high glucose, and PBS. The following chemicals were purchased from other companies as indicated: BCA protein assay kit (Pierce biotechnology), CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS assay) (Promega, Melbourne, Australia), foetal calf serum (FCS) (CSL Ltd., Melbourne, Australia), trifluoroacetic acid (Auspep, Melbourne, Australia), and acetonitrile (Merck, Darmstadt, Germany).

Analysis of results and statistics. Percentage yield of the pre-synaptic neurotoxins in the venoms was determined by measuring the area under the curve of the size-exclusion FPLC venom elution profiles. These data were compared using unpaired Student's *t*-test. 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 initial twitch height (i.e. prior to the addition of venom, toxin or vehicle). Change in baseline tension was measured as change from the original baseline prior to the administration of venoms/toxins. Data were compared by a one-way analysis of variance (ANOVA) on the change in twitch height and baseline tension at the 180-min. time point.

In cell-based assays, the concentration-response curves were displayed as a percentage of maximal cell growth (% cell viability) *versus* venom/toxin concentration and graphed using the Graph Pad Prism 5 program (GraphPad Software Inc, La Jolla, CA, USA). A one-way ANOVA was carried out to compare venom/toxin concentration. For LDH study, % LDH release was compared between the treatment (venoms/toxins) group, untreated cell control (negative control) and maximum LDH release control (lysis solution) using a one-way ANOVA and a two-way ANOVA. All ANOVAS were followed by a Tukey's multiple comparison test. Statistical significance was indicated by $p < 0.05$.

Results

Fractionation of venoms and isolation of pre-synaptic neurotoxins.

Size-exclusion FPLC. Acanthophis rugosus and *Oxyuranus scutellatus* venoms were fractionated by size-exclusion FPLC. The venom profile of each snake identified 7–8 major peaks that were individually collected (fig. 1A,B). Screening in the CBCNM preparation indicated that fraction 1 from *A. rugosus* venom (fig. 1A, highlighted sections) and fraction 2 from *O. scutellatus* venom (fig. 1B, highlighted sections) displayed pre-synaptic neurotoxic activity (i.e. inhibition of indirect twitches). Analysis of the chromatographs indicated that cannitoxin represented 15.9 ± 0.2% of *O. scutellatus* venom, which was a significantly higher proportion compared to P-EPTX-Ar1a (5.7 ± 0.1%) in *A. rugosus* venom ($p < 0.05$, n = 5, Student's unpaired *t*-test).

RP-HPLC. Acanthophis rugosus and *Oxyuranus scutellatus* venoms were also profiled using RP-HPLC on a Phenomenex Jupiter analytical column (fig. 1C,D). The venom profile of each snake identified 10–11 peaks that indicated the presence of a myotoxin at 30 min.

Fraction 1 (i.e. P-EPTX-Ar1a) from *A. rugosus* venom (fig. 1E) eluted as 4 peaks that were collected for molecular weight and N-terminal amino acid determinations (see below). Fraction 2 (i.e. cannitoxin) from *O. scutellatus* venom (fig. 1F) also yielded 4 peaks indicating the likely presence of the previously identified α, γ, β1 and β2 subunits [32].

Molecular mass determination. The 4 subunits of P-EPTX-Ar1a and cannitoxin obtained from RP-HPLC were examined using MALDI-TOF. The nominal mass present at the apex of each peak indicated the molecular mass of the subunit. The subunits of P-EPTX-Ar1a were found to be of the following molecular masses: 13,639 Da (α), 13,359 Da (β_1), 13,459 Da (β_2) and 17,045 Da (γ) (fig. 2A–D). MALDI-TOF analysis showed the γ subunit (17,045 DA) to be heterogeneously glycosylated as indicated by broadening of the peak (fig. 2D). The subunits of cannitoxin were found to be of the following molecular masses: 13,174 Da (β_2), 13,765 Da (α), 13,212 Da (β_1) and 17,812 Da (γ) (fig. 2E–H).

The sum of the molecular masses of α, γ and β_1 or β_2 subunits resulted in average masses of 44,093 Da for P-EPTX-Ar1a and 44,770 Da for cannitoxin, respectively.

Amino acid sequence determination. Partial N-terminal amino acid sequence of the isolated subunits of P-EPTX-Ar1a was determined (table 1). The sequence was compared with other protein sequences at the National Centre for Biotechnology Information (NCBI) database using a BLAST service.

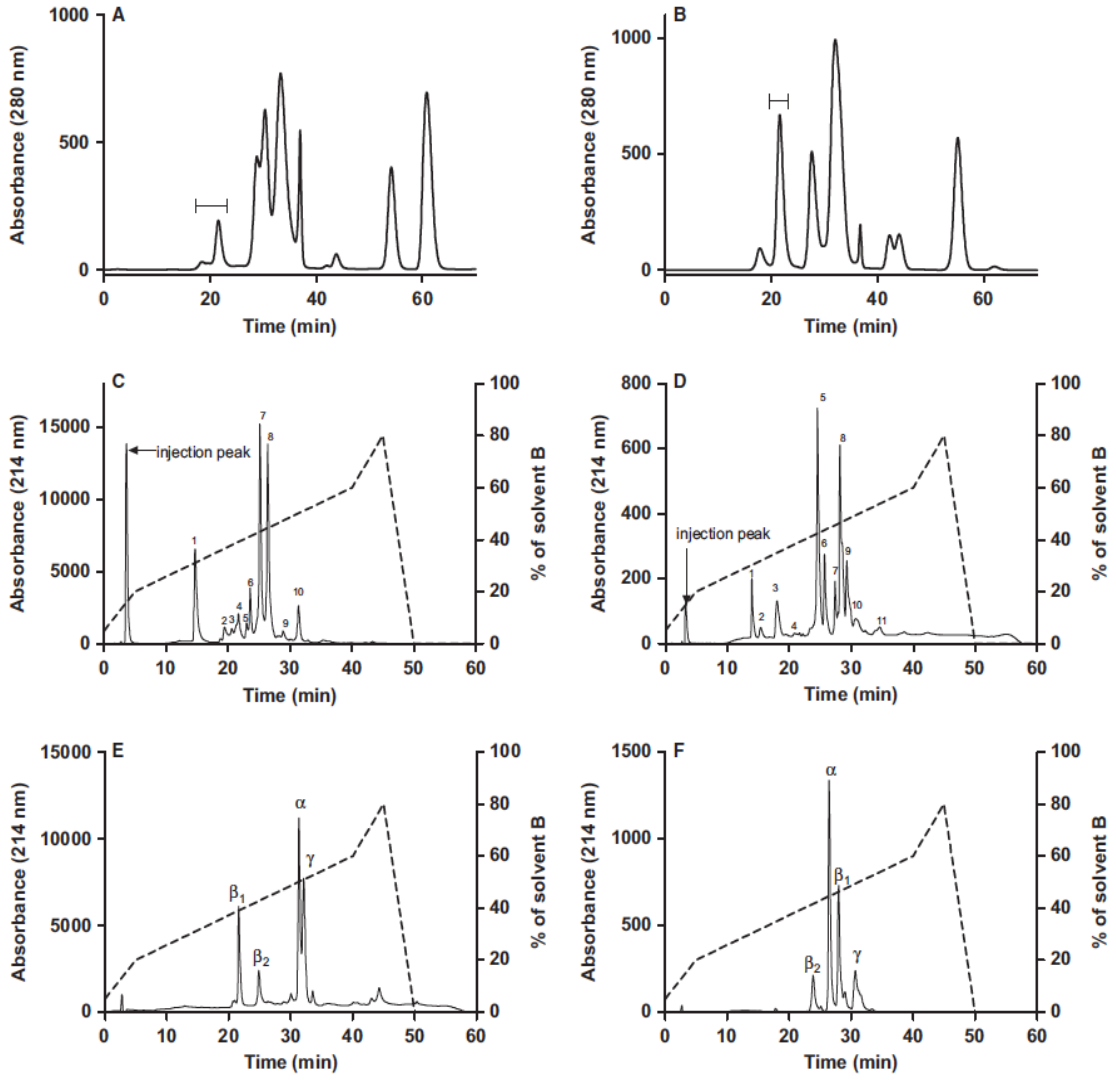

Fig. 1. Size-exclusion chromatograph of (A) *Acanthophis rugosus* venom or (B) *Oxyuranus scutellatus* venom on a Superdex G-75 column equilibrated with ammonium acetate (0.1 M; pH 6.8) at a flow rate of 0.5 ml/min. Highlighted sections indicate the pre-synaptic neurotoxin in the venom (i.e. P-EPTX-Ar1a for the *A. rugosus* and cannitoxin for *O. scutellatus*). RP-HPLC chromatograph of (C) *A. rugosus* venom, (D) *O. scutellatus* venom, (E) P-EPTX-Ar1a or (F) cannitoxin on a Phenomenex Jupiter analytical C18 column, equilibrated with solvent A (0.1% TFA) and eluted with varied gradient conditions of solvent B (90% ACN in 0.09% TFA) at a flow rate of 0.2 ml/min. Gradient conditions used: 0–20% over 5 min., 20–60% for 5–40 min., and 60–80% for 40–45 min.

Cysteine residues were determined by blank Edman cycles and compared with relative toxin subunits. β_1-P-EPTX-Ar1a and β_2-P-EPTX-Ar1a share 85% sequence identity with P-EPTX-Aa1a beta chain, isolated from *A. antarcticus* venom [34], and 70 and 75% sequence identity with the β_1 and β_2 subunits of cannitoxin [32], respectively. α-P-EPTX-Ar1a shares 94% sequence identity with P-EPTX-Aa1a alpha chain, a PLA_2 toxin isolated from *A. antarcticus* venom [34], and 84% sequence identity with the α-subunit of cannitoxin [32]. The γ-subunit of P-EPTX-Ar1a showed 57% sequence identity with the γ-subunit of cannitoxin and 69% sequence identity with the P-EPTX-Aa1a γ-subunit.

Chick biventer cervicis nerve-muscle preparation.

Acanthophis rugosus and *Oxyuranus scutellatus* venoms (30 μg/ml; n = 5) had a significant effect on baseline tension compared with the vehicle (one-way ANOVA, $p < 0.05$; fig. 3A); however, the increase in baseline tension induced by *O. scutellatus* venom was significantly less than that of *A. rugosus* venom (one-way ANOVA, $p < 0.05$). *Acanthophis rugosus* venom, but not *O. scutellatus* venom, significantly inhibited direct twitches in the CBCNM preparation (n = 5; one-way ANOVA, $p < 0.05$; fig. 3B).

Both cannitoxin and P-EPTX-Ar1a (1 μM) caused significant increases in baseline tension and significant inhibition of

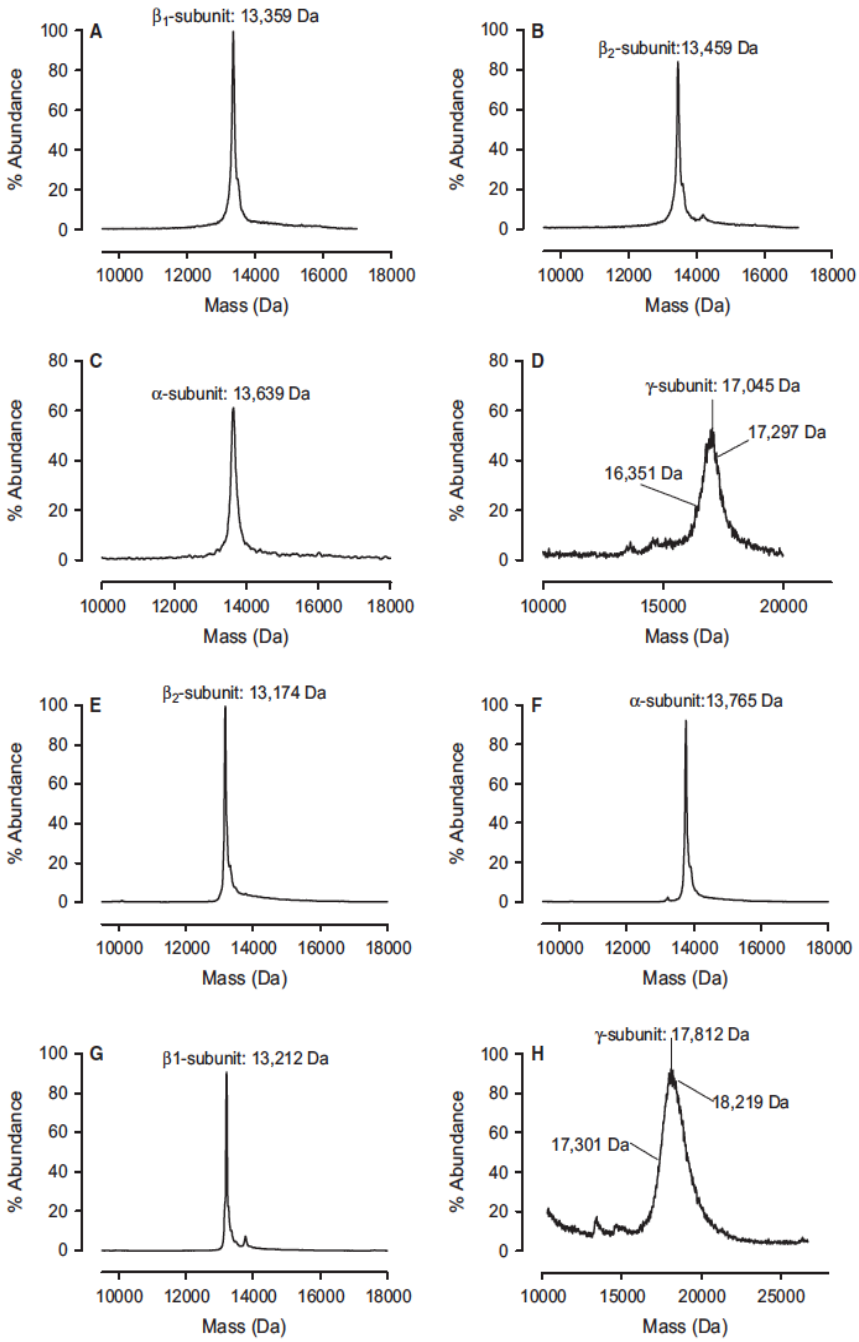

Fig. 2. MALDI-TOF mass spectrometry showing nominal masses of the P-EPTX-Ar1a (A) β_1-subunit, (B) β_2-subunit and (C) α-subunit. (D) MALDI-TOF mass spectrometry of the γ-subunit indicating a spectrum consistent with heterogeneous glycosylation, with a nominal mass of 17,045 Da. MALDI-TOF mass spectrometry, indicating the molecular masses of the cannitoxin (E) β_2-subunit, (F) α-subunit, (G) β_1-subunit and (H) γ-subunit (17,812 Da).

direct twitches compared with vehicle (n = 6; one-way ANOVA, $p < 0.05$; fig. 3C,D).

Phospholipase A_2 activity.

Oxyuranus scutellatus venom had significantly higher PLA_2 activity (373 ± 32.6 µmol/min/mg) compared to *A. rugosus* venom (291.8 ± 10.8 µmol/min/mg; n = 4; one-way ANOVA, $p < 0.05$). In contrast, the PLA_2 activity of the pre-synaptic neurotoxins was not significantly different, that is, P-EPTX-Ar1a (317.3 ± 6.1 µmol/min/mg; n = 4) and cannitoxin (309.8 ± 4.5 µmol/min/mg; n = 4; one-way ANOVA, $p > 0.05$).

Table 1.
N-terminal sequence of the subunits of P-EPTX-Ar1a and PLA_2 components isolated from other elapid snake venoms.

Species	PLA_2 component	N-terminal sequence
Acanthophis rugosus	β1-P-EPTX-Ar1a	DLAQFGCMIE CANCGSRPSL
	β2-P-EPTX-Ar1a	DLAQFGCMIE CANCGSRPS
A. antarcticus[1]	P-EPTX-Aa1a β-subunit	DLFQFGKMIE CANKGSRPSL DYMNYGCYCGK
Oxyuranus scutellatus[2]	cannitoxin β1-subunit	NLVQFGKMIE
	cannitoxin β2-subunit	NLVQFGFMIE CAIRN RQPAL DFMNY GCYCG….
A. rugosus	α-P-EPTX-Ar1a	NLLQFGFMIR CANCRRRPVC HY
A. antarcticus[1]	P-EPTX-Aa1a α-subunit	NLLQFGFMIR CANKRRRPVW PYEESGC
A. rugosus[3]	acanmyotoxin-1	NLLQIGIMKR CANKRRRPVF HYRDYGCYC
O. scutellatus[2]	cannitoxin α-subunit	NLLQFGYMIR CANGRSRPVW
A. rugosus	γ-P-EPTX-Ar1a	AIPLPSLNNE EQNNMMQQTT PPEEESL
A. antarcticus[1]	P-EPTX-Aa1a γ -subunit	SIPLPSLNFE QFGNMIQCTI
O. scutellatus[2]	cannitoxin γ -subunit	SEIPQPSLDF EQFSNMIQCT IPPGE..

[1]Data from Blacklow *et al.* [34].
[2]Data from Kuruppu *et al.* [32].
[3]Data from Wickramaratna *et al.* [23].

Cell viability assay.
Serially diluted *O. scutellatus* and *A. rugosus* (50–300 μg/ml) venoms were incubated with L6 cells for 1 hr or 24 hr (fig. 4). *Oxyuranus scutellatus* venom (50–300 μg/ml) had no significant effect on L6 cells after incubation for 1 hr (fig. 4C). However, cell viability was significantly reduced after 24-hr incubation with *O. scutellatus* venom (300 μg/ml) (fig. 4D). In contrast, *A. rugosus* venom had a significant inhibitory effect after incubation for 1 hr (150–300 μg/ml; fig. 4A) and 24 hr (50–300 μg/ml; n = 4, $p < 0.05$, one-way-repeated measures ANOVA; fig. 4B).

Serially diluted cannitoxin (0.1–2 μM) and P-EPTX-Ar1a (0.1–2 μM) were incubated with L6 cells for 1 and 24 hr (fig. 5). P-EPTX-Ar1a (0.7–2 μM; fig. 5B) and cannitoxin (0.7–2 μM; fig. 5D) significantly affected L6 cell viability (n = 4, $p < 0.05$, one-way-repeated measures ANOVA) after 24-hr incubation. However, P-EPTX-Ar1a displayed more significant cytotoxic activity than cannitoxin ($p < 0.05$, Student's unpaired *t*-test).

Lactate dehydrogenase assay.
Acanthophis rugosus venom (50–250 μg/ml) produced significantly greater LDH release following 1-hr incubation than *O. scutellatus* venom (50–250 μg/ml) (n = 4, $p < 0.0001$, two-way ANOVA; fig. 6A).

P-EPTX-Ar1a (1–2 μM) incubation for 1 hr induced significant LDH release (n = 4, $p < 0.05$, one-way ANOVA). However, LDH release induced by cannitoxin (1–2 μM) was not significantly different from cell control (n = 4, $p > 0.05$, one-way ANOVA; fig. 6B).

Discussion

In this study, we have shown that the pre-synaptic neurotoxin from *A. rugosus* venom, P-EPTX-Ar1a, induces *in vitro* myotoxicity and cytotoxicity. In contrast, the pre-synaptic neurotoxin from *O. scutellatus* venom, cannitoxin, displayed weak myotoxic and minimal cytotoxic activities. To determine the correlation between the activity of the pre-synaptic neurotoxins and the whole venoms, we also compared the effects of both venoms and the proportion of each toxin within their respective venom. In support of our above observations, *A. rugosus* venom displayed marked myotoxicity and cytotoxicity while *O. scutellatus* venom displayed small but measurable myotoxic and cytotoxic activities.

The snake pre-synaptic neurotoxins (i.e. β-neurotoxins) interrupt acetylcholine release from the nerve terminal causing flaccid paralysis due to transmission disturbance at the somatic neuromuscular junction. These toxins are present in a range of Australasian elapid venoms. Therefore, β-neurotoxins from *A. rugosus* and *O. scutellatus* venoms, P-EPTX-Ar1a and cannitoxin, respectively, were isolated. The proportion of P-EPTX-Ar1a in *A. rugosus* venom (i.e. 6%) was significantly less than cannitoxin (i.e. 16%) in *O. scutellatus* venom. Both venoms were profiled using RP-HPLC with each venom displaying the presence of a myotoxin eluting around 25–30 min., consistent with myotoxic components isolated from the venoms of snakes in genus *Acanthophis* [23,24].

Pre-synaptic neurotoxin complexes are normally comprised of 2–6 subunits [34,35]. RP-HPLC analysis of P-EPTX-Ar1a indicated the presence of 4 subunits. These subunits were further examined for molecular mass to enable comparison with cannitoxin. P-EPTX-Ar1a and cannitoxin consist of 4 subunits: β-1, β-2, α and γ. The α-subunit eluted around 30 min., indicating the PLA_2 component in each toxin. Previous studies reported that taipoxin acts as a potent pre-synaptic neurotoxin and also exhibits myolytic and necrotic activity [16,36]. The basic α-taipoxin was myotoxic, and its potency was enhanced by the presence of the γ-subunit, whereas the role of the neutral β-subunit was unclear [16]. This suggests that the α-subunit is required to generate myotoxicity.

It has been documented that snake venom PLA_2 components have masses in the range of 12–14 kDA. The α-P-EPTX-Ar1a is similar in molecular weight to that of α-P-EPTX-Aa1a [34], the toxin isolated from *A. antarcticus*. Molecular mass of P-EPTX-Ar1a subunit 4 (17,045 DA) was similar to γ-subunit of cannitoxin [32] and γ-subunit of P-EPTX-Aa1a [34]. In addition, MALDI-TOF analysis also revealed subunit 4 of P-EPTX-Ar1a to be a glycoprotein as evidenced by the areas of heterogenous glycosylation observed in MALDI spectra [32] indicating subunit 4 as the γ-subunit of P-EPTX-Ar1a.

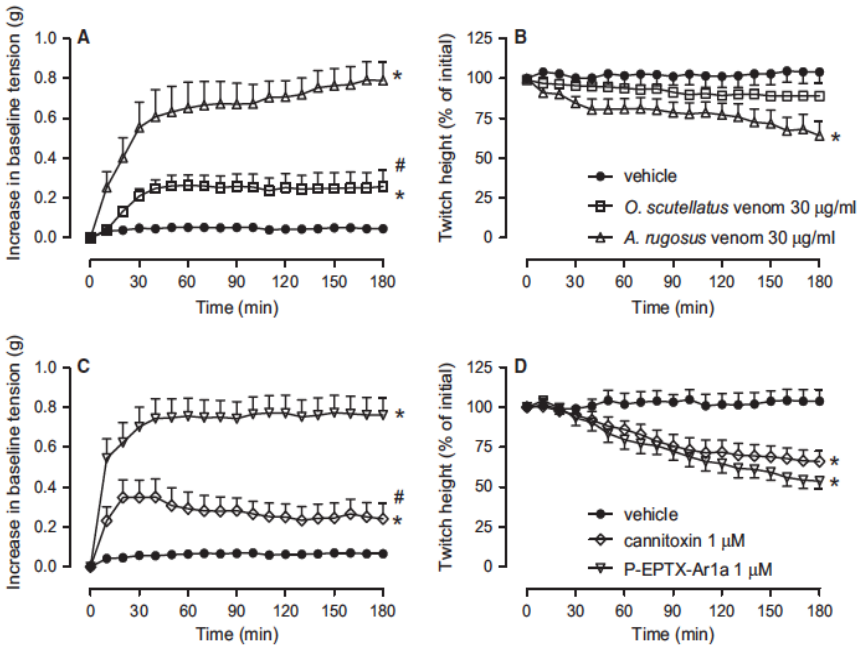

Fig. 3. The effect of *Acanthophis rugosus* venom and *Oxyuranus scutellatus* venom (30 µg/ml; n = 5) or vehicle (n = 5) on (A) baseline tension or (B) direct twitches of CBCNM preparation at the 180-min. time point. The effect of P-EPTX-Ar1a and cannitoxin (1 µM; n = 6) or vehicle (n = 5) on (C) baseline tension or (D) direct twitches of CBCNM preparation at the 180-min. time point. *$p < 0.05$, significantly different from vehicle, one-way ANOVA. #$p < 0.05$, is significantly different from *A. rugosus* venom or P-EPTX-Ar1a, one-way ANOVA.

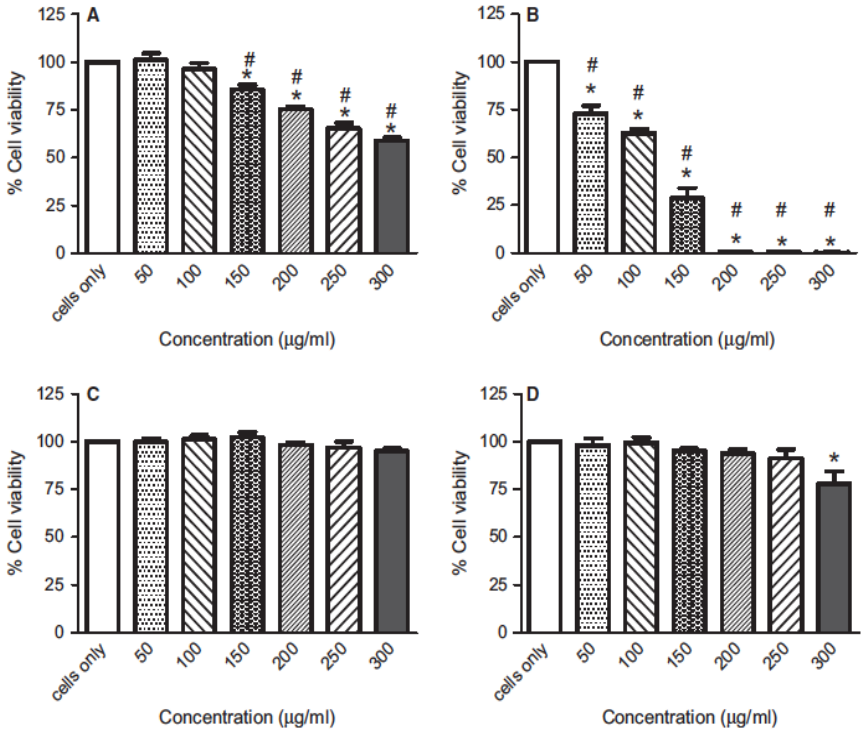

Fig. 4. Cell viability of L6 rat skeletal muscle cells after incubation with *Acanthophis rugosus* venom (50–300 µg/ml; n = 4) for (A) 1 hr or (B) 24 hr and *Oxyuranus scutellatus* venom (50–300 µg/ml; n = 4) for (C) 1 hr or (D) 24 hr. *$p < 0.05$, significantly different from cells only (one-way ANOVA). #$p < 0.05$, significantly different from *O. scutellatus* venom, Student's unpaired *t*-test.

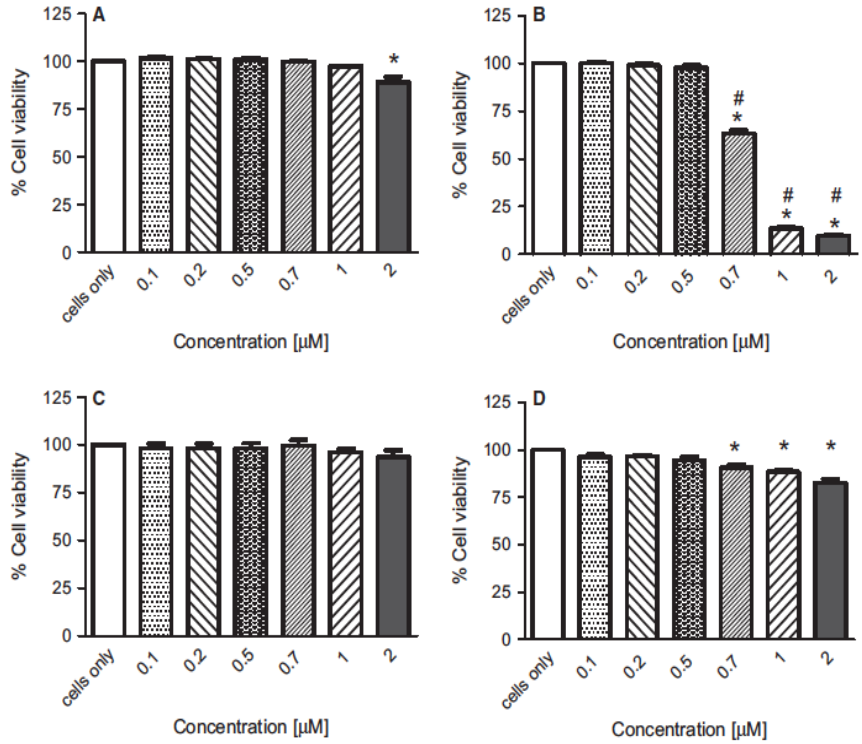

Fig. 5. Cell viability of L6 rat skeletal muscle cells after incubation with P-EPTX-Ar1a (0.1–2 μM; n = 4) for (A) 1 hr or (B) 24 hr and cannitoxin (0.1–2 μM; n = 4) for (C) 1 hr or (D) 24 hr *$p < 0.05$, significantly different from cell only (one-way ANOVA). #$p < 0.05$, significantly different from cannitoxin, Student's unpaired *t*-test.

Inhibition of direct twitches and a rise in baseline tension have been postulated to be indicative of myotoxic activity [37]. In this study, *A. rugosus* (10–30 μg/ml) venom, P-EPTX-Ar1a and cannitoxin (0.1–1 μM) showed myotoxic effects in the chick biventer cervicis skeletal muscle preparation. Despite having a greater proportion of pre-synaptic neurotoxin in the whole venom, *O. scutellatus* venom failed to induce a significant increase in baseline tension and had less inhibitory effect on direct twitches.

Numerous studies indicate that PLA_2s of classes I (elapid venoms) and II (viperid venoms) [4,38] generate a rapid functional and structural disruption of plasma membranes [39]. We measured PLA_2 activity of the venoms and pre-synaptic neurotoxins to determine whether PLA_2 activity correlates with myotoxic/cytotoxic activities. *Oxyuranus scutellatus* venom displayed the greatest PLA_2 activity. Interestingly, no direct relationship was found between the degree of PLA_2 activity and the myotoxic/cytotoxic activities of venom suggesting that the pharmacological effects induced by PLA_2 are independent of its catalytic activity [40] or that the toxin may contain a membrane-damaging region separate from the catalytic site [41].

An MTS assay in the rat skeletal muscle cell line (L6) was used to determine cell viability. This assay has previously been used by our laboratory to examine the cytotoxicity of a range of elapid snake venoms [25]. *Oxyuranus scutellatus* venom was markedly less cytotoxic than *A. rugosus* venom. *Acanthophis rugosus* venom (150–300 μg/ml) affected cells after 1-hr incubation suggesting the possibility of rapid membrane disruption or tissue necrosis. After 24-hr incubation, *A. rugosus* venom reduced cell viability at all concentrations (50–300 μg/ml) tested. P-EPTX-Ar1a was more cytotoxic than cannitoxin after 24-hr incubation, indicating that the cytotoxic effects of the pre-synaptic neurotoxin do not depend on PLA_2 activity. In this study, large concentrations of whole venoms were required to produce cytotoxicity, suggesting the venom component(s) responsible for cell death is only a minor component of the whole venom [25].

LDH levels were measured as an indicator of cytotoxic activity. The cytosolic enzyme 'LDH' is released from cells as a result of the loss of membrane integrity from necrosis, as the leakage of cell contents. The integrity of the cell membrane is lost as a result of the binding of the cytotoxin to membrane proteins, rendering the cell susceptible to actions by PLA_2, which may influence a wide range of membrane mechanisms [42]. *Acanthophis rugosus* venom and P-EPTX-Ar1a induced a significant increase in LDH release, which appears to suggest plasma membrane disruption. In contrast, only a small proportion of cells were lysed following 1-hr exposure to *O. scutellatus* venom or cannitoxin supporting the apparent lack of rapid cytotoxic activity for this species.

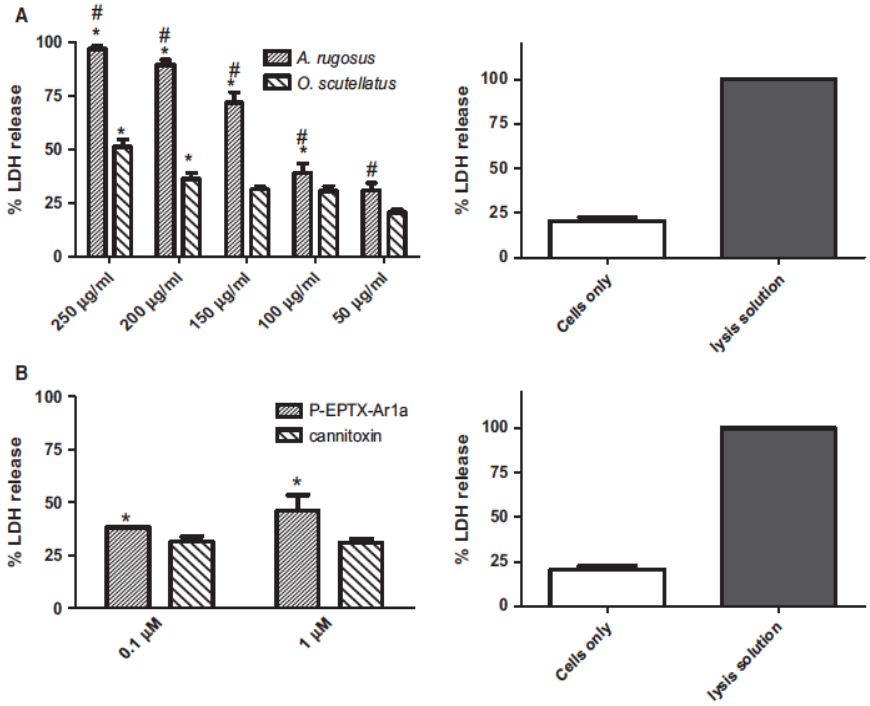

Fig. 6. Lactate dehydrogenase (LDH) release in L6 rat skeletal muscle cells following 1-hr incubation with (A) *Acanthophis rugosus* venom or *Oxyuranus scutellatus* venom (50–250 µg/ml; n = 4) and (B) P-EPTX-Ar1a and cannitoxin (1–2 µM; n = 4). A lysis control was used to display maximum cell lysis. $^{*}p < 0.05$ compared to cell control; one-way ANOVA followed by Tukey's multiple-comparison test. $^{\#}p < 0.0001$ compared to *O. scutellatus* venom; two-way ANOVA.

In conclusion, *A. rugosus* venom displayed marked *in vitro* myotoxic and cytotoxic activities while *O. scutellatus* venom displayed a mild effect on the CBCNM preparation and L6 skeletal muscle cell line. The isolated pre-synaptic neurotoxins (i.e. cannitoxin and P-EPTX-Ar1a) induced significant effects on the CBCNM preparation. The myotoxic/cytotoxic activities were not related to PLA_2 activity or the proportion of toxin in the whole venom. This study exhibited the myotoxic activity of pre-synaptic PLA_2 neurotoxins of Australasian elapids. To understand the mechanism behind the cytotoxicity of these isolated toxins, the role of extracellular Ca^{2+} and an electrophysiological examination are required to determine Ca^{2+} influx after membrane disruption.

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References

1 Currie BJ, Sutherland SK, Hudson BJ, Smith MA. An epidemiological study of snake bite envenomation in Papua New Guinea. Med J Aust 1991;**154**:266–8.
2 Currie BJ. Snakebite in tropical Australia, Papua New Guinea and Irian Jaya. Emergency Medicine 2000;**12**:285–94.
3 Lomonte B, Rangel J. Snake venom Lys49 myotoxins: from phospholipases A(2) to non-enzymatic membrane disruptors. Toxicon 2012;**60**:520–30.
4 Mebs D, Ownby CL. Myotoxic components of snake venoms: their biochemical and biological activities. Pharmacol Ther 1990;**48**:223–36.
5 Kuruppu S, Isbister GK, Hodgson WC. Phospholipase A_2-dependent effects of the venom from the New Guinean small-eyed snake *Micropechis ikaheka*. Muscle Nerve 2005;**32**:81–7.
6 Tedesco E, Rigoni M, Caccin P, Grishin E, Rossetto O, Montecucco C. Calcium overload in nerve terminals of cultured neurons intoxicated by alpha-latrotoxin and snake PLA_2 neurotoxins. Toxicon 2009;**54**:138–44.
7 Ponce-Soto LA, Barros JC, Marangoni S, Hernandez S, Dal Belo CA, Corrado AP *et al.* Neuromuscular activity of BaTX, a presynaptic basic PLA_2 isolated from *Bothrops alternatus* snake venom. Comp Biochem Physiol C: Toxicol Pharmacol 2009;**150**:291–7.
8 Mora R, Valverde B, Diaz C, Lomonte B, Gutierrez JM. A Lys49 phospholipase A(2) homologue from *Bothrops asper* snake venom induces proliferation, apoptosis and necrosis in a lymphoblastoid cell line. Toxicon 2005;**45**:651–60.

9 Lomonte B, Gutierrez JM. A new muscle damaging toxin, myotoxin II, from the venom of the snake *Bothrops asper* (terciopelo). Toxicon 1989;**27**:725–33.

10 Leite RS, Franco W, Ownby CL, Selistre-de-Araujo HS. Effects of ACL myotoxin, a Lys49 phospholipase A_2 from *Agkistrodon contortrix laticinctus* snake venom, on water transport in the isolated toad urinary bladder. Toxicon 2004;**43**:77–83.

11 Wuster W, Dumbrell AJ, Hay C, Pook CE, Williams DJ, Fry BG. Snakes across the Strait: trans-Torresian phylogeographic relationships in three genera of Australasian snakes (Serpentes: Elapidae: *Acanthophis*, *Oxyuranus*, and *Pseudechis*). Mol Phylogenet Evol 2005;**34**:1–14.

12 Vargas M, Segura A, Herrera M, Villalta M, Estrada R, Cerdas M *et al.* Preclinical evaluation of caprylic acid-fractionated IgG antivenom for the treatment of Taipan (*Oxyuranus scutellatus*) envenoming in Papua New Guinea. PLoS Negl Trop Dis 2011;**5**:e1144.

13 Lalloo DG, Trevett AJ, Owens D, Minei J, Naraqi S, Saweri A *et al.* Coagulopathy following bites by the Papuan taipan (*Oxyuranus scutellatus canni*). Blood Coagul Fibrinolysis 1995;**6**:65–72.

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

15 Lalloo DG, Trevett AJ, Korinhona A, Nwokolo N, Laurenson IF, Paul M *et al.* Snake bites by the Papuan taipan (*Oxyuranus scutellatus canni*): paralysis, hemostatic and electrocardiographic abnormalities, and effects of antivenom. Am J Trop Med Hyg 1995;**52**:525–31.

16 Harris JB, Maltin CA. Myotoxic activity of the crude venom and the principal neurotoxin, taipoxin, of the Australian taipan, Oxyuranus scutellatus. Br J Pharmacol 1982;**76**:61–75.

17 Crachi MT, Hammer LW, Hodgson WC. A pharmacological examination of venom from the Papuan taipan: (*Oxyuranus scutellatus canni*). Toxicon 1999;**37**:1721–34.

18 Currie BJ. An epidemiological study of snake bite envenomation in Papua New Guinea [3]. Med J Aust 1992;**156**:290.

19 Mebs D, Samejima Y. Purification, from Australian elapid venoms, and properties of phospholipases A which cause myoglobinuria in mice. Toxicon 1980;**18**:443–54.

20 Sutherland SK, Coulter AR, Harris RD, Lovering KE, Roberts ID. A study of the major australian snake venoms in the monkey (*Macaca fascicularis*). I. The movement of injected venom, methods which retard this movement, and the response to antivenoms. Pathology 1981;**13**:13–27.

21 Lalloo DG, Trevett AJ, Black J, Mapao J, Saweri A, Naraqi S *et al.* Neurotoxicity, anticoagulant activity and evidence of rhabdomyolysis in patients bitten by death adders (*Acanthophis* sp.) in southern Papua New Guinea. QJM 1996;**89**:25–35.

22 Wickramaratna JC, Fry BG, Hodgson WC. Species-dependent variations in the vitro myotoxicity of death adder (*Acanthophis*) venoms. Toxicol Sci 2003;**74**:352–60.

23 Wickramaratna JC, Fry BG, Aguilar MI, Kini RM, Hodgson WC. Isolation and pharmacological characterization of a phospholipase A(2) myotoxin from the venom of the Irian Jayan death adder (*Acanthophis rugosus*). Br J Pharmacol 2003;**138**:333–42.

24 Hart AJ, Smith AI, Reeve S, Hodgson WC. Isolation and characterisation of acanmyotoxin-2 and acanmyotoxin-3, myotoxins from the venom of the death adder *Acanthophis* sp. Seram. Biochem Pharmacol 2005;**70**:1807–13.

25 Kalam Y, Isbister GK, Mirtschin P, Hodgson WC, Konstantakopoulos N. Validation of a cell-based assay to differentiate between the cytotoxic effects of elapid snake venoms. J Pharmacol Toxicol Methods 2010;**63**:137–42.

26 Isbister GK, Halkidis L, O'Leary MA, Whitaker R, Cullen P, Mulcahy R *et al.* Human anti-snake venom IgG antibodies in a previously bitten snake-handler, but no protection against local envenoming. Toxicon 2010;**55**:646–9.

27 Chang CC, Su MJ. Mutual potentiation, at nerve terminals, between toxins from snake venoms which contain phospholipase A activity: [beta]-bungarotoxin, crotoxin, taipoxin. Toxicon 1980;**18**:641–8.

28 Harris JB, Johnson MA. Further observations on the pathological responses of rat skeletal muscle to toxins isolated from the venom of the Australian tiger snake, *Notechis scutatus scutatus*. Clin Exp Pharmacol Physiol 1978;**5**:587–600.

29 Dixon RW, Harris JB. Myotoxic activity of the toxic phospholipase, notexin, from the venom of the Australian tiger snake. J Neuropathol Exp Neurol 1996;**55**:1230–7.

30 Lipps BV. Isolation of subunits, alpha, beta and gamma of the complex taipoxin from the venom of Australian taipan snake (*Oxyuranus s. scutellatus*): characterization of beta taipoxin as a potent mitogen. Toxicon 2000;**38**:1845–54.

31 Melo PA, Burns CF, Blankemeyer JT, Ownby CL. Membrane depolarization is the initial action of crotoxin on isolated murine skeletal muscle. Toxicon 2004;**43**:111–9.

32 Kuruppu S, Reeve S, Banerjee Y, Kini RM, Smith AI, Hodgson WC. Isolation and pharmacological characterization of cannitoxin, a presynaptic neurotoxin from the venom of the papuan taipan (*Oxyuranus scutellatus canni*). J Pharmacol Exp Ther 2005;**315**:1196–202.

33 Chaisakul J, Konstantakopoulos N, Smith AI, Hodgson WC. Isolation and characterisation of P-EPTX-Ap1a and P-EPTX-Ar1a: presynaptic neurotoxins from the venom of the northern (*Acanthophis praelongus*) and Irian Jayan (*Acanthophis rugosus*) death adders. Biochem Pharmacol 2010;**80**:895–902.

34 Blacklow B, Escoubas P, Nicholson GM. Characterisation of the heterotrimeric presynaptic phospholipase A(2) neurotoxin complex from the venom of the common death adder (*Acanthophis antarcticus*). Biochem Pharmacol 2010;**80**:277–87.

35 Aquilina JA. The major toxin from the Australian Common Brown Snake is a hexamer with unusual gas-phase dissociation properties. Proteins 2009;**75**:478–85.

36 Harris JB, Johnson MA, Macdonell C. Taipoxin, a presynaptically active neurotoxin, destroys mammalian skeletal muscle [proceedings]. Br J Pharmacol 1977;**61**:133P.

37 Harvey AL, Barfaraz A, Thomson E, Faiz A, Preston S, Harris JB. Screening of snake venoms for neurotoxic and myotoxic effects using simple in vitro preparations from rodents and chicks. Toxicon 1994;**32**:257–65.

38 Harris JB, Cullen MJ. Muscle necrosis caused by snake venoms and toxins. Electron Microsc Rev 1990;**3**:183–211.

39 Gutierrez JM, Ownby CL. Skeletal muscle degeneration induced by venom phospholipases A_2: insights into the mechanisms of local and systemic myotoxicity. Toxicon 2003;**42**:915–31.

40 Kini RM. Excitement ahead: structure, function and mechanism of snake venom phospholipase A_2 enzymes. Toxicon 2003;**42**:827–40.

41 Bultron E, Gutierrez JM, Thelestam M. Effects of *Bothrops asper* (terciopelo) myotoxin III, a basic phospholipase A_2, on liposomes and mouse gastrocnemius muscle. Toxicon 1993;**31**:217–22.

42 Dufton MJ, Hider RC. Structure and pharmacology of elapid cytotoxins. Pharmacol Ther 1988;**36**:1–40.

1. Introduction

The following work raised interesting questions regarding time-dependent viability of cultured cell and morphological changes in the skeletal muscle preparation treated with venoms/purified toxins. Therefore, addition experiments were carried out. This section includes additional work not included in the manuscript published in Basic & Clinical Pharmacology & Toxicology.

2. Material and Methods (continued)

2.1 Cell proliferation assay

As per the manuscript.

2.2 Morphological Studies

Following the *in vitro* experiments, the tissues were placed in Tissue Tek, frozen with liquid nitrogen and stored at -80°C until required. The tissues were cut into transverse sections (14 μm) using a Leica CM1800 cryostat and placed onto gelatin-coated slides. Tissue sections were post fixed for 15 min in a solution containing 4% paraformaldehyde in distilled water, stained with haematoxylin and eosin and examined under a light microscope (Olympus BX 51, Olympus Optical Co., Japan). Slides were photographed using an Olympus C-4040ZOOM (Olympus Optical Co., Japan) digital camera.

2.3 Chemicals in Morphological Studies

The following chemicals were used: eosin and Mayer's Haemalum (Sigma Chemical Co., St. Louis, MO, U.S.A.).

2.4 Data analysis

Statistical analysis of the cell proliferation assay data is as per manuscript.

3. Results

3.1 Cell Viability assay

A. rugosus venom (200-300 µg/ml) significantly ($P < 0.05$, $n = 4$) inhibited cell proliferation of L6 cells following a period of incubation of 2, 3 or 5 h (Figures 1a-c). *O. scutellatus* venom (50-300 µg/ml) did not reduce cell viability after 2, 3 or 5 h incubation (Figures 1d-f).

3.2 Morphological Studies

Tissues exposed to *A. rugosus* venom (30 µg/ml; Figure 2b) showed slight morphological changes compared with tissues exposed to *O. scutellatus* venom (30 µg/ml; Figure 2c) or vehicle (Figure 2a). In contrast, P-EPTX-Ar1a (1 µM; Figure 2d) and cannitoxin (1 µM; Figure 2e) induced marked morphological changes. These changes included edema, vacuoles and disintegration of myofibers.

4. Discussion (continued)

Further discussion concerning the additional data is included in the General Discussion (Chapter 9).

Figures

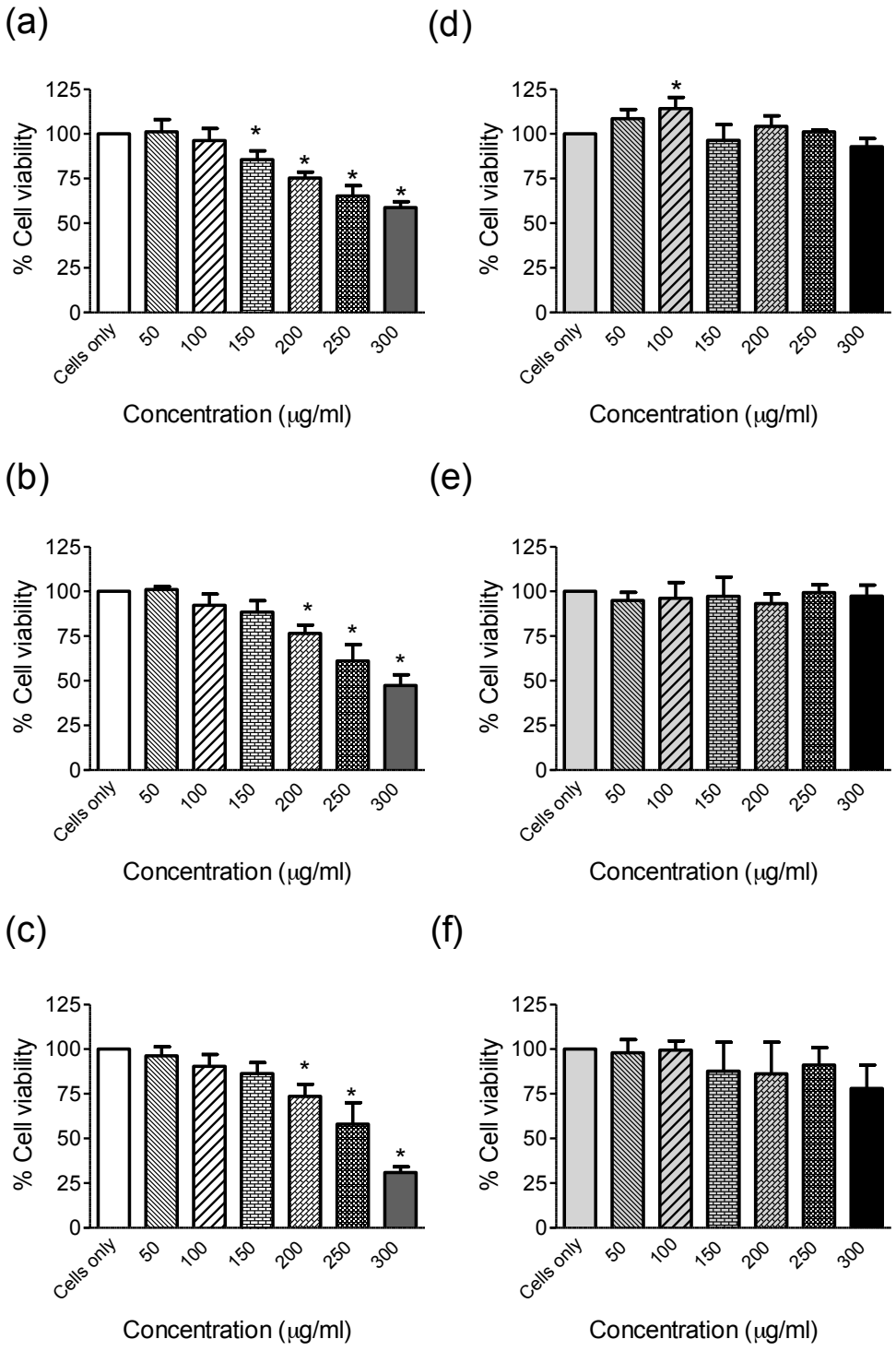

Figure 1: *Cell viability of L6 rat skeletal muscle cells after incubation with A. rugosus venom (50-300 μg/ml; n = 4) for 2 (a), 3 (b) or 5 hours (c), and cell viability of L6 rat skeletal muscle cells after incubation with O. scutellatus venom (50-300 μg/ml; n = 4) for 2 (d), 3 (e) or 5 hours (f).* ***P** < 0.05, significantly different from cells only (one-way-repeated measures ANOVA).*

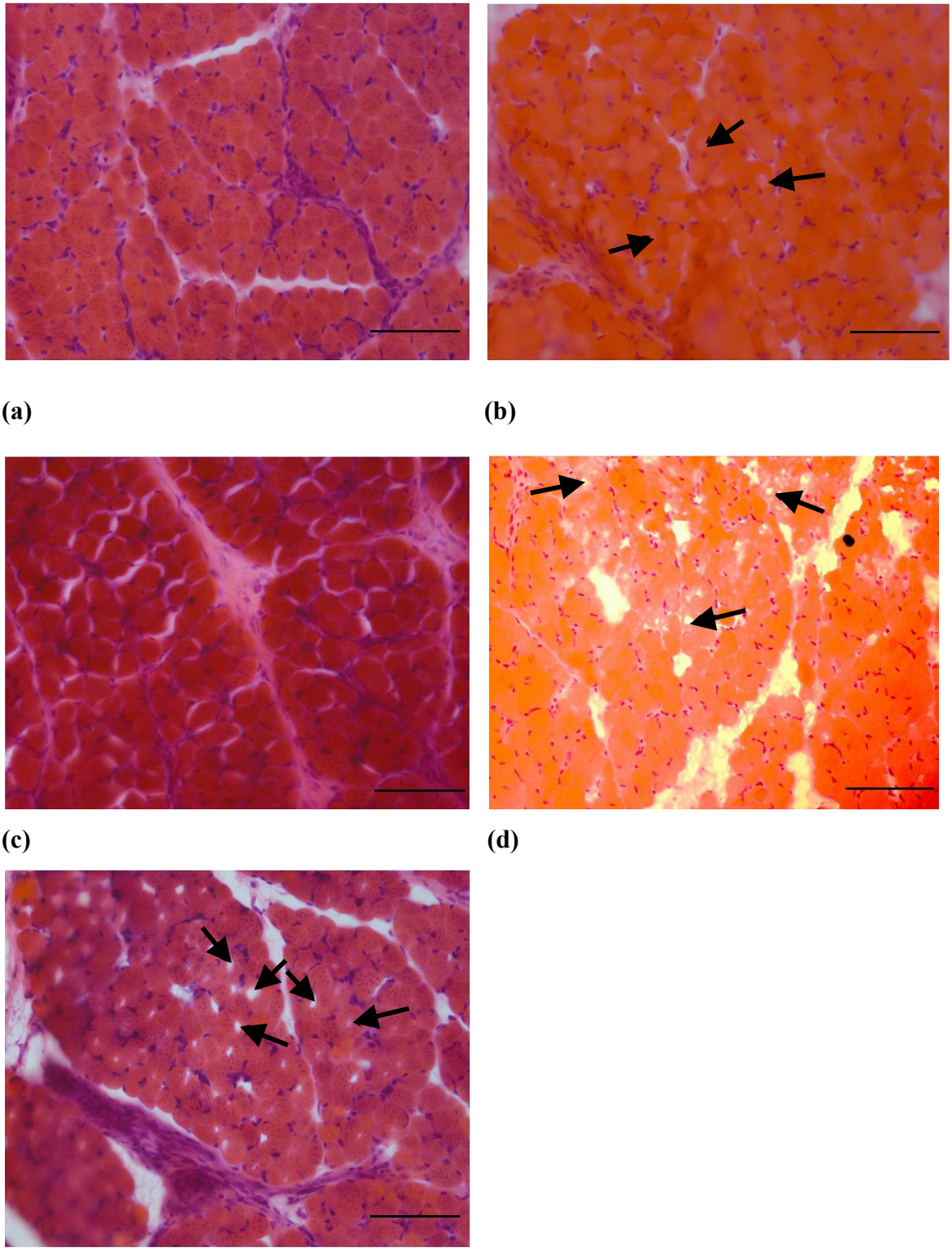

Figure 2: *Transverse sections of CBCNM preparation exposed to (a) vehicle, (b) A. rugosus venom (30 µg/ml), (c) O. scutellatus venom (30 µg/ml), (d) P-EPTX-Ar1a (1 µM) or (e) cannitoxin (1 µM). Scale bars, 50 µm in all micrographs. Arrows indicate prominent vacuoles.*

CHAPTER 4

THE EFFECT OF THE PRE-SYNAPTIC NEUROTOXIN FROM IRIAN JAYAN DEATH ADDER VENOM ON CYTOPLASMIC CALCIUM IN MOUSE DORSAL ROOT GANGLION NEURONS

Monash University

Declaration for Thesis Chapter 4

This chapter is made up of the following publication.

Declaration by candidate

In the case of Chapter 4, the nature and extent of my contribution to the work was the following:

Nature of Contribution	Extent of Contribution (%)
I declare that experiments and writing of this manuscript were solely undertaken by the myself, taking into consideration the advice and recommendations of co-authors	70 %

The following co-authors contributed to the work.

Name	Nature of Contribution
Ms Mary A Tonta	Performed experiments and data analysis
Professor Wayne C Hodgson	Manuscript preparation
Dr Harold A Coleman	Provision of expertise, data analysis, development of ideas, manuscript preparation
Professor Helena C Parkington	Provision of expertise, development of ideas, manuscript preparation

Candidate's signature: [redacted]: 07.06.2013

Declaration by co-authors

The undersigned hereby certify that:

(1) the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors;
(2) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(3) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(4) there are no other author of the publication according to these criteria;
(5) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journal or other publications and (c) the head of the responsible academic unit; and
(6) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location (s) Department of Physiology and Department of Pharmacology, Monash University

		Date
Ms Mary A Tonta	[redacted]	06.06.2013
Professor Wayne C Hodgson	[redacted]	06.06.2013
Dr Harold A Coleman	[redacted]	06.06.2013
Professor Helena C Parkington	[redacted]	06.06.2013

Irian Jayan death adder (*Acanthophis rugosus*) neurotoxin activates rapid cytoplasmic Ca^{2+} influx after cationic channel activation in mouse neurons

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****This manuscript has been submitted to the British Journal of Pharmacology.**

Summary

BACKGROUND AND PURPOSE. Snake neurotoxins induce neurotoxic effects that include disruption of ion channel activity and changes in membrane potential. P-EPTX-Ar1a, a neurotoxin from Irian Jayan death adder (*Acanthophis rugosus*) venom, previously isolated and characterized by us, induces neurotoxicity. Here we investigated the mechanisms mediating this neurotoxic effect.

EXPERIMENTAL APPROACH. We studied embryonic day 16-18 mouse primary dorsal root ganglion (DRG) neurons plated onto glass coverslips and studied within 6-8 hours. The cells were loaded with the Ca^{2+} fluorophore Fluo-4-AM, and membrane currents were recorded using the patch-clamp technique in whole-cell mode.

KEY RESULTS. P-EPTX-Ar1a (69-230 nM) evoked a rapid increase in intracellular Ca^{2+} which, at the highest concentrations, induced retraction of projections and dissociation of neurons from the glass coverslips. Ca^{2+} influx did not occur in Ca^{2+}-free solution containing EGTA but persisted in the presence of an endoplasmic reticulum-specific Ca^{2+}ATPase inhibitor, cyclopiazonic acid. Ca^{2+} influx was significantly reduced by tetrodotoxin, nifedipine or agatoxin indicating a role for voltage-gated Na^{+} and L-type and P/Q-type Ca^{2+} channels. In whole cell patch clamp, P-EPTX-Ar1a (0.17 μM) rapidly and reversibly induced an inward current in DRG neurons which was significantly reduced by the cationic channel blocker SKF 96365 or upon removal of extracellular Na^{+}, but not by changing the Cl^{-} gradient.

CONCLUSIONS AND IMPLICATIONS. P-EPTX-Ar1a induces membrane depolarization following activation of a cationic channel that mainly carries Na^{+} in DRG neurons. Depolarization increases Ca^{2+} influx through voltage-gated L- and P/Q-type channels, resulting in a neurotoxin-induced increase in cytoplasmic Ca^{2+}.

Key words: cytoplasmic calcium**,** venom, dorsal root ganglion, pre-synaptic neurotoxin

Abbreviations: CPA, cyclopiazonic acid; DRG, dorsal root ganglia; EGTA, ethylene glycol-bis (β-aminoethyl ether)-*N,N,N,N'*-tetraacetic acid; PLA_2, phospholipase A_2; TTX, tetrodotoxin; TRP, transient receptor potential; BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid tertrakis (acetoxymethyl ester); NMDA, N-methyl-D-aspartate; SKF 96365 (1-{β-[3-(4-methoxyphenyl)-propoxy]-4-methoxyphenethyl}-1H-imidazole hydro-chloride).

Introduction

An abundant component of many elapid venoms, is phospholipase A_2 (PLA_2, EC 3.1.1.4) (Fry, 1999). Snake pre-synaptic PLA_2 neurotoxins (SPANs) are key toxic components of snake venoms of families Elapidae (e.g. taipoxin from Australian taipan, *Oxyuranus scutellatus*, β-bungarotoxin from banded krait, *Bungarus multicinctus*), Crotalidae (e.g. crotoxin from South American rattlesnake, *Crotalus durissus terrificus*) and Viperidae (e.g. ammodytoxin A from the viper, *Vipera ammodytes ammodytes*). PLA_2 hydrolyses the *sn*-2 ester bond of 3-*sn*-phosphoglycerides, releasing lysophospholipids (LysoPLs) and fatty acids (FAs) (Harris, 1985; Rossetto *et al.*, 2004). However, as pointed out elegantly by Kini, specific pharmacological sites on the SPAN molecules bind at specific target recognition sites on the surfaces of target tissues or membranes, but this binding does not necessarily involve activation of PLA_2 enzyme activity (Kini, 2003). Thus, the immediate visible effects of envenomation might be generated by mechanisms which are independent of PLA_2 enzymatic activity and, instead, reflect initial toxin binding with other membrane proteins e.g. ion channels. The pharmacological activity of SPANs is characterized by disruption of acetylcholine (ACh) transmission at motor nerve terminals which can either facilitate, as with dendrotoxin from *Dendroaspis angusticeps*, or block neurotransmitter release, as with β-bungarotoxin (Rowan, 2001). Moreover, SPANs also induce myotoxicity or peripheral muscle damage (Harris and Maltin, 1982) due to persistent plasma membrane depolarization (Melo *et al.*, 2004).

Effects of SPANs that may be independent of PLA_2 enzyme activation include, for example: Inhibition of voltage-gated K^+ channels which can delay repolarization of the nerve terminal after the action potential, resulting in a prolonged opening of voltage-gated Ca^{2+} channels, Ca^{2+} influx in the nerve terminal, and hence transmitter release. Dendrotoxin (Penner *et al.*, 1986) and β-bungarotoxin (Petersen *et al.*, 1986) reduced a slow, non-inactivating component of the K^+ current in guinea-pig dorsal root ganglion (DRG) neurons. However, not all SPANs have inhibitory effects on voltage-gated K^+ channels (Fathi *et al.*, 2001; Hodgson *et al.*, 2007).

A rise in cytoplasmic Ca^{2+} in response to SPANs has been demonstrated in mammalian neurons (Rigoni *et al.*, 2007; Rigoni *et al.*, 2004; Tedesco *et al.*, 2009). β-bungarotoxin induces an increase in cytoplasmic Ca^{2+} and neurotoxicity in cultured cerebellar granule neurons (Tseng and Lin-Shiau, 2003b). These effects are attenuated by BAPTA-AM, EGTA, MK801 and diltiazem suggesting that the neurotoxic effect was generated by activation of NMDA receptors and opening of L-type Ca^{2+} channels, resulting in intracellular Ca^{2+} overload (Tseng and Lin-Shiau, 2003b). This also triggers nitric oxide (NO) production, as NO synthase inhibitors were neuroprotective against β-bungarotoxin (Tseng and Lin-Shiau, 2003a). This suggests that NO may contribute to neuronal cell death.

We have previously purified a pre-synaptic neurotoxin (P-EPTX-Ar1a) from Irian Jayan death adder (*Acanthophis rugosus*) venom (Chaisakul *et al.*, 2010). This toxin caused neurotoxicity, indicated by an inhibition of nerve-induced twitches in the chick biventer cervicis nerve-muscle preparation (Chaisakul *et al.*, 2010). Furthermore, we have reported that P-EPTX-Ar1a induces myotoxic and cytotoxic activities in skeletal muscle tissue and L6 cells, a skeletal muscle cell line (Chaisakul *et al.*, 2013). These disturbances may involve an increase in cytoplasmic Ca^{2+}. In addition, evidence that snake PLA_2 neurotoxins change neuronal membrane potential is limited. Here, we hypothesized that P-EPTX-Ar1a induces disruption of function through membrane depolarization which leads to rapid Ca^{2+} influx. Numerous studies have used cerebellar granule neurons (CGNs) to examine the neurotoxicity of snake PLA_2 neurotoxins. However, snake PLA_2 toxins do not cross the blood-brain barrier (Montecucco *et al.*, 2008). Therefore, mouse DRGs were used in the current study since they represent a peripheral sensory neuron which is more likely to be a target of snake venoms/toxins. P-EPTX-Ar1a rapidly and reversibly activated a cationic channel and evoked a rise in intracellular Ca^{2+} as a result of extracellular Ca^{2+} influx.

Methods

Toxin isolation

Freeze-dried venom of *A. rugosus* was purchased from Venom Supplies (Venom Supplies Pty Ltd, Tanunda, SA, Australia). Venom was dissolved in Milli-Q H_2O and insoluble material was removed by centrifugation at 10,000 x g at 4°C for 8 min. The supernatant was subjected to chromatographic separation using a Shimadzu (Kyoto, Japan) high-performance liquid chromatography system (LC-10ATvp pump and SPD-10AVP detector). Venom (3 mg) was applied to a Superdex G-75 column (13 µm; 10 × 300mm; GE Healthcare, Buckinghamshire, UK) equilibrated with ammonium acetate buffer (0.1 M; pH 6.8). The sample was eluted at a flow rate of 0.5 ml/min. The eluent was monitored at 280 nm. Fractions were collected and freeze-dried prior to study. Subunits of P-EPTX-Ar1a were disassociated by RP-HPLC. Protein content of the isolated toxin was determined utilizing a BCA protein assay kit according to the manufacturer's instructions (Pierce Biotechnology, IL, USA). Briefly, isolated toxin was added in triplicate to a 96-well micro-titer plate. Bovine serum albumin solutions, diluted from 0.025 - 1 mg/ml, were used as reference standards and distilled water was used as a blank. Absorbance was measured at 562 nm utilizing a fusion α microplate reader (Perkin Elmer, MA, USA). To confirm the purity of P-EPTX-Ar1a, the molecular mass was determined using MALDI-TOF mass spectrometry, the amino acid sequence was determined using Edman degradation, phenylthiohydantoin derivatization chemistry, and separation of derivatized amino acids by RP-HPLC was performed (Chaisakul *et al.*, 2013). Neurotoxic activity of P-EPTX-Ar1a was confirmed using the isolated chick biventer cervicis nerve-muscle preparation as described previously (Chaisakul *et al.*, 2010).

Cell isolation

Swiss albino mice (34 animals) were deeply anaesthetized using isoflurane followed by decapitation on day 16 to 18 of pregnancy. The anaesthetized fetuses (E16-18) were rapidly placed on ice and decapitated. These procedures are in accordance with the National Health and Medical Research Council of Australia guidelines for the treatment and management of experimental

animals. Ethics approval was obtained from Monash University Animal Research Platform Ethics Committee-1 prior to commencement of the study. All thoracic and lumbar DRG from each fetus were isolated and gently triturated mechanically in 200 µl Hank's solution. DMEM/F12 (800 µl) that included 5% heat-inactivated fetal calf serum (FCS), nerve growth factor (NGF, 0.5 ng/ml), N-2 supplement (10 µl/1 ml), and glia-derived neurotrophic factor (GDNF, 2 ng/ml) was then added to the cell triturate. The mixture was plated onto 9 mm glass coverslips, previously coated with poly-L-ornithine and laminin (~50,000 cells per coverslip), and placed in an incubator for 1h at 36°C to settle. The medium in each well (containing one coverslip) was then topped up with 1ml of warmed DMEM/F12 and incubated for a further 30-60 min before starting experiments. The cells were used within 6 h of plating.

Ca^{2+} imaging

DRG cells were loaded with Fluo-4-AM (5µM) for 15 min at room temperature. Coverslips with loaded cells were placed in a Warner tissue bath (220-250 µl volume, Warner Instruments, Hamden, CT, USA) mounted on a confocal inverted microscope (Olympus IX71) and continuously superfused with Hank's solution containing (mM): NaCl 137; KCl 5.4; $NaHCO_4$ 4; glucose 5.6; KH_2PO_4 0.44; $MgSO_4$ 0.4; $CaCl_2$ 1.5; $MgCl_2$ 0.5; Na_2HPO_4 0.3; HEPES 10, at 3 ml/min and 33°C.

To measure cytoplasmic free calcium $[Ca^{2+}]_i$, the cells were excited by a krypton/argon laser at 488 nm and emission was recorded at 525nm, with the light passing through a Yokogawa CSU22 Nipkow spinning disk (Yokogawa Electric Corp, Tokyo, Japan) and captured by a high-sensitivity electron-multiplying Andor iXon CCD camera (Andor Technology, Belfast, Ireland). Full frames were collected at 1 frame per s. The effects of P-EPTX-Ar1a on cells were tested under different experimental conditions including normal Ca^{2+} and Ca^{2+}-free Hank's solutions. Analysis consisted of randomly selecting 10-12 cells from bright field images of cells on each coverslip and

an average of the fluorescence was obtained for the treatment response in that animal. The treatment was tested independently in 'n' animals.

A non-lethal concentration of P-EPTX-Ar1a for DRG cells was first determined. The level of fluorescence was recorded in 10-12 DRG cells by selecting regions of interest (ROI) over individual cells, using the facility on the Andor iQ computer software. Cells were tested with a 10 s exposure to high K^+ (100 mM, isosmotic Na^+ replacement) at the end of each experiment.

Electrophysiological recording

Coverslips of cells were transferred to a Warner bath and were continuously superfused with Hank's solution at room temperature. In some experiments, extracellular Na^+ was reduced and this solution contained (mM): tetraethyl ammonium (TEA) 102; NaCl 35; KCl 5.4; $NaHCO_4$ 4; glucose 5.6; KH_2PO_4 0.44; $MgSO_4$ 0.4; $CaCl_2$ 1.5; $MgCl_2$ 0.5; Na_2HPO_4 0.3; HEPES 10. Membrane currents were recorded using the patch-clamp technique in whole cell mode. Electrodes were pulled from glass (PG150T-15, Harvard Apparatus Ltd, UK) using a Sutter P-87 puller (Sutter Instruments, Novato, CA, USA). The tips of the electrodes were fire polished using a micro forge (Narishige MF-830, Japan) and the electrodes were back-filled with solution. One of two patch solutions was used: normal Cl^- solution containing (mM): KCl 135; HEPES 10; $MgCl_2$ 1.2; ATP 3; EGTA 0.3. Low Cl^- solution contained (mM): CsCl 15.4; Cs_2SO_4 72.4; HEPES 10; $MgCl_2$ 1.2; ATP 1; EGTA 0.3. Electrodes had resistances of 3-5 MΩ.

The cells were held at −70 mV before and during application of P-EPTX-Ar1a. Current-voltage relationships were determined by ramping the membrane potential between −110 and 0 or +20 mV over 200 ms. The current attributed to P-EPTX-Ar1a was determined by subtracting the current response to voltage ramps before P-EPTX-Ar1a application from the maximum current response, and in the continued presence of P-EPTX-Ar1a. Recordings were made using an Axopatch-200 amplifier and the data were analysed using pClamp (Axon Instruments, Foster City, CA USA).

The use of sulphate in the patch electrode as a substitute for chloride affected the junction potentials. The magnitude of the liquid junction potential was calculated and the reversal potentials were corrected accordingly.

Data analysis

The area under the curve of the Fluo-4 $[Ca^{2+}]_i$ signal response of P-EPTX-Ar1a for each cell was normalized in terms of the response to 10 s exposure to 100 mM K^+ solution. 10-12 cells per plate were analysed. A mean value from the 10-12 individual cells was used for statistical analysis. The response to P-EPTX-Ar1a is reported in terms of the % of cells responding to toxin over time of exposure and beyond. Thus, the % of cells responding for the first time in the first, second and third minutes, and not until after 4 min are shown separately (see Figs 2 and 3). Multiple comparisons were made using a one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. It was deemed unwise to expose the same cells to P-EPTX-Ar1a on two occasions, since the effect of P-EPTX-Ar1a did not wash out clearly. Thus, the effects of P-EPTX-Ar1a alone and in the presence of blockers of ion channels or transporter were studied in different plates. A P value of < 0.05 was accepted as significant. Data are expressed as mean ± SEM, with n = number of independently repeated plates.

Chemicals

The following reagents were used: Dulbecco's modified Eagle's medium (DMEM) and Hank's balanced salt solution (HBSS) (Gibco, Mt Waverley, Victoria, Australia); Fluo-4-AM (Molecular Probes, Eugene, OR, USA); ω-conotoxin GVIA (CTX GVIA) (Alomone Laboratories, Jerusalem, Israel); ω-agatoxin TK (AgTX TK) (Peptides International, Louisville, KY, USA); SKF-96365, EGTA, nifedipine, cyclopiazonic acid (CPA), MK801, poly-L-ornithine (Sigma-Aldrich, MO, USA); GDNF, NGF, N-2 and laminin (Invitrogen, Victoria, Australia); FCS (Bovogen Biologicals Pty. Ltd, Keilor, Vic. Australia).

Results

Cytoplasmic calcium increase in mice DRG neurons exposed to P-EPTX-Ar1a

When a lethal concentration of P-EPTX-Ar1a (0.23 μM or 10 μg/ml) was applied to DRG neurons for 4 min, a large increase in $[Ca^{2+}]_i$ occurred, with withdrawal of projections and dissociation of the soma from the glass coverslips (Fig. 1).

When the concentration of P-EPTX-Ar1a was reduced to 69 nM (3 μg/ml) a lethal effect was not observed (Fig. 2a), $[Ca^{2+}]_i$ increased in 83 ± 3% of neurons in each coverslip (n = 28 plates from 28 mice). Of those, 23 ± 4% of cells responded initially within the first minute, and this was joined by an additional 23 ± 4% of cells in the second minute and 18 ± 3% of cells in the third minute. In 36 ± 4% of cells there was no response until at least 4 min had elapsed, by which time the P-EPTX-Ar1a had been removed from the chamber (Fig. 2b). Following removal of P-EPTX-Ar1a, Ca^{2+} oscillations abated slowly (Fig. 2a; the blue line).

Effect of extracellular Ca^{2+} on P-EPTX-Ar1a induced increase in $[Ca^{2+}]_i$

Experiments using Hank's solution from which Ca^{2+} had been omitted for 1 min prior to and during the 4 min application of P-EPTX-Ar1a (69 nM) showed that approximately 3-10 % of cells displayed an increase in $[Ca^{2+}]_i$, while 77 ± 5% responded upon restoration of Ca^{2+} (Fig. 3b). However, including EGTA (150 μM) in the Ca^{2+}-free Hank's solution resulted in complete abolition of the $[Ca^{2+}]_i$ response to P-EPTX-Ar1a (Figs. 3a, c). Reintroduction of Ca^{2+} at the time of P-EPTX-Ar1a removal resulted in a prompt and large increase in $[Ca^{2+}]_i$ in 100% of cells (Figs. 3a, c).

Effects of ion channel blockers on the $[Ca^{2+}]_i$ response to P-EPTX-Ar1a

The increase in $[Ca^{2+}]_i$ induced by P-EPTX-Ar1a (69 nM) was unaffected by pre-incubation in ω-conotoxin (n = 6), which inhibits N-type voltage-gated Ca^{2+} channels. The P-EPTX-Ar1a-induced increase in $[Ca^{2+}]_i$ was reduced to 55 ± 6 % of cell in the presence of

nifedipine (n = 12) and to 46 ± 15 % by agatoxin (n = 5) suggesting the involvement of L- and P/Q-type voltage-gated Ca^{2+} channels, respectively (Fig. 4a). A combination of nifedipine plus agatoxin (n = 4) reduced the number of cells responding to P-EPTX-Ar1a to 22.5 ± 2.5 % (P < 0.0001, one-way ANOVA). Interestingly, blockade of voltage-sensitive Na^+ channels by TTX (n = 14) reduced the number of cells responding to P-EPTX-Ar1a to 41 ± 5 % (Fig. 4b).

To investigate the effects of P-EPTX-Ar1a on internal Ca^{2+} stores, the neurons were incubated in Hank's solution containing CPA (10 μM) for 2 min prior to and during application of P-EPTX-Ar1a (69 nM) for 4 min. The oscillations in $[Ca^{2+}]_i$ in response to P-EPTX-Ar1a persisted in the presence of CPA, suggesting releasing of Ca^{2+} from endoplasmic reticulum was not responsible for the rise in $[Ca^{2+}]_i$.

SKF 96365 blocks a variety of cationic channels (Iouzalen *et al.*, 1996), including Ca^{2+}-conducting transient receptor potential (TRP) channels (Dietrich *et al.*, 2006; Rae *et al.*, 2012) and, alone, it induced a small but sustained increase in $[Ca^{2+}]_i$. Incubation with SKF 96365 (10 μM) for 4 min before and during P-EPTX-Ar1a (69 nM) application significantly reduced the rise in $[Ca^{2+}]_i$ to 34 ± 6 % of cells (n = 5, P < 0.0001, one-way ANOVA, Fig. 4b).

Blockade of NMDA receptors with MK801 (1 μM) significantly reduced the increase in $[Ca^{2+}]_i$ evoked by P-EPTX-Ar1a (69 nM) (Fig. 4b).

Electrophysiological recording (patch-clamp)

From a holding potential of −70 mV, depolarizing voltage ramps (from −110 to 0 or +20 mV) were applied before and during application of P-EPTX-Ar1a (0.17 μM or 7 μg/ml). In normal Hank's solution, P-EPTX-Ar1a evoked an inward current within 1-2 min that was associated with an increase in membrane conductance. This current was reversed following washing out of the toxin (n = 5) (Fig. 5a). When Na^+ in the Hank's solution was reduced to 35 mM, the P-EPTX-Ar1a-induced inward current was promptly reversed (n = 8, Fig. 5b), suggesting an increase in Na^+ conductance by P-EPTX-Ar1a.

The membrane current was plotted against membrane potential during the voltage ramp to give the current-voltage (I-V) relationships. The current activated by P-EPTX-Ar1a was all but abolished in low Na^+ Hank's solution indicating the large Na^+ contribution (Fig. 5c).

SKF 96365 (10 μM) abolished the inward currents induced by P-EPTX-Ar1a (*n* = 6) (Fig. 6). The ionic conductance involved in the P-EPTX-Ar1a response was further probed using electrodes in which K^+ was replaced by the impermeant Cs^+, or Cl^- was replaced by impermeant SO_4^{2-} (Fig. 7). The reversal potential of the current evoked by P-EPTX-Ar1a, extrapolated from the Goldman-Hodgkin-Katz (GHK) equation of best fit, was not influenced by these substitutions, confirming the major contribution of Na^+ to the conductance.

Discussion

In this study, we have demonstrated that P-EPTX-Ar1a, a characterized neurotoxin from *A. rugosus* venom (Chaisakul *et al.*, 2010), induces a substantial increase in $[Ca^{2+}]_i$ in sensory neurons of mice. This rise in $[Ca^{2+}]_i$ results from the brisk activation of a cationic channel which depolarizes the neurons, increasing the probability of opening of voltage-gated L- and P/Q-type Ca^{2+} channels. While neurons recovered from more modest rises in $[Ca^{2+}]_i$, large increases in $[Ca^{2+}]_i$ resulted in retraction of growth cones or processes and detachment of the neuron from the substrate and, presumably, cell death.

Envenomation by snakes of the genus *Acanthophis* (i.e. death adders) can be a cause of severe morbidity in Australia (Johnston *et al.*, 2012) and Papua New Guinea (Currie, 2000). This results from neurotoxicity and myotoxicity (Isbister *et al.*, 2010). Pre-synaptic neurotoxins, which disrupt ACh release from nerve terminals, have been identified in many death adder venoms (Blacklow *et al.*, 2010a; Blacklow *et al.*, 2010b). SPAN-induced cellular disruptions may be generated by an increase in cell permeability, direct depolarization of the nerve terminal (Rugolo *et al.*, 1986), or enhanced Ca^{2+} entry during the action potential (Su and Chang, 1984). The significant rise in $[Ca^{2+}]_i$ in CGN neurons evoked by β-bungarotoxin which is responsible for massive depletion of ATP, culminating in cell death (Tseng and Lin-Shiau, 2003b). We have

recently reported myotoxic and cytotoxic activities induced by P-EPTX-Ar1a (Chaisakul *et al.*, 2013). In the current study, we investigated the possibility that P-EPTX-Ar1a might induce an increase in $[Ca^{2+}]_i$ and, if so, the nature of the ion channels involved. P-EPTX-Ar1a caused a rapid, though sometimes delayed, significant increase in $[Ca^{2+}]_i$. Maitotoxin, a non-peptide toxin from the dinoflagellate (*Gambierdiscus toxicus*), also increases $[Ca^{2+}]_i$ in a variety of cell types via influx mechanisms (Choi *et al.*, 1990; Gusovsky and Daly, 1990; Sinkins *et al.*, 2009) and not resulting from release of intracellular Ca^{2+} stores (Gutierrez *et al.*, 1997). In the present study, we considered the source of the increase in $[Ca^{2+}]_i$ evoked by P-EPTX-Ar1a in DRG neurons. Prevention of Ca^{2+} filling of the endoplasmic reticulum (ER) using CPA (or thapsigargin, data not shown) had no effect, indicating that the release of Ca^{2+} from ER is unlikely to be responsible for the increase in $[Ca^{2+}]_i$ by P-EPTX-Ar1a.

In the present study, P-EPTX-Ar1a application to DRG neurons under voltage clamp evoked a prompt inward current. This current was reversible following removal of P-EPTX-Ar1a. Reversal of the current was also achieved upon lowering extracellular Na^+, or by application to the superfusate of SKF 96365, a poorly-selective blocker of cation channels. These results suggest the possibility of a very rapid binding of the toxin to a cation channel, and is in keeping with the specific target recognition site theory outlined by Kini (Kini, 2003). The increase in Ca^{2+} influx in brainstem neurons in response to maitotoxin when external Na^+ was lowered (Kakizaki *et al.*, 2006) could also indicate involvement of the SKF 96365-sensitive TRPC1 channels, for which Na^+ and Ca^{2+} compete. Activation of such a cation channel would give rise to depolarization and would explain the slower recruitment of voltage-gated L- and P/Q-type Ca^{2+} channels and Ca^{2+} influx observed in the present study. A previous study also demonstrated the role of slow recruitment of L-type Ca^{2+} channels in the Ca^{2+} influx and neurotoxicity evoked in CGN neurons by β-bungarotoxin (Tseng and Lin-Shiau, 2003b). On a cautionary note, SKF 96365 has been reported to block channels of very different classes, e.g. T-type Ca^{2+} channels (Singh *et al.*, 2010), K^+ channels (Schwarz *et al.*, 1994), store-operated channels (Varnai *et al.*, 2009), and any of these channels could contribute to $[Ca^{2+}]_i$, directly or indirectly.

Inhibition of voltage gated K^+ channels has been identified as the target of some snake neurotoxins (e.g. β-bungarotoxin or dendrotoxin) which would induce depolarization and could account for the release of neurotransmitter (Penner *et al.*, 1986; Petersen *et al.*, 1986). Chloride channels may also participate in determining membrane potential in some cells (Tsunoda and Matsumiya, 1987). We therefore tested the possible involvement of these channels in the response of DRG neurons to P-EPTX-Ar1a by using electrodes in which K^+ and/or Cl^- were substituted with Cs^+ and/or SO_4^{2-}, respectively. Our results indicated that P-EPTX-Ar1a-induced depolarization is not likely to be due to an effect on K^+ or Cl^- channels, as the ion replacement patch solution did not alter the reversal potential on an I-V plot.

The rise in $[Ca^{2+}]_i$ induced by maitotoxin is inhibited by imidazoles, including SKF 96365 (Daly *et al.*, 1995). In our study, while the combination of agatoxin and nifedipine summed together to inhibit most of the rise in $[Ca^{2+}]_i$, Ca^{2+} influx persisted in some 20% of cells in the presence of these blockers. The P-EPTX-Ar1a-evoked rise in $[Ca^{2+}]_i$ was also blocked by SKF 96365, as was the inward current. SKF 96365 blocks most transient receptor potential (TRP) channels of the C-type (Alexander *et al*., 2011), and all members of the TRPC family conduct Ca^{2+} as well as Na^+, suggesting the possibility of a direct involvement of these channels in the increase in $[Ca^{2+}]_i$ observed. However, this interpretation must be viewed with caution, as Na^+ played a major role in the current recorded here, with little scope for Ca^{2+}. In addition, selective blockade of individual members of the TRPC family using pharmacological means is not possible due to poor selectivity of the blockers (and see discussion above).

Ca^{2+} is essential for the hydrolysis of phospholipids by snake PLA_2 enzymes (Pieterson *et al.*, 1974), since Ca^{2+} has a significant role in binding the negatively charged phosphate group on the substrate and allowing appropriate orientation of the substrate around the active site (Verheij *et al.*, 1980). In fact, SPANs can induce an increase in $[Ca^{2+}]_i$ in neurons by an action of hydrolysed products of PLA_2 i.e. LysoPLs (located on the external leaflet of plasma membrane) and FAs (on both sides of the membrane) (Rigoni *et al.*, 2007). These PLA_2 products can induce a change in membrane fluidity and membrane-bound protein causing plasma membrane leakage and cell

damage. In addition, PLA_2 products such as arachidonic acid can stimulate extracellular Ca^{2+} entry via specific channels (arachidonate-regulated Ca^{2+} (ARC) channels), characterized in a number of cell lines (Holmes *et al.*, 2007; Shuttleworth and Thompson, 1998) including pancreatic β-cell (Yeung-Yam-Wah *et al.*, 2010). Thus, hydrolysed products of snake PLA_2 could contribute to the residual increase in $[Ca^{2+}]_i$ that persisted in the presence of ion channel blockers.

In our current study (Fig.3), P-EPTX-AR1a-induced increase in $[Ca^{2+}]_i$ in DRG neurons was prompt and large when normal Ca^{2+} was re-introduced, especially in those neurons that had been exposed to the Ca^{2+} chelator, EGTA. This response was also observed in the β-bungarotoxin-induced elevation in $[Ca^{2+}]_i$, a result which was interpreted in terms of an irreversible neurotoxin-induced signalling cascade that remains active even following removal of toxin from the medium (Tseng and Lin-Shiau, 2003b). Such a possibility cannot be excluded in the present study.

In conclusion, our data indicate that P-EPTX-Ar1a, a neurotoxin purified from Irian Jayan death adder venom, plays an important role in the generation of a rapid increase in $[Ca^{2+}]_i$ via Ca^{2+} influx. P-EPTX-Ar1a activates a cationic channel which would cause membrane depolarization. This depolarization opens voltage-sensitive Na^+ channels and the ensuing Na^+ action potential activates voltage-gated Ca^{2+} channels, resulting in Ca^{2+} influx which, when sufficiently large and prolonged, causes significant pharmacological effects.

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References

Alexander SP, Mathie A, Peters JA (2011). Guide to Receptors and Channels (GRAC), 5th edition. Br J Pharmacol 164 (Suppl. 1): S1-S324.

Blacklow B, Escoubas P, Nicholson GM (2010a). Characterisation of the heterotrimeric presynaptic phospholipase A_2 neurotoxin complex from the venom of the common death adder (*Acanthophis antarcticus*). Biochem Pharmacol 80: 277-287.

Blacklow B, Konstantakopoulos N, Hodgson WC, Nicholson GM (2010b). Presence of presynaptic neurotoxin complexes in the venoms of Australo-Papuan death adders (*Acanthophis* spp.). Toxicon 55: 1171-1180.

Chaisakul J, Konstantakopoulos N, Smith AI, Hodgson WC (2010). Isolation and characterisation of P-EPTX-Ap1a and P-EPTX-Ar1a: pre-synaptic neurotoxins from the venom of the northern (*Acanthophis praelongus*) and Irian Jayan (*Acanthophis rugosus*) death adders. Biochem Pharmacol 80: 895-902.

Chaisakul J, Parkington HC, Isbister GK, Konstantakopoulos N, Hodgson WC (2013). Differential myotoxic and cytotoxic activities of pre-synaptic neurotoxins from Papuan taipan (*Oxyuranus scutellatus*) and Irian Jayan death adder (*Acanthophis rugosus*) venoms. Basic Clin Pharmacol Toxicol 112: 325-334.

Choi OH, Padgett WL, Nishizawa Y, Gusovsky F, Yasumoto T, Daly JW (1990). Maitotoxin: effects on calcium channels, phosphoinositide breakdown, and arachidonate release in pheochromocytoma PC12 cells. Mol Pharmacol 37: 222-230.

Currie BJ (2000). Snakebite in tropical Australia, Papua New Guinea and Irian Jaya. Emerg Med 12: 285-294.

Daly JW, Lueders J, Padgett WL, Shin Y, Gusovsky F (1995). Maitotoxin-elicited calcium influx in cultured cells. Effect of calcium-channel blockers. Biochem Pharmacol 50: 1187-1197.

Dietrich A, Chubanov V, Kalwa H, Rost BR, Gudermann T (2006). Cation channels of the transient receptor potential superfamily: Their role in physiological and pathophysiological processes of smooth muscle cells. Pharmacol Ther 112: 744-760.

Fathi HB, Rowan EG, Harvey AL (2001). The facilitatory actions of snake venom phospholipase A_2 neurotoxins at the neuromuscular junction are not mediated through voltage-gated K^+ channels. Toxicon 39: 1871-1882.

Fry BG (1999). Structure-function properties of venom components from Australian elapids. Toxicon 37: 11-32.

Gusovsky F, Daly JW (1990). Maitotoxin: a unique pharmacological tool for research on calcium-dependent mechanisms. Biochem Pharmacol 39: 1633-1639.

Gutierrez D, Diaz de Leon L, Vaca L (1997). Characterization of the maitotoxin-induced calcium influx pathway from human skin fibroblasts. Cell Calcium 22: 31-38.

Harris JB (1985). Phospholipases in snake venoms and their effects on nerve and muscle. Pharmacol Ther 31: 79-102.

Harris JB, Maltin CA (1982). Myotoxic activity of the crude venom and the principal neurotoxin, taipoxin, of the Australian taipan, *Oxyuranus scutellatus*. Br J Pharmacol 76: 61-75.

Hodgson WC, Dal Belo CA, Rowan EG (2007). The neuromuscular activity of paradoxin: a presynaptic neurotoxin from the venom of the inland taipan (*Oxyuranus microlepidotus*). Neuropharmacology 52: 1229-1236.

Holmes AM, Roderick HL, McDonald F, Bootman MD (2007). Interaction between store-operated and arachidonate-activated calcium entry. Cell Calcium 41: 1-12.

Iouzalen L, Lantoine F, Pernollet MG, Millanvoye-Van Brussel E, Devynck MA, David-Dufilho M (1996). SK&F 96365 inhibits intracellular Ca^{2+} pumps and raises cytosolic Ca^{2+} concentration without production of nitric oxide and von Willebrand factor. Cell Calcium 20: 501-508.

Isbister GK, Halkidis L, O'Leary MA, Whitaker R, Cullen P, Mulcahy R *et al.* (2010) Human anti-snake venom IgG antibodies in a previously bitten snake-handler, but no protection against local envenoming. *Toxicon* 55: 646-649.

Johnston CI, O'Leary MA, Brown SG, Currie BJ, Halkidis L, Whitaker R *et al.* (2012). Death adder envenoming causes neurotoxicity not reversed by antivenom - Australian Snakebite Project (ASP-16). PLoS Negl Trop Dis 6: e1841.

Kakizaki A, Takahashi M, Akagi H, Tachikawa E, Yamamoto T, Taira E *et al.*(2006). Ca^{2+} channel activating action of maitotoxin in cultured brainstem neurons. Eur J Pharmacol 536: 223-231.

Kini RM (2003). Excitement ahead: structure, function and mechanism of snake venom phospholipase A_2 enzymes. Toxicon 42: 827-840.

Montecucco C, Gutierrez JM, Lomonte B (2008). Cellular pathology induced by snake venom phospholipase A_2 myotoxins and neurotoxins: common aspects of their mechanisms of action. Cell Mol Life Sci 65: 2897-2912.

Penner R, Petersen M, Pierau FK, Dreyer F (1986). Dendrotoxin: a selective blocker of a non-inactivating potassium current in guinea-pig dorsal root ganglion neurones. Pflugers Arch 407: 365-369.

Petersen M, Penner R, Pierau FK, Dreyer F (1986). Beta-bungarotoxin inhibits a non-inactivating potassium current in guinea pig dorsal root ganglion neurones. Neurosci Lett 68: 141-145.

Pieterson WA, Volwerk JJ, de Haas GH (1974). Interaction of phospholipase A_2 and its zymogen with divalent metal ions. Biochemistry 13: 1439-1445.

Rae MG, Hilton J, Sharkey J (2012). Putative TRP channel antagonists, SKF 96365, flufenamic acid and 2-APB, are non-competitive antagonists at recombinant human a1b2g2 GABAA receptors. Neurochem Int 60: 543-554.

Rigoni M, Pizzo P, Schiavo G, Weston AE, Zatti G, Caccin P *et al.* (2007). Calcium influx and mitochondrial alterations at synapses exposed to snake neurotoxins or their phospholipid hydrolysis products. J Biol Chem 282: 11238-11245.

Rigoni M, Schiavo G, Weston AE, Caccin P, Allegrini F, Pennuto M *et al.* (2004). Snake presynaptic neurotoxins with phospholipase A_2 activity induce punctate swellings of neurites and exocytosis of synaptic vesicles. J Cell Sci 117: 3561-3570.

Rossetto O, Rigoni M, Montecucco C (2004). Different mechanism of blockade of neuroexocytosis by presynaptic neurotoxins. Toxicol Lett 149: 91-101.

Rowan EG (2001). What does beta-bungarotoxin do at the neuromuscular junction? Toxicon 39: 107-118.

Rugolo M, Dolly JO, Nicholls DG (1986). The mechanism of action of beta-bungarotoxin at the presynaptic plasma membrane. Biochem J 233: 519-523.

Schwarz G, Droogmans G, Nilius B (1994). Multiple effects of SK&F 96365 on ionic currents and intracellular calcium in human endothelial cells. Cell calcium 15: 45-54.

Shuttleworth TJ, Thompson JL (1998). Muscarinic receptor activation of arachidonate-mediated Ca^{2+} entry in HEK293 cells is independent of phospholipase C. J Biol Chem 273: 32636-32643.

Singh A, Hildebrand ME, Garcia E, Snutch TP (2010). The transient receptor potential channel antagonist SKF96365 is a potent blocker of low-voltage-activated T-type calcium channels. Br J Pharmacol 160: 1464-1475.

Sinkins WG, Estacion M, Prasad V, Goel M, Shull GE, Kunze DL *et al.* (2009). Maitotoxin converts the plasmalemmal Ca^{2+} pump into a Ca^{2+}-permeable nonselective cation channel. Am J Physiol Cell Physiol 297: 1533-1543.

Su MJ, Chang CC (1984). Presynaptic effects of snake venom toxins which have phospholipase A_2 activity (beta-bungarotoxin, taipoxin, crotoxin). Toxicon 22: 631-640.

Tedesco E, Rigoni M, Caccin P, Grishin E, Rossetto O, Montecucco C (2009). Calcium overload in nerve terminals of cultured neurons intoxicated by alpha-latrotoxin and snake PLA_2 neurotoxins. Toxicon 54: 138-144.

Tseng WP, Lin-Shiau SY (2003a). Calcium-activated NO production plays a role in neuronal death induced by beta-bungarotoxin in primary cultures of cerebellar granular neurons. Naunyn Schmiedebergs Arch Pharmacol 367: 451-461.

Tseng WP, Lin-Shiau SY (2003b). Neuronal death signaling by beta-bungarotoxin through the activation of the N-methyl-D-aspartate (NMDA) receptor and L-type calcium channel. Biochem Pharmacol 65: 131-142.

Tsunoda Y, Matsumiya H (1987). Calcium-activated membrane depolarization via modulation of chloride efflux from parietal cells during gastrin stimulation. FEBS Letters 222: 149-153.

Varnai P, Hunyady L, Balla T (2009). STIM and Orai: the long-awaited constituents of store-operated calcium entry. Trends Pharmacol Sci 30: 118-128.

Verheij HM, Volwerk JJ, Jansen EH, Puyk WC, Dijkstra BW, Drenth J *et al.* (1980). Methylation of histidine-48 in pancreatic phospholipase A_2. Role of histidine and calcium ion in the catalytic mechanism. Biochemistry 19: 743-750.

Yeung-Yam-Wah V, Lee AK, Tse FW, Tse A (2010). Arachidonic acid stimulates extracellular Ca^{2+} entry in rat pancreatic cells via activation of the noncapacitative arachidonate-regulated Ca^{2+} (ARC) channels. Cell Calcium 47: 77-83.

Figures

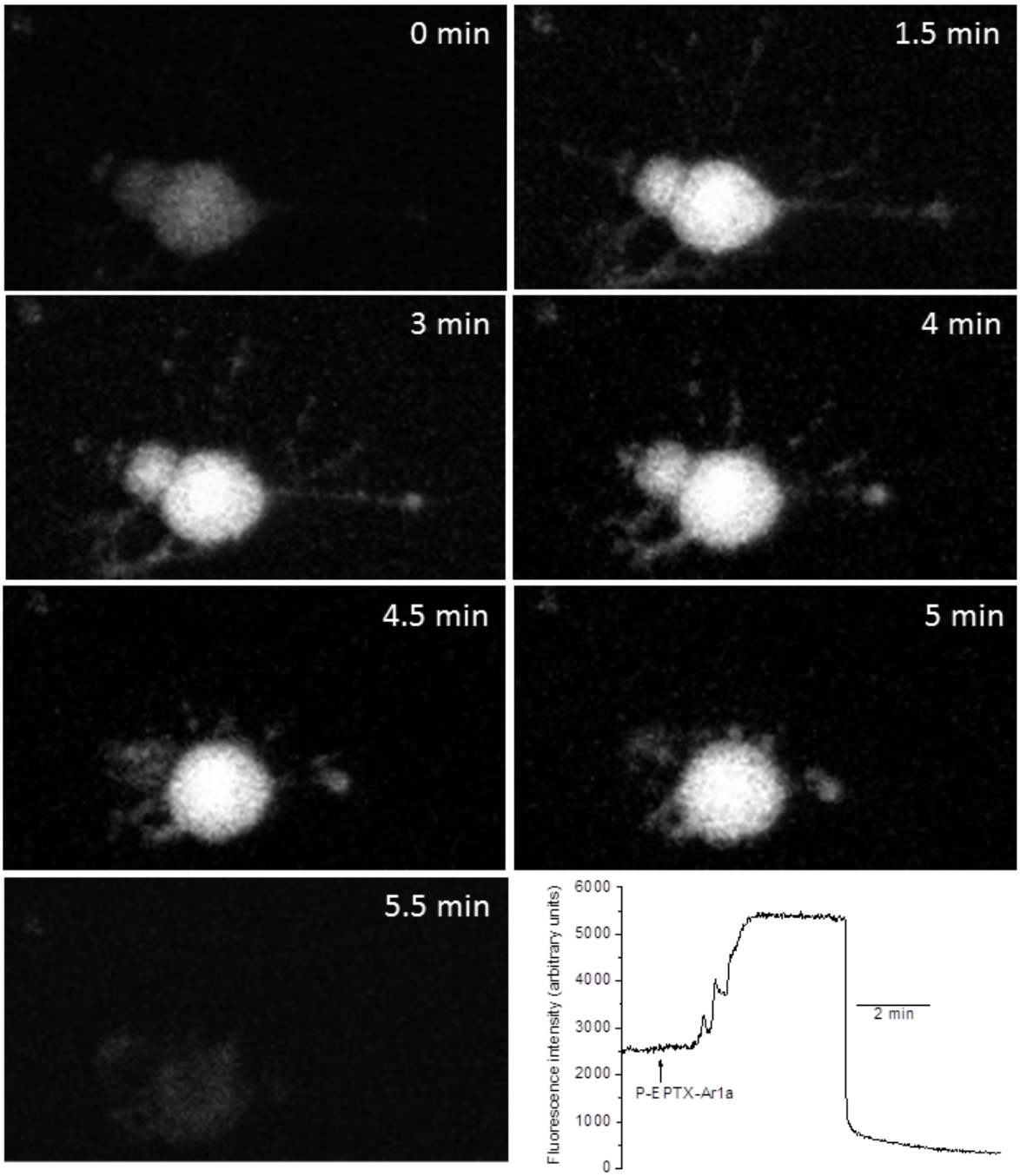

Figure 1: *Effect of P-EPTX-Ar1a (0.23 µM) on a mouse DRG neuron in normal Ca^{2+} (1.3 mM) Hank's solution. DRG neuron was loaded with the intracellular Ca^{2+} detector fluo-4 AM. Fluorescent image before the incubation of the toxin is shown at t = 0 min. Retraction of neuron projections occurred at 4 min and cell death at t = 5.5 min.*

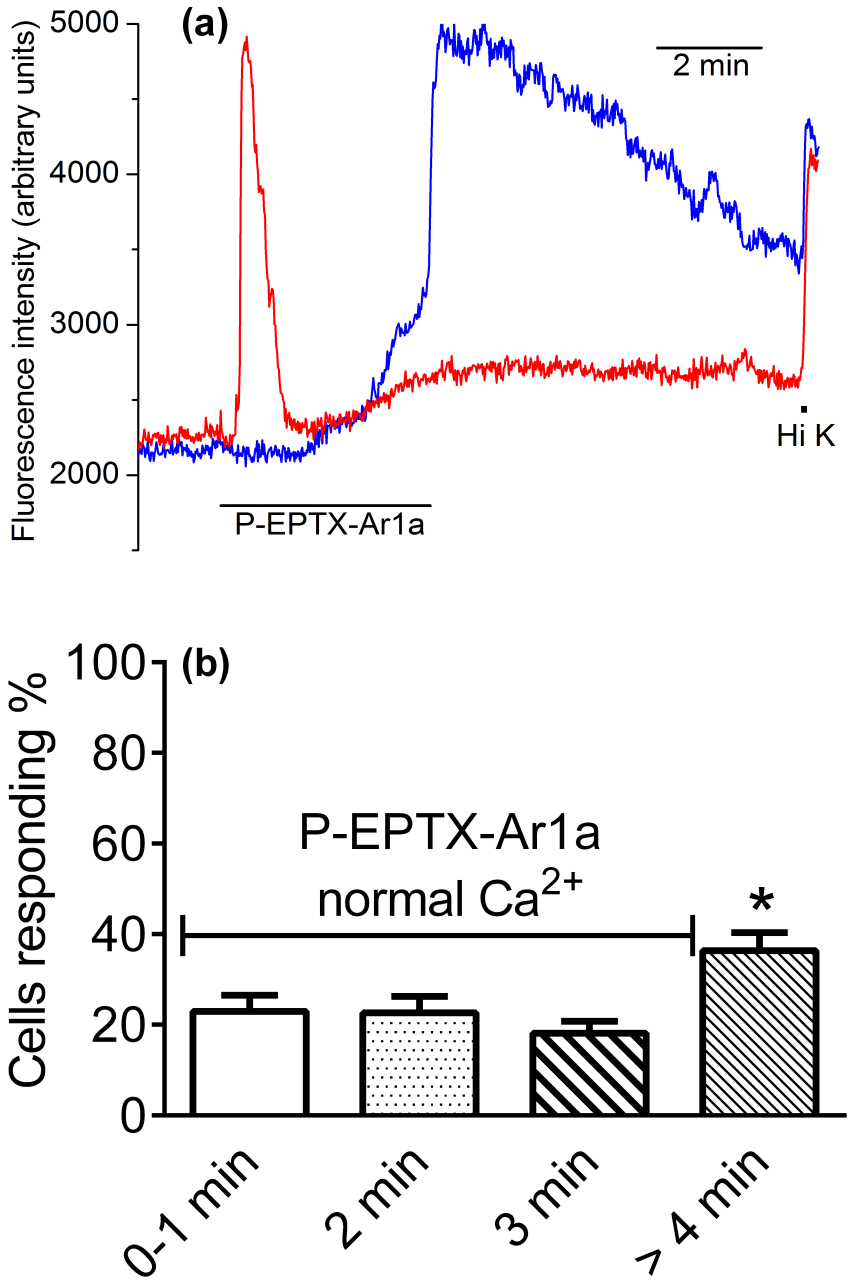

Figure 2: *Intracellular Ca^{2+} fluorescence levels in response to P-EPTX-Ar1a (69 nM) for 4 min in mouse DRG neurons. (a) Examples of the rise in cytoplasmic Ca^{2+} in neurons exposed to P-EPTX-Ar1a, Ca^{2+} spikes occurred in some neurons (red), while in others the response was gradual and lacked oscillations (blue). (b) P-EPTX-Ar1a induced an increase in fluorescence in 20-25 % of cells within 1 min, and in an additional 20-25 % of cells in the 2nd, and again in the 3rd min, while approximately 40 % of cells did not respond until after 4 min had elapsed. (*P = 0.002, one-way ANOVA, n = 28 mice).*

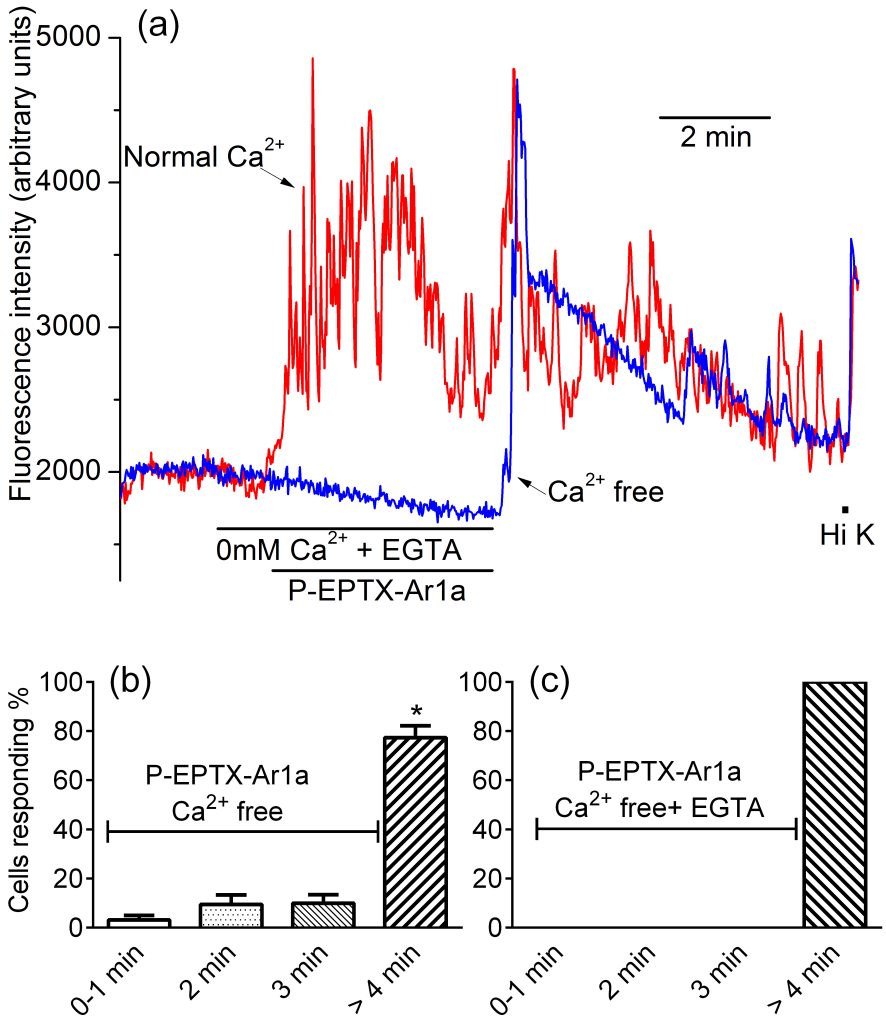

Figure 3: *Effect of removing extracellular Ca^{2+} on the increase in cytoplasmic Ca^{2+} induced by exposure of mouse DRG neurons to P-EPTX-Ar1a (69 nM) for 4 min (blue). (a) Raw traces of the response in $[Ca^{2+}]_i$ fluorescence levels. (b) The toxin induced an increase in Ca^{2+} in 5, 10 and 10 %, progressively, of neurons in solution from which Ca^{2+} had merely been omitted. (c) When EGTA (150 µM) was included in the nominally Ca^{2+} free superfusate no cell responded to P-EPTX-Ar1a with a rise in cytoplasmic Ca^{2+}. However, when extracellular Ca^{2+} was re-introduced there was a rapid increase in cytoplasmic Ca^{2+} in all neurons. *P < 0.0001, significantly different compared to the responses during incubation period with toxin, one-way ANOVA (n = 9-16 mice, and 12 cells from each mouse).*

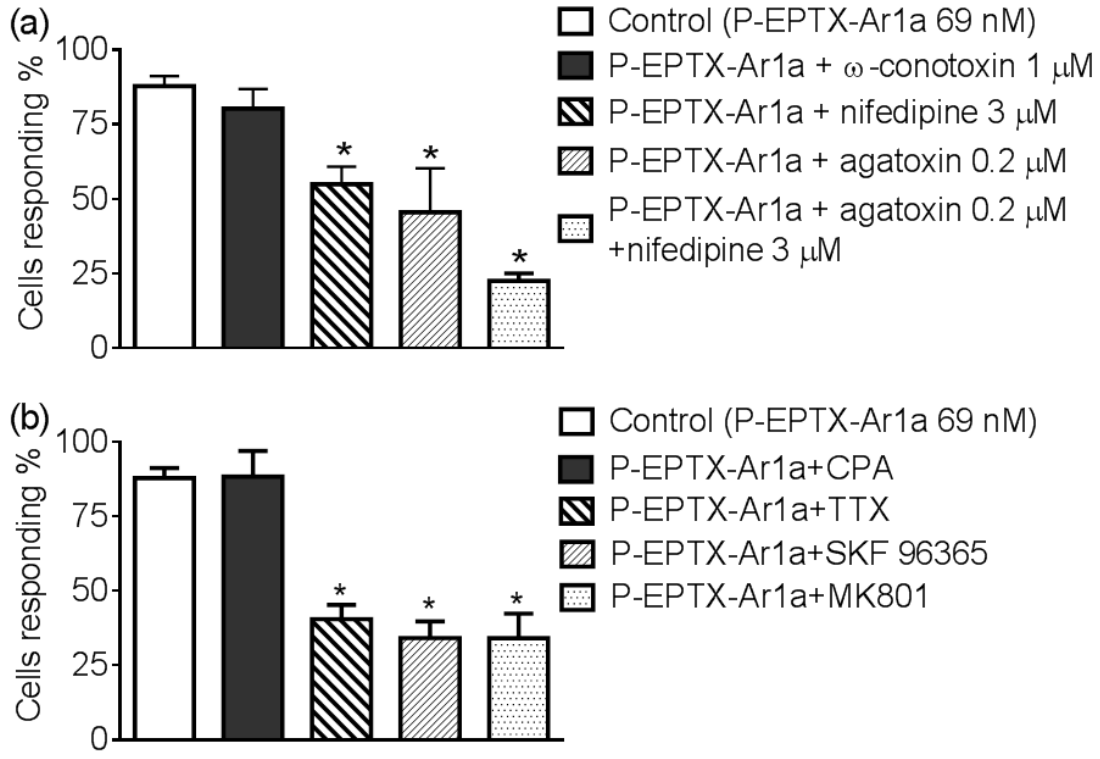

Figure 4: *Effects of ion channel blockers on the increase in cytoplasmic Ca^{2+} evoked by P-EPTX-Ar1a (69 nM) in mice DRG neurons. (a) Blockade of Ca^{2+} channels with ω-conotoxin against N-type, nifedipine against L-type, and agatoxin against P/Q-type Ca^{2+} channels. (b) CPA (10μM), the endoplasmic reticulum-specific Ca^{2+} ATPase inhibitor, TTX (1μM) which blocks voltage-gated Na^{+} channels, the cation channel antagonist, SKF 96365 (10 μM), and the NMDA receptors antagonist MK801 (1 μM). * $P < 0.05$, significantly different compared with P-EPTX-Ar1a alone, one-way ANOVA (n = 4-18 mice).*

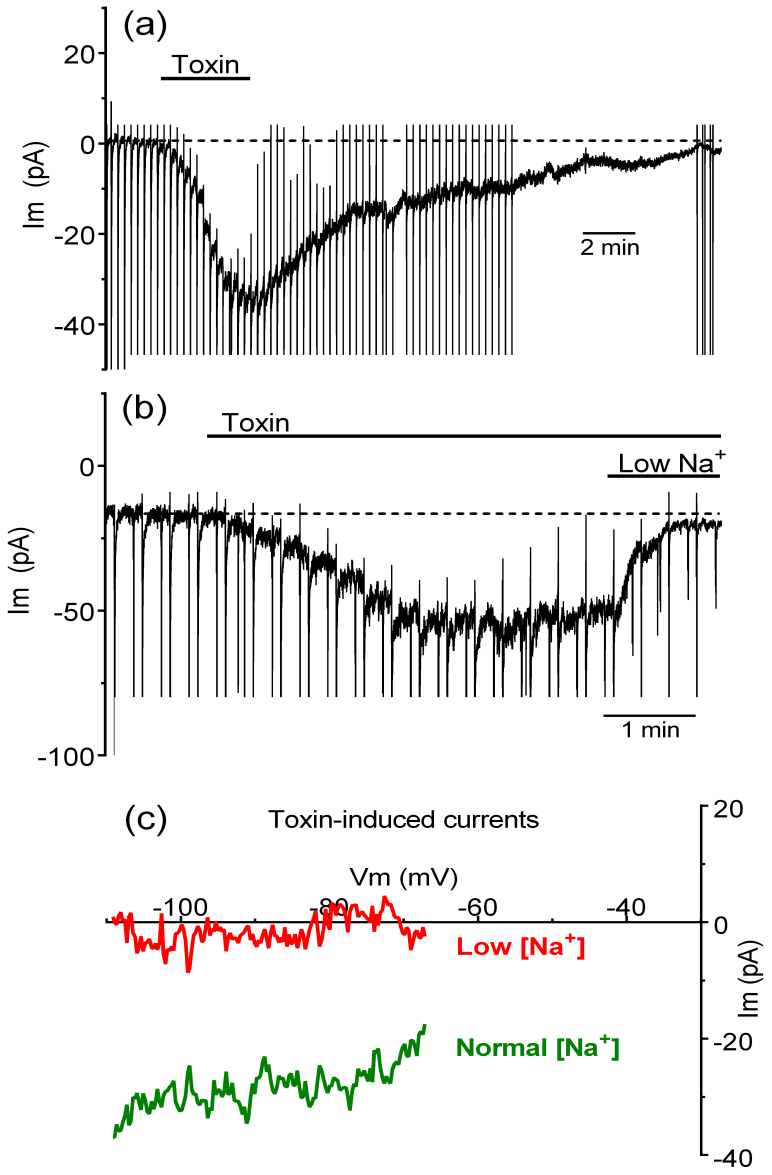

Figure 5: *Depolarizing voltage ramps (-110 to 0 or + 20 mV for 200 ms) were applied from a holding potential of -70 mV. P-EPTX-Ar1a (0.17 µM) induced an inward current that was abolished following removal of P-EPTX-Ar1a (a). Lowering extracellular Na^+ (35 mM) reversed the P-EPTX-Ar1a induced inward current (b). Current-voltage relationship from the voltage ramps indicates a large Na^+ contribution to the current activated by P-EPTX-Ar1a (c). The periodic transients in (a) and (b) are the responses to voltage ramps and have been truncated for reasons of clarity (n = 5-8).*

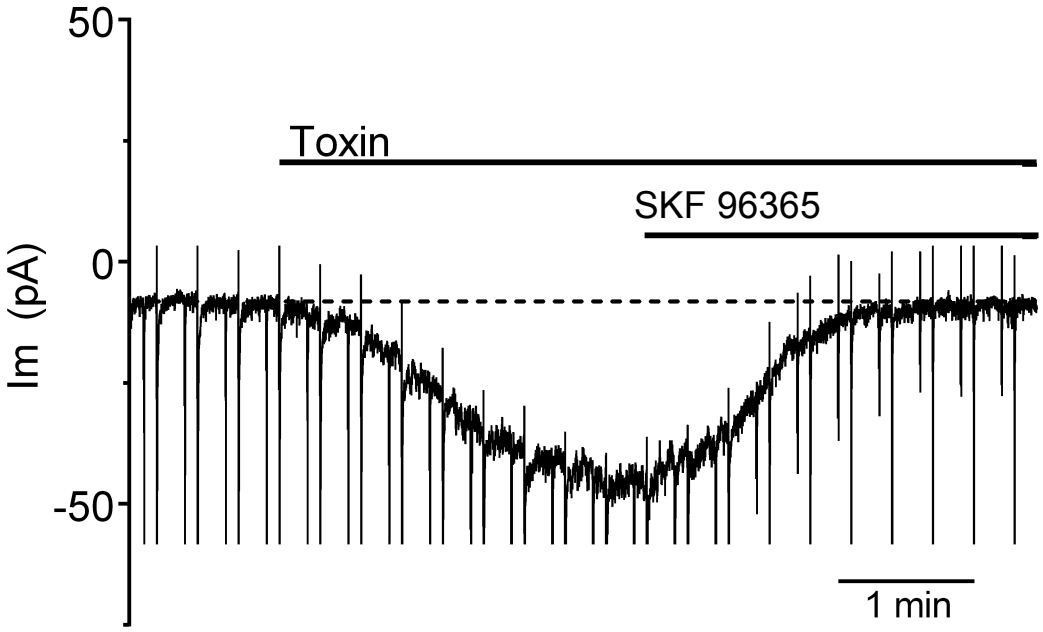

Figure 6: *P-EPTX-Ar1a (0.17 µM)-induced inward current was restored to initial conductance by the application of SKF 96365 (10 µM) (n = 6).*

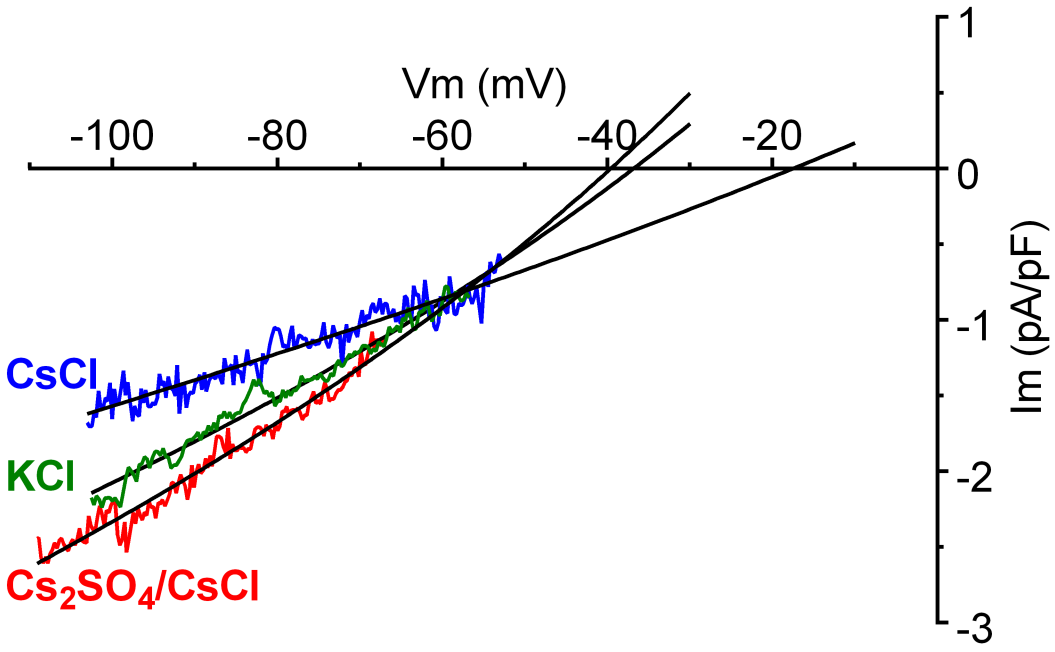

Figure 7: *Current-voltage relationships for the inward current evoked by P-EPTX-Ar1a in mouse DRG neurons. Current in normal Cl^- (KCl) patch solution (green) indicated reversal potential –24 ± 13 mV (n = 3). With Cs replacement (blue) or low Cl^- (Cs_2SO_4/CsCl) (red) patch solutions, reversal potentials were recorded at –4 ± 12 (n = 2) and –37 ± 7 (n = 16) mV, respectively. A significant difference in reversal potential was not observed (P > 0.05, one-way ANOVA) indicating that Cl^- is not involved.*

CHAPTER 5

SUDDEN CARDIOVASCULAR COLLAPSE FOLLOWING ENVENOMING BY PAPUAN TAIPAN (*OXYURANUS SCUTELLATUS*)

Monash University

Declaration for Thesis Chapter 5

This chapter is made up of the following publication and additional work undertaken but not included in the manuscript.

Declaration by candidate

In the case of Chapter 5, the nature and extent of my contribution to the work was the following:

Nature of Contribution	Extent of Contribution (%)
I declare that experiments and writing of this manuscript were solely undertaken by the myself, taking into consideration the advice and recommendations of co-authors	85%

The following co-authors contributed to the work.

Name	Nature of Contribution
Associate Professor Geoffrey K Isbister	Development of ideas, manuscript preparation
Dr Nicki Konstantakopoulos	Development of ideas, manuscript preparation
Dr Marianne Tare	Provision of expertise and facilities
Professor Helena C Parkington	Provision of expertise, development of ideas, manuscript preparation and facilities
Professor Wayne C Hodgson	Development of ideas, manuscript preparation

Candidate's signature: [redacted] e: 07.06.2013

Declaration by co-authors

The undersigned hereby certify that:

(1) the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors;
(2) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(3) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(4) there are no other author of the publication according to these criteria;
(5) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journal or other publications and (c) the head of the responsible academic unit; and
(6) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location (s) Department of Physiology and Department of Pharmacology, Monash University

		Date
Associate Professor Geoffrey K Isbister	[redacted]	06.06.2013
Dr Nicki Konstantakopoulos	[redacted]	04.06.2013
Dr Marianne Tare	[redacted]	06.06.2013
Professor Helena C Parkington	[redacted]	06.06.2013
Professor Wayne C Hodgson	[redacted]	06.06.2013

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In vivo and in vitro cardiovascular effects of Papuan taipan (*Oxyuranus scutellatus*) venom: Exploring "sudden collapse"

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HIGHLIGHTS

- 'Sudden collapse' after elapid envenoming is a poorly understood cause of mortality.
- Papuan taipan venom induces cardiovascular collapse in anaesthetized rats.
- This effect is inhibited by prior exposure to small priming doses of venom.
- Venom produced relaxation in vascular tissue but was without effect in cardiac tissue.
- Collapse appears to be mediated by the release of endogenous mediators.

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ABSTRACT

'Sudden collapse' following envenoming by some Australasian elapids is a poorly understood cause of mortality. We have previously shown that *Oxyuranus scutellatus* venom causes cardiovascular collapse in anaesthetized rats. Prior administration of a sub lethal dose of venom attenuated the response to subsequent administration of higher (lethal) venom doses. In this study, we investigated the possible mechanisms mediating this 'protective effect'. Papuan taipan venom (5 μg/kg, i.v.) produced a small transient hypotension in anaesthetized rats, while 10 μg/kg resulted in a 73 ± 12% decrease in arterial pressure. Venom (20 μg/kg or 50 μg/kg) produced cardiovascular collapse in all animals tested ($n = 12$). Cardiovascular collapse by 50 μg/kg venom was prevented by prior administration of 'priming' doses of venom (5, 10 and 20 μg/kg). Also, prior administration of indomethacin (30 mg/kg, i.v.) or heparin (300 units/kg, i.v.) prevented sudden collapse induced by venom (20 μg/kg). Venom was without effect in isolated hearts indicating that a direct cardiac effect was unlikely to be responsible for 'sudden collapse'. Venom induced endothelium-dependent and -independent relaxation in pre-contracted rat mesenteric artery rings which was inhibited by indomethacin, IbTx and Rp-8-CPT-cAMPs. This relaxation was markedly reduced upon second exposure. Our results indicate that cardiovascular collapse induced by *O. scutellatus* venom may be due to a combination of release of dilator autacoids and to direct relaxation of vascular smooth muscle involving the cAMP/protein kinase A cascade. Further work will involve identification of the venom component(s) responsible for this action and may provide insight into the management of envenomed patients.

1. Introduction

Snake bite is an important clinical issue in Australasia (Currie, 2000), and medically important snakes in this region belong to the family Elapidae. Many of these elapids have highly potent venoms, based on murine LD_{50} values (Broad et al., 1979). The major clinical syndromes resulting from envenoming by Australasian elapids include coagulopathy, neurotoxicity and myotoxicity (Ireland et al., 2010). With modern intensive care, and the early use of antivenoms, the majority of patients envenomed by snakes have uncomplicated

Abbreviations: ACh, acetylcholine; ANOVA, analysis of variance; i.v., intravenous; i.p., intraperitoneal; MAP, mean arterial pressure; NO, nitric oxide; IbTx, iberiotoxin; Rp-8-CPT-cAMPs, 8-(4-chlorophenylthio)adenosine-3′,5′-cyclic monophosphorothioate,Rp-isomer sodium salt.

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hospital admissions and deaths are rare. The majority of deaths result from an early collapse and associated cardiac arrest. The pathology of this early collapse is poorly understood. The early collapse often occurs in pre-hospital settings (i.e. in a remote area or on the way to hospital) within an hour of the bite. In most cases the collapse is transient and victims often respond well to basic life support (Johnston et al., 2002). Early collapse has been most commonly associated with envenoming by brown snakes (*Pseudonaja* spp.), and less commonly with taipans (*Oxyuranus* spp.) and tiger snakes (*Notechis* spp.) (Currie, 2000). Although rapid and severe hypotension following the administration of snake venom has been described in whole animal experiments, the response has not been investigated fully. Most studies of Elapid venoms have focused on their coagulopathic, neurotoxic and myotoxic effects (Connolly et al., 1995; Isbister et al., 2006; Sutherland et al., 1981; Wickramaratna et al., 2003). Little work has been done to define the pathology of 'sudden collapse' and a number of different mechanisms and toxins have been suggested to be responsible.

Taipans are a highly venomous genus consisting of the inland taipan (*O. microlepidotus*), coastal taipan (*O. scutellatus*) and, more recently, the Western Desert taipan (*O. temporalis*). Although the Papuan taipan (*O. s. canni*) has been regarded as a separate subspecies to Australian populations, recent taxonomic studies have indicated no significant differentiation between the two populations (Vargas et al., 2011; Wuster et al., 2005).

We have previously reported that Papuan taipan venom (10 μg/kg, i.v.) produces cardiovascular collapse in anaesthetized rats. Interestingly, prior administration of a lower dose of venom (i.e. 5 μg/kg), which only caused a small hypotensive response, enabled the subsequent administration of 10 and 100 μg/kg venom without cardiovascular collapse (Crachi et al., 1999). We hypothesize that incrementally increasing the dose of venom may prevent the cardiovascular collapse consequent to release of endogenous compounds such as histamine and bradykinin as a possible mechanism (Myint et al., 1985).

In this study we investigated the pathology of the early collapse and whether this is mediated primarily in cardiac or vascular tissue. We also aimed to elucidate the mechanism behind the 'self protective' effect of Papuan taipan venom using in vivo and in vitro experiments in order to understand mechanisms for development of future treatments.

2. Materials and methods

2.1. Venom preparation and storage

Freeze dried Papuan taipan (*O. scutellatus*) venom was purchased from Venom Supplies (Tanunda, South Australia). Venom was dissolved in MilliQ water and stored at −20 °C until required. Thawed solutions were kept on ice during experiments. Stock venom solutions were filter sterilized using a 0.22 μm membrane and the protein content determined via a BCA Protein Assay Kit (Pierce biotechnology; IL, USA) as per manufacturer's instructions.

2.2. Anaesthetized rat preparation

Male Sprague-Dawley rats (250–300 g) were anaesthetized with pentobarbitone sodium (60–100 mg/kg, i.p., supplemented as required) (Jurox Pty Ltd., NSW, Australia). Cannulae were inserted into the trachea, jugular vein and carotid artery, for artificial respiration (if required), administration of drugs/venom and measurement of blood pressure, respectively. Arterial blood pressure was recorded using a Gould Statham P23 pressure transducer connected to a Power Lab system. At the conclusion of the experiment animals were killed by an overdose of pentobarbitone. Pulse pressure was defined as the difference between systolic and diastolic blood pressures. MAP was defined as diastolic blood pressure plus one-third of pulse pressure.

Where indicated, 300 units/kg heparin (Tibballs and Sutherland, 1992); 2 mg/kg mepyramine (Rash et al., 1998) and 5 mg/kg atropine (Barrett, 1971) were used to inhibit coagulation, H1-receptors and muscarinic receptors, respectively. The concentrations of antagonists/inhibitors were altered and chosen based on previous experiments. NO production was inhibited by the bolus injection of L-NAME 10 mg/kg (i.v.) (Parkington et al., 2002) and cyclooxygenase production was inhibited by indomethacin 30 mg/kg (i.v.) (Huang, 1984).

2.3. Isolation and study of rat mesenteric artery

Rats (300–400 g) were killed by isoflurane and decapitated, the mesenteric bed removed and placed in ice-cold physiological saline solution (PSS). The mesenteric artery was cleared of loose connective tissue and fat, and cut into 1–2 mm lengths. A stainless steel wire (diameter ~40 μm) was carefully threaded through the lumen of each ring and connected to the movable jaw of a wire myograph (Danish MyoTechnology). A second wire was threaded through the lumen, and attached to a jaw connected to the force transducer. Where indicated, the endothelium was removed by gently rubbing the intimal surface with thin roughened wire. Pre-heated PSS (5 ml) was added to the bath and bubbled with carbogen (95% O_2/5% CO_2) to maintain a pH of 7.4. The composition of PSS was (mM): NaCl 120; KCl 5; $NaHCO_3$ 25; glucose 11; KH_2PO_4 1.0; $MgSO_4$ 1.2; $CaCl_2$ 2.5. The solution was maintained at 35.5 °C. The solution was changed every 15 min. Incremental increases in tension were applied until the resting tension was equivalent to ~70 mmHg. An intact endothelium was confirmed by a maximal relaxation to ACh (10 μM) in tissues precontracted with a sub-maximal concentration of phenylephrine (3 μM). Discrete additions of venom were performed with each concentration being investigated in separate tissues.

L-NAME (0.2 mM; Crachi et al., 1999), indomethacin (10 μM; Crachi et al., 1999) and mepyramine (1 μM; Krstic et al., 1996; DalBo et al., 2008) were used to inhibit NO production, prostanoid production and H_1 receptor activation, respectively. To investigate the effect of venom on vascular smooth muscle, the endothelium was removed and IbTx (0.1 μM) was used to block large-conductance Ca^{2+}-activated K^+ channels (Parkington et al., 2002; Lin et al., 2010), and Rp-8-CPT-cAMPs (10 μM) to block protein kinase A (PKA).

2.4. Langendorff heart preparation

Rats were anaesthetized with 60–100 mg/kg pentobarbitone and then injected with heparin (5000 U in 0.2 ml). Hearts were removed and placed in cold Ca^{2+}-free PSS. The heart and the aortic arch were cleared of surrounding tissues, the pulmonary vein perforated to allow free perfusion of the heart and the heart mounted on a Landendorff apparatus (ML870B2 Langendorff System, ADInstruments, Bella Vista, NSW, Australia). The heart was perfused through the aorta and coronary sinus with PSS (36 °C) of the following composition: NaCl 118; KCl 4.7; $NaHCO_3$ 25; glucose 11; KH_2PO_4 1.2; $MgSO_4$ 1.2; $CaCl_2$ 1.2 mM. The flow was gradually increased to 6–8 ml/min and then a Millar pressure catheter (Millar instruments Inc.) was introduced into the left ventricle through a small puncture at the junction of the left atrium and ventricle, and connected to a Power Lab system (ADInstruments). Perfusion pressure was monitored via a pressure transducer in the perfusion line. Perfusion pressure was clamped at 65 mmHg, and the preparation left to equilibrate for 20 min.

2.5. Chemical and drugs

The following drugs and solutions were used: histamine HCl, phenylephrine HCl, N^{ω}-nitro-L-arginine methylester (L-NAME), Rp-8-pCPT-cAMPs, IbTx and indomethacin, all from Sigma Chemical Co. (St. Louis, MO, USA); and mepyramine maleate (May & Baker, Dagenham, UK); heparin sodium (Hospira, VIC, Australia).

2.6. Data analysis and statistics

Statistical analysis was performed using Prism 5.0 software (GraphPad Software, San Diego, CA, USA). Student's unpaired *t*-test was performed on venom responses in the presence of venoms in different animals and tissues, and paired *t*-tests were used to compare responses before and after venom/agonist in the same tissues. Multiple comparisons were made using a one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. A two-way ANOVA was performed on the data from the rat mesenteric artery in the presence and absence of endothelium. Values of $P < 0.05$ were accepted as significant. Data were expressed as mean ± SEM.

2.7. Animal ethics

All animal experiments used in this study were approved by the SOBS-B Monash University Animal Ethics Committee.

3. Results

3.1. Anaesthetized rats

3.1.1. Cardiovascular collapse – single doses

In separate experiments, *O. scutellatus* venom (20 μg/kg or 50 μg/kg, i.v.) caused rapid cardiovascular collapse, as defined by the absence of recordable blood pressure (Fig. 1a), when administered to anaesthetized rats (Fig. 2a, $n = 6$). However, venom did

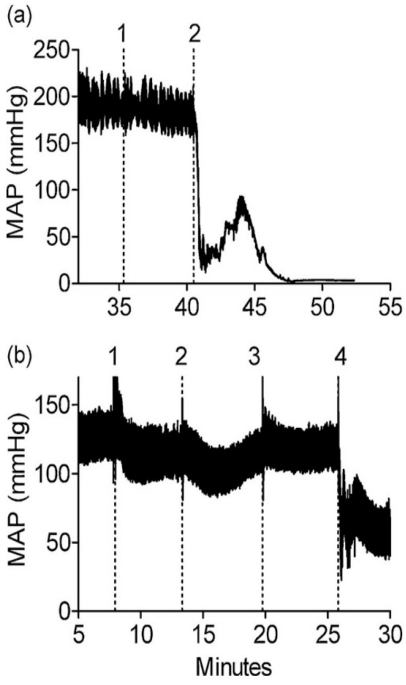

Fig. 1. (a) Effect of saline (at time point 1) or *O. scutellatus* venom (20 μg/kg, i.v., at time point 2) on mean arterial pressure (MAP) in an anaesthetized rat. (b) Sequential addition of venom in the same anaesthetized rat (5, 10, 20 and 50 μg/kg, i.v., added at time points 1, 2, 3 and 4, respectively) on MAP.

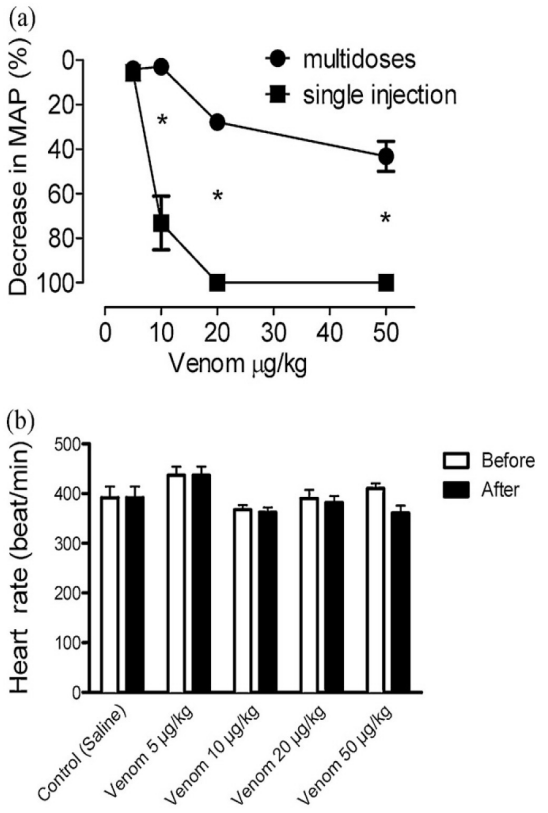

Fig. 2. (a) Effect of *O. scutellatus* venom after single injection (5, 10, 20 and 50 μg/kg, i.v., $n = 6$–9) in different rats and sequential addition of venom (5, 10, 20 and 50 μg/kg, i.v., $n = 8$) in the same rat on MAP. *$P < 0.05$, significantly different from single injection, Student's unpaired *t*-test. (b) A comparison of heart rate before and after (measured at the point when MAP had decreased by 50%) single injection (5, 10, 20 and 50 μg/kg, i.v., $n = 3$–11) in different rats, of *O. scutellatus* venom.

not significantly affect heart rate as indicated by a comparison of heart rate immediately prior to venom addition and then again at the time point where MAP had decreased by 50% (Fig. 2b, $n = 3$–11). Lower doses of venom caused either a small hypotensive effect (i.e. 5 μg/kg, $6 \pm 1\%$, $n = 9$) or a more marked effect (i.e. 10 μg/kg, $73 \pm 12\%$, $n = 6$, Fig. 2a) without cardiovascular collapse.

3.1.2. Cardiovascular collapse – multiple doses

When the larger doses (20 μg/kg or 50 μg/kg) of *O. scutellatus* venom were added following a small priming dose of 5 μg/kg or 10 μg/kg, the large dose failed to produce collapse but induced mild-moderate hypotension (i.e. 20 μg/kg, $27 \pm 3\%$ and 50 μg/kg, $43 \pm 7\%$, $n = 8$, Figs. 1b and 2). The priming dose only prevented cardiovascular collapse when given 30 min prior to, but not 1 h prior to, the administration of the larger dose of venom which caused collapse.

To examine the effect of 'priming' doses on cardiovascular collapse, multiple additions of 5 μg/kg were administered prior to the administration of 50 μg/kg i.e. a dose which, when administered alone, produced collapse in 100% of rats tested ($n = 6$). Neither 3 nor 4 discrete additions of 5 μg/kg prevented collapse induced by subsequent administration of 50 μg/kg ($n = 5$, Table 1). However, 5 discrete additions of 5 μg/kg were successful in preventing collapse by subsequent administration of 50 μg/kg ($n = 4$, Table 1).

3.1.3. Effects of antagonists on cardiovascular collapse

After prior administration of indomethacin (30 mg/kg, i.v., $n = 8$) or heparin (300 U/kg, i.v., $n = 7$), *O. scutellatus* venom (20 μg/kg, i.v.) still produced a marked hypotensive effect ($74 \pm 6\%$ or $42 \pm 10\%$, respectively), but collapse did not occur. In contrast, cardiovascular collapse was not prevented by prior administration of atropine (5 mg/kg, i.v., $n = 4$), L-NAME (10 mg/kg, i.v., $n = 4$) or mepyramine (2 mg/kg, i.v., $n = 6$) (Fig. 3, $P > 0.05$, one-way ANOVA). The 'protective' effect of heparin (300 U/kg, i.v.) was not potentiated by concomitant administration of mepyramine (2 mg/kg, i.v.) (i.e. $25 \pm 10\%$, $n = 4$, $P = 0.345$, two-way ANOVA, Fig. 3). Mepyramine (2 mg/kg, i.v.) significantly inhibited the hypotensive response to histamine (5 μg/kg, i.v.; $n = 6$; $P < 0.05$, paired *t*-test) confirming the efficacy of this dose (data not shown).

3.2. Isolated mesenteric artery

Venom (3–10 μg/ml) induced almost complete relaxation (97%) in isolated phenylephrine pre-contracted mesenteric arteries (Fig. 4). The relaxation was markedly diminished on second addition of the venom in the same preparation (Fig. 4a, $n = 5$, $P < 0.05$, Student's paired *t*-test). In endothelium denuded arteries, the dose-relaxation curve was significantly shifted to the right (Fig. 4b,

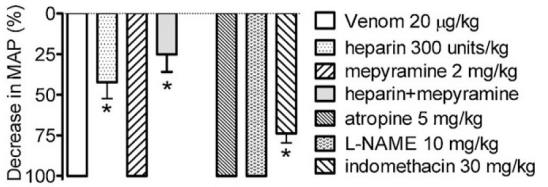

Fig. 3. The effect of *O. scutellatus* venom (20 μg/kg, i.v., $n = 6$) alone and in the presence of heparin (300 units/kg, i.v., $n = 7$), mepyramine (2 mg/kg, i.v., $n = 3$) or a combination of heparin and mepyramine ($n = 4$); atropine (5 mg/kg, i.v., $n = 4$), L-NAME (10 mg/kg, i.v., $n = 4$), or indomethacin (30 mg/kg, i.v., $n = 8$) on MAP. *$P < 0.05$, significantly different from venom alone, one-way ANOVA.

Table 1
Sudden collapse after a single dose versus sequential priming doses of *O. scutellatus* venom.

Group (s)	Number of collapse	% collapse
O. scutellatus venom 50 μg/kg, i.v. single injection in different rats	6	100 (n=6)
Sequential addition of *O. scutellatus* venom 5, 10, 20 and 50 μg/kg, i.v., in same rat	0	0 (n=8)
Sequential addition of *O. scutellatus* venom 5 μg/kg, i.v., 3 times prior 50 μg/kg, i.v. in same rat	5	100 (n=5)
Sequential addition of *O. scutellatus* venom 5 μg/kg, i.v., 4 times prior 50 μg/kg, i.v. in same rat	3	60 (n=5)
Sequential addition of *O. scutellatus* venom 5 μg/kg, i.v., 5 times prior 50 μg/kg, i.v. in same rat	0	0 (n=4)

n=4–5, P<0.05, two-way ANOVA). However, a concentration-dependent relaxation persisted in denuded arteries, demonstrating a significant direct effect of venom on vascular smooth muscle (Fig. 4b, n=4–5, P<0.05, two-way ANOVA).

The relaxant effects of venom (10 μg/ml) on endothelium-intact arteries were significantly inhibited by indomethacin (10 μM) (Fig. 5a, n=5–6, P<0.05, one-way ANOVA) but not by L-NAME (0.2 mM, n=5) or mepyramine (1 μM, n=5) (Fig. 5a).

In endothelium-denuded arteries, the relaxant effect of venom (10 μg/ml, n=10) was significantly reduced by indomethacin (10 μM, n=10), 8-CPT-cAMP-RPI (10 μM, n=5), IbTx (0.1 μM, n=9) (Fig. 5b, P<0.05, one-way ANOVA) and prior administration of a dose of venom (10 μg/ml, n=5, data not shown).

In separate experiments, the effect of venom on endothelial cell function was examined. An ACh concentration–relaxation curve was performed followed by 30 min rest. Then 10 μg/ml of venom was applied for 10 min and, following 30 min equilibrium, a second ACh concentration–relaxation curve was performed. Endothelium-dependent relaxations to ACh were identical before and following venom exposure (n=5, Fig. 6).

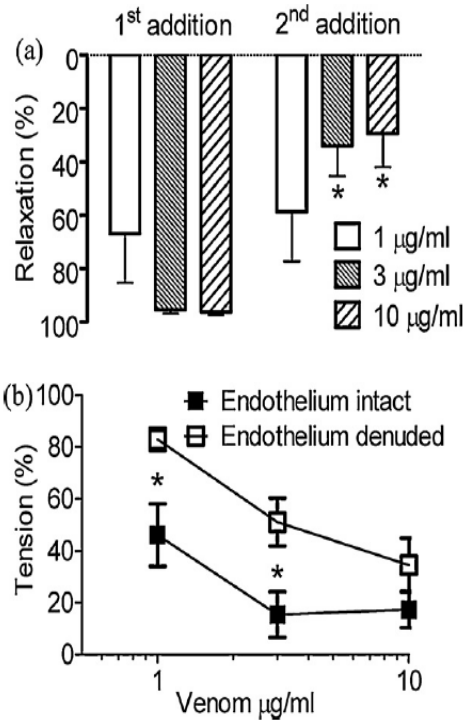

Fig. 4. (a) Relaxation effects of a 2nd addition of *O. scutellatus* venom on endothelium-intact rat mesenteric arteries (n=4–5), *P<0.05, is significantly different from the 1st addition, Student's paired t-test. (b) Relaxant effect of venom (1–10 μg/ml) on relaxation of pre-constricted mesenteric artery ring in the presence and absence of the endothelium (n=4–5). *P<0.05, is significantly different, two way ANOVA.

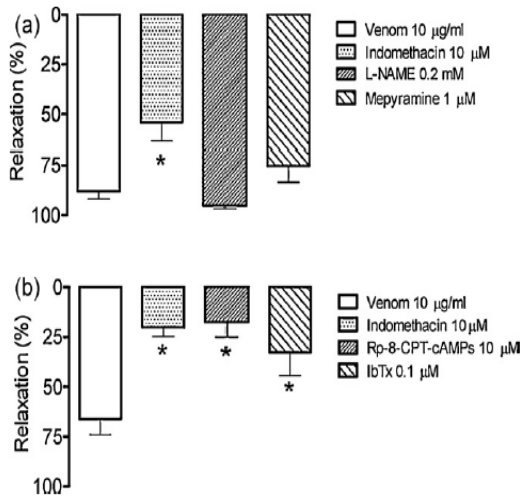

Fig. 5. (a) Relaxation effects of *O. scutellatus* venom (10 μg/ml) on endothelium-intact rat mesenteric arteries in control solution and in the presence of mepyramine (1 μM, n=5); indomethacin (10 μM, n=6) or L-NAME (0.2 mM, n=5). (b) Relaxation effects of *O. scutellatus* venom (10 μg/ml) on the endothelium-denuded rat mesenteric arteries in the presence of indomethacin (10 μM, n=10), Rp-8-CPT-cAMPs (10 μM, n=5) or IbTx (0.1 μM, n=9). *P<0.05, significantly different, one-way ANOVA.

3.3. Heart function (Langendorff heart)

Venom (20–100 μg) had no significant effects on heart rate or left ventricular developed pressure (LVDP) in isolated Langendorff heart preparations (n=3–4, Fig. 7), indicating the apparent absence of direct cardiotoxic components in the venom.

4. Discussion

Sudden collapse following snake envenoming is an uncommon but life-threatening phenomenon. The mechanism(s) behind this effect are unknown although it has been postulated that intravascular coagulation contributes to the collapse (Tibballs et al., 1989,

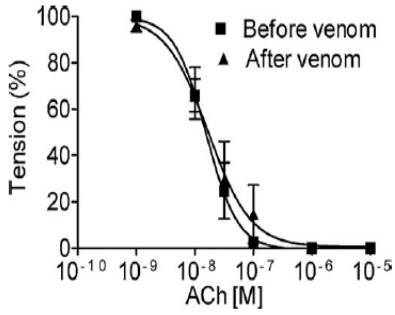

Fig. 6. Concentration relaxation curves to ACh obtained before and after addition of venom (10 μg/ml; n=5).

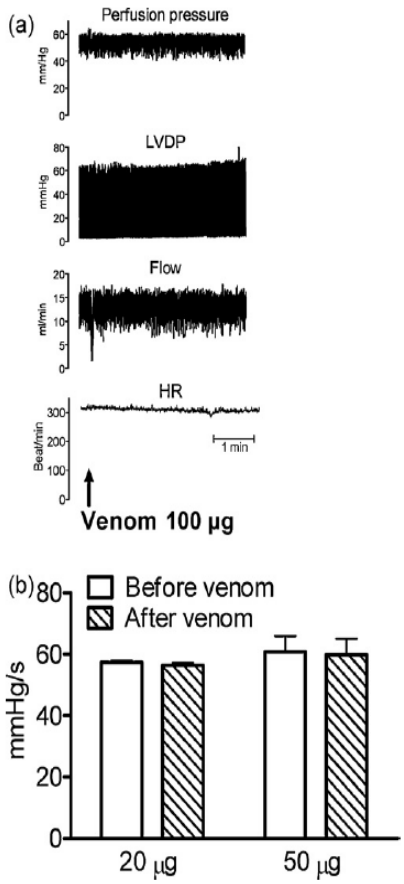

Fig. 7. (a) Effect of *O. scutellatus* venom (100 µg) on perfusion pressure, left ventricular developed pressure (LVDP), coronary flow and heart rate (HR), and (b) of *O. scutellatus* venom (20–50 µg) on LVDP in isolated Langendorff heart preparations (n = 3–4).

1992). However, other hypotensive components found in snake venoms can have a profound effect on the cardiovascular system and remain as potential candidates (Hodgson and Isbister, 2009).

We have previously shown that Papuan taipan venom at 10 µg/kg causes a large rapid hypotensive response and cardiovascular collapse in anaesthetized rats. The 'collapse' was not prevented by artificial respiration, indicating that it is not due to respiratory failure via the action of the numerous neurotoxins present in the venom. The hypotensive response was markedly attenuated by prior application of a single small, 5 µg/kg, 'priming' dose of venom (Crachi et al., 1999). In the current study we have extended observations to a supramaximal dose of venom, 50 µg/kg. While three discrete injections of 5 µg/kg were not protective against 50 µg/kg, five discrete applications of 5 µg/kg (i.e. a total 25 µg/kg) were 'protective'. Thus, it appears that multiple 'priming' doses of venom show a 'threshold' point beyond which even a supramaximal dose (e.g. 50 µg/kg) no longer causes 'collapse'. This indicates that the collapse may be due to the release of endogenous mediators which can be depleted by incremental addition of sublethal amounts of venom, or that a 'resistance' can be achieved by the incremental addition (i.e. a tachyphylactic effect) (Adams et al., 1981). Interestingly, the protective effect of the 'priming' doses is transient (i.e. up to about 30 min) and fails to protect when given 1 h after the priming dose. This supports a role for a depletable mediator in this effect, and indicates that the endogenous substance involved is restored over a period of time.

The protective effects of small doses of venom were explored further. Atropine, L-NAME and mepyramine were unable to prevent collapse suggesting that acetylcholine, nitric oxide (NO) and histamine, respectively, are not major mediators in the collapse. Indomethacin prevented cardiovascular collapse and death, although only reducing the hypotensive effects by approximately 25%. This indicates that cyclooxygenase metabolites (e.g. prostaglandins, prostacyclin and/or thromboxane) may contribute. Indeed, PLA_2 enzymes are common components of many snake venoms and contribute to a range of activities (e.g. neurotoxicity, myotoxicity and coagulopathy). PLA_2 fractions from sand viper (*Vipera ammodytes*) (Sket and Gubensek, 1976) and cobra (*Naja naja*) venoms (Cicala and Cirino, 1993) have been shown to cause hypotension in anaesthetized rats. The hypotensive effect induced by a PLA_2 component isolated from *Vipera russelli* was also inhibited by indomethacin (Ho and Lee, 1981; Huang, 1984).

Pretreatment with heparin also inhibited the collapse induced by Papuan taipan venom. While this would appear to support earlier reports that intravascular coagulation contributes to the collapse (Tibballs et al., 1989, 1992), interpretation of our data is complicated by the fact that heparin has been reported to inhibit histamine release from canine mast cells (Inase et al., 1993). Therefore, the effects of heparin observed in the current study possibly relate to the inhibition of release/synthesis of a range of vasoactive mediators including histamine. Further work is required to determine if consumptive coagulopathy contributes to the early collapse.

We have previously reported that Papuan taipan venom causes endothelium-independent relaxation in rat aortic rings. This effect was independent of nitric oxide and was not mediated by cyclooxygenase metabolites (Crachi et al., 1999). A previous study has suggested that PLA_2 contributes to hypotension through a complex mechanism that may involve a direct action of PLA_2 on endothelial cells (Cicala and Cirino, 1993). In the present study, the effects of Papuan taipan venom on resistance arteries (i.e. rat mesenteric arteries) were examined. The venom did not appear to have an adverse effect on endothelial cells as evidenced by a lack of effect of venom exposure on relaxation responses to ACh. In addition, removal of the endothelium reduced the venom-induced relaxation by about half, demonstrating that the venom possesses both endothelium-dependent and -independent relaxing effects. Indomethacin inhibited relaxation in both endothelium intact and -denuded arteries suggesting the involvement of prostaglandins produced in both endothelial and smooth muscle cells. This result correlates to the data from in vivo experiments which confirm the involvement of prostaglandin production rat cardiac collapse. The prostanoids prostaglandin E_2 (acting via EP2 and 4 receptors) and prostacyclin (PGI_2) cause relaxation via the cAMP/PKA cascade (Catalan et al., 1980). Blockade of PKA using Rp-8-CPT-cAMPs abolished the indomethacin-sensitive component of relaxation. Relaxation was also markedly reduced by IbTx, a selective blocker of large-conductance Ca^{2+}-activated K^+ channels, which may also be involved in PKA induced relaxation in vascular smooth muscle. A previous study suggests that injection of PGI_2 in vivo can cause rapid cAMP depletion in neurons of rats (Schmid et al., 1987) and this supports the depletion of vascular mediators after second addition of venom.

The progressive cardiovascular failure induced by Gaboon viper venom may be due to damage to the endoplasmic system and/or intracellular calcium movement e.g. increase of calcium efflux in cardiomyocytes (Marsh et al., 1979). However, our isolated heart experiments failed to detect any adverse effect of venom on heart rate or mean ventricular pressure.

In conclusion, in vivo administration of Papuan taipan can produce sudden cardiovascular collapse which is similar to the early collapse seen in human envenomation. This collapse can be

prevented by prior administration of sublethal doses of venom indicating the likely release of endogenous factors which can be depleted. In addition, a marked decrease in blood pressure appears to be mediated by an effect on the vasculature rather than on cardiac tissue. Fractionation of the venom will allow us to isolate and characterize the toxins responsible for these effects.

Conflict of interest

None.

Acknowledgements

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References

Adams, Z.S., Gattullo, D., Losano, G., Marsh, N.A., Vacca, G., Whaler, B.C., 1981. The effect of *Bitis gabonica* (gaboon viper) snake venom on blood pressure, stroke volume and coronary circulation in the dog. Toxicon 19, 263–270.

Barrett, A.M., 1971. The effects of some autonomic blocking agents on the heart rates of anaesthetized and pithed rats. European Journal of Pharmacology 15, 267–273.

Broad, A.J., Sutherland, S.K., Coulter, A.R., 1979. The lethality in mice of dangerous Australian and other snake venom. Toxicon 17, 661–664.

Catalan, R.E., Aragones, M.D., Martinez, A.M., Armijo, M., Pina, M., 1980. Effect of indomethacin on the cyclic AMP-dependent protein kinase. European Journal of Pharmacology 63, 187–190.

Cicala, C., Cirino, G., 1993. Phospholipase A_2-induced hypotension in the rat and its pharmacological modulation. General Pharmacology 24, 1197–1202.

Connolly, S., Trevett, A.J., Nwokolo, N.C., Lalloo, D.G., Naraqi, S., Mantle, D., Schofield, I.S., Fawcett, P.R., Harris, J.B., Warrell, D.A., 1995. Neuromuscular effects of Papuan Taipan snake venom. Annals of Neurology 38, 916–920.

Crachi, M.T., Hammer, L.W., Hodgson, W.C., 1999. A pharmacological examination of venom from the Papuan taipan: (*Oxyuranus scutellatus canni*). Toxicon 37, 1721–1734.

Currie, B.J., 2000. Snakebite in tropical Australia, Papua New Guinea and Irian Jaya. Emergency Medicine 12, 285–294.

DalBo, S., Moreira, E.G., Brandao, F.C., Horst, H., Pizzolatti, M.G., Micke, G.A., et al., 2008. Mechanisms underlying the vasorelaxant effect induced by proanthocyanidin-rich fraction from *Croton celtidifolius* in rat small resistance arteries. Journal of Pharmacological Sciences 106, 234–241.

Ho, C.-L., Lee, C.Y., 1981. Cardiovascular effects of phospholipases A_2 purified from various snake venoms. Proceedings of the National Science Council – Republic of China 5, 181–189.

Hodgson, W.C., Isbister, G.K., 2009. The application of toxins and venoms to cardiovascular drug discovery. Current Opinion in Pharmacology 9, 173–176.

Huang, H.C., 1984. Effects of phospholipases A_2 from *Vipera russelli* snake venom on blood pressure, plasma prostacyclin level and renin activity in rats. Toxicon 22, 253–264.

Inase, N., Schreck, R.E., Lazarus, S.C., 1993. Heparin inhibits histamine release from canine mast cells. American Journal of Physiology 264, L387–L390.

Ireland, G., Brown, S.G., Buckley, N.A., Stormer, J., Currie, B.J., White, J., Spain, D., Isbister, G.K., 2010. Changes in serial laboratory test results in snakebite patients: when can we safely exclude envenoming? Medical Journal of Australia 193, 285–290.

Isbister, G.K., Williams, V., Brown, S.G., White, J., Currie, B.J., 2006. Clinically applicable laboratory end-points for treating snakebite coagulopathy. Pathology 38, 568–572.

Johnston, M.A., Fatovich, D.M., Haig, A.D., Daly, F.F., 2002. Successful resuscitation after cardiac arrest following massive brown snake envenomation. Medical Journal of Australia 177, 646–649.

Krstic, M.K., Stepanovic', R., Ilic', K., Krstic', S.K., 1996. Endothelium-independent contractile and relaxant responses to histamine in the rabbit aorta and common carotid, mesenteric, renal, and femoral arteries. General Pharmacology: The Vascular System 27, 529–533.

Lin, Y.-L., Dai, Z.-K., Lin, R.-J., Chu, K.-S., Chen, I.-J., Wu, J.-R., et al., 2010. Baicalin, a flavonoid from *Scutellaria baicalensis Georgi*, activates large-conductance Ca^{2+}-activated K^+ channels via cyclic nucleotide-dependent protein kinases in mesenteric artery. Phytomedicine 17, 760–770.

Marsh, N.A., Smith, I.C., Whaler, B.C., 1979. Effects of envenomation on cardiac cell permeability [proceedings]. Journal of Physiology 291, 72P–73P.

Myint, L., Warrell, D.A., Phillips, R.E., Tin Nu, S., Tun, P., Maung Maung, L., 1985. Bites by Russell's viper (*Vipera russelli siamensis*) in Burma: haemostatic, vascular, and renal disturbances and response to treatment. Lancet 2, 1259–1264.

Parkington, H.C., Chow, J.A., Evans, R.G., Coleman, H.A., Tare, M., 2002. Role for endothelium-derived hyperpolarizing factor in vascular tone in rat mesenteric and hindlimb circulations in vivo. Journal of Physiology 542, 929–937.

Rash, L.D., King, R.G., Hodgson, W.C., 1998. Evidence that histamine is the principal pharmacological component of venom from an Australian wolf spider (*Lycosa godeffroyi*). Toxicon 36, 367–375.

Schmid, G., Bahner, U., Palkovits, M., Geiger, H., Heidland, A., 1987. Prostacyclin (PGI_2) induces rapid depletion of cyclic AMP levels in brain nuclei of rats. Life Sciences 41, 2547–2553.

Sket, D., Gubensek, F., 1976. Pharmacological study of phospholipase A from *Vipera ammodytes* venom. Toxicon 14, 393–396.

Sutherland, S.K., Campbell, D.G., Stubbs, A.E., 1981. A study of the major Australian snake venoms in the monkey (*Macaca fascicularis*). II. Myolytic and haematological effects of venoms. Pathology 13, 705–715.

Tibballs, J., Sutherland, S., Kerr, S., 1989. Studies on Australian snake venoms. Part 1: the haemodynamic effects of brown snake (*Pseudonaja*) species in the dog. Anaesthesia and Intensive Care 17, 466–469.

Tibballs, J., Sutherland, S.K., Rivera, R.A., Masci, P.P., 1992. The cardiovascular and haematological effects of purified prothrombin activator from the common brown snake (*Pseudonaja textilis*) and their antagonism with heparin. Anaesthesia and Intensive Care 20, 28–32.

Tibballs, J., Sutherland, S.K., 1992. The efficacy of heparin in the treatment of common brown snake (*Pseudonaja textilis*) envenomation. Anaesthesia and Intensive Care 20, 33–37.

Vargas, M., Segura, A., Herrera, M., Villalta, M., Estrada, R., Cerdas, M., Paiva, O., Matainaho, T., Jensen, S.D., Winkel, K.D., Leon, G., Gutierrez, J.M., Williams, D.J., 2011. Preclinical evaluation of caprylic acid-fractionated IgG antivenom for the treatment of Taipan (*Oxyuranus scutellatus*) envenoming in Papua New Guinea. PLoS Neglected Tropical Diseases 5, e1144.

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

Wuster, W., Dumbrell, A.J., Hay, C., Pook, C.E., Williams, D.J., Fry, B.G., 2005. Snakes across the Strait: trans-Torresian phylogeographic relationships in three genera of Australasian snakes (Serpentes: Elapidae: *Acanthophis*, *Oxyuranus*, and *Pseudechis*). Molecular Phylogenetics and Evolution 34, 1–14.

1. Introduction

The present study raised interesting questions regarding the potential 'protective effect' of antivenom and histamine receptor antagonists on snake venom-induced cardiovascular collapse. Therefore, addition experiments were carried out. This additional work was not included in the manuscript published in Toxicology Letters.

2. Materials and Methods (continued)

2.1 Sodium dodecyl sulphate (SDS)-PAGE

Electrophoresis was performed according to Laemmli (1970). *O. scutellatus* venom (20 µg) was resolved on a 15% SDS-PAGE under reducing conditions (5% β-mercaptoethanol in Laemmli's sample buffer (62.5 mM Tris-hydrochloride, 25% glycerol, 2% SDS, 0.01% bromophenol blue). All samples were heated for 5 min at 95^{o}C prior to resolving on SDS-PAGE. Protein bands were visualized by staining with BioSafe Coomassie G-250 solution (Bio-Rad Laboratories; Hercules, CA USA), followed by de-staining in distilled water. Gel image was captured utilizing Typhoon Trio scanner (GE Healthcare; Uppsala, Sweden).

2.2 Western blot

Venom (20 µg) proteins were resolved on a 15% SDS-PAGE and transferred onto a PVDF membrane. The membrane was then incubated with 5% skim milk in TBST (20 mM Tris, 0.5 M NaCl, 0.5 % Tween-20) to prevent non specific binding. The membrane was then incubated with primary antibody (CSL polyvalent snake antivenom or taipan antivenom diluted 1:500-fold in TBST with 5% skim milk) and allowed to incubate overnight at 4˚C. Immunoreactive bands were visualized using appropriate secondary antibodies (goat-anti-horse-IgG-HRP, Santa Cruz biotechnology, TX, USA) and ECL^{TM} Western Blotting Detection Reagent (GE Healthcare, Uppsala, Sweden).

2.3 Anaesthetized rat preparation

As per the manuscript.

2.4 Isolation and study of rat mesenteric artery

As per the manuscript.

2.5 Chemicals and drugs

The following chemicals were purchased from Bio-Rad Laboratories, Hercules, CA, USA; 30% acrylamide-base, beta-mercaptoethanol, Coomassie G250, Laemmli sample buffer, skim milk and sodium dodecyl sulphate (SDS).

The following chemicals were purchased from Sigma-Aldrich, St. Louis, MO, USA; Bromophenol blue, TEMED (*N,N,N',N'*-tetramethylethylenediamine), Tris- hydrochloride and H-89. Goat anti-horse IgG-HRP (Santa Cruz biotechnology, CA, USA) and Polyvalent snake antivenom (CSL Ltd., Melbourne, Australia) were also used.

2.6 Analysis of Results and Statistics

As per the manuscript.

3. Results

3.1 SDS-PAGE and Western blot

SDS-PAGE analysis indicated that *O. scutellatus* venom is comprised of protein bands ranging from 10 kDa to 200 kDa (Figure 1, lane A). Western blot analysis using CSL polyvalent snake antivenom (Figure 1, lane B), and CSL taipan antivenom (Figure 1, lane C) showed that both antivenoms were able to detect the majority of the protein bands in the venom. There was no apparent difference between polyvalent snake antivenom and taipan antivenom.

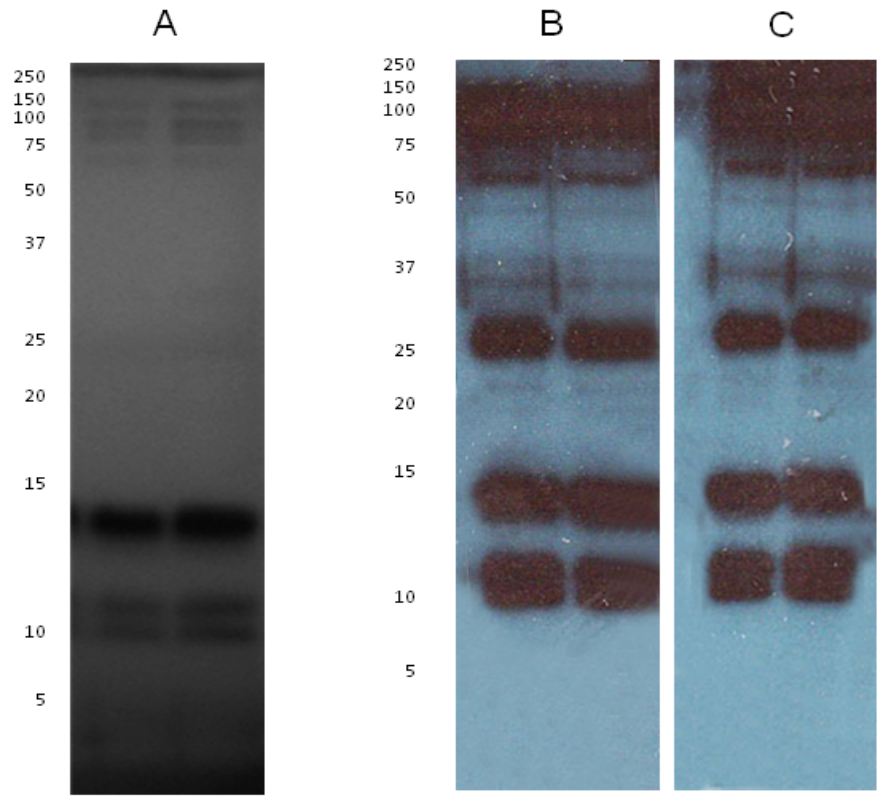

Figure 1: *SDS-PAGE of O. scutellatus venom (lane A), Western blot of O. scutellatus venom with CSL polyvalent snake antivenom (lane B), Western blot of O. scutellatus venom with CSL taipan antivenom (lane C).*

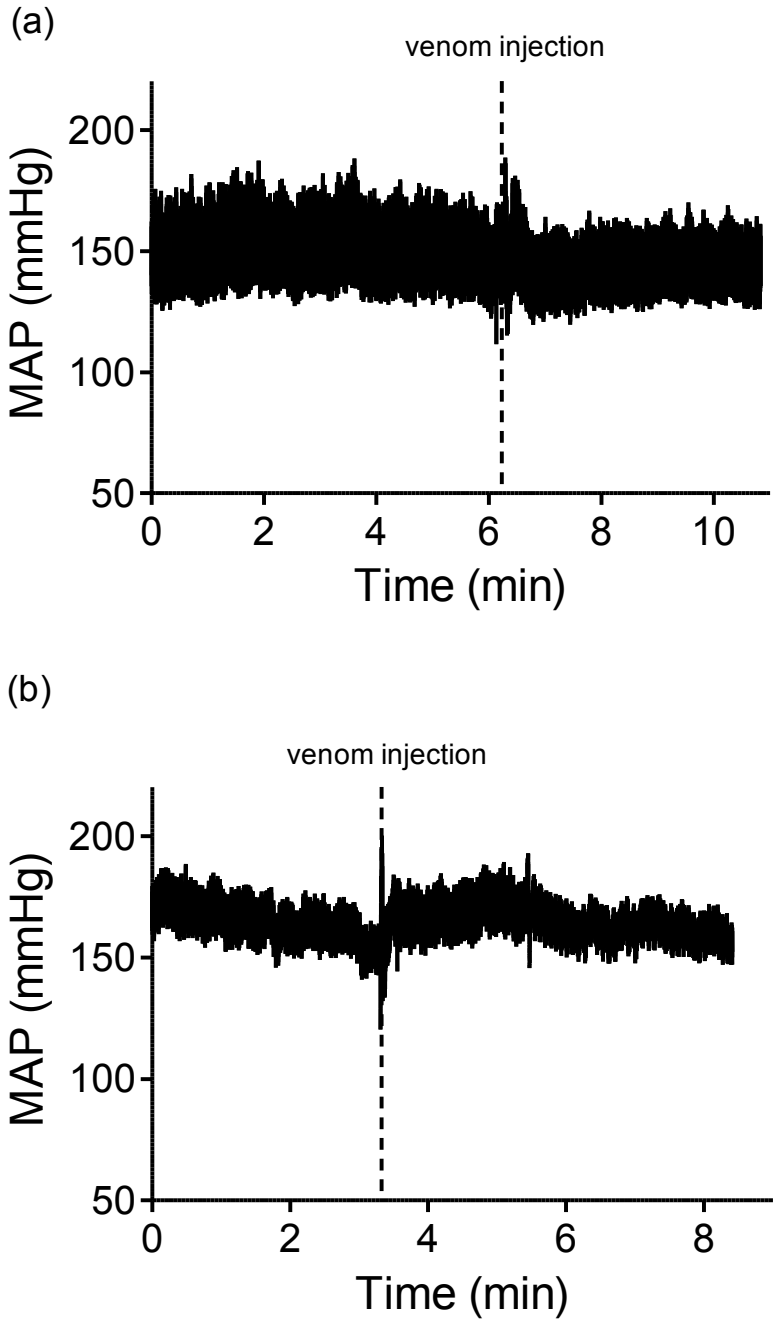

Figure 2: *The effect of O. scutellatus (20 µg/kg, i.v.) venom in anaesthetized rats in the presence of (a) CSL polyvalent snake antivenom (500 units/kg, i.v.) or (b) CSL polyvalent snake antivenom (1000 units/kg, i.v.).*

3.2 Anaesthetized rat experiments

3.2.1 The protective effect of polyvalent snake antivenom on sudden cardiovascular collapse following administration of O. scutellatus venom

Prior administration of 500 or 1000 units/kg of CSL polyvalent snake antivenom (i.v., Figures 2a and 2b) prevented *O. scutellatus*-induced early cardiovascular collapse. However, only the administration of 1000 units/kg of CSL polyvalent snake antivenom significantly inhibited the decrease in MAP following the administration of the venom ($P < 0.05$, one-way ANOVA, Figure 3).

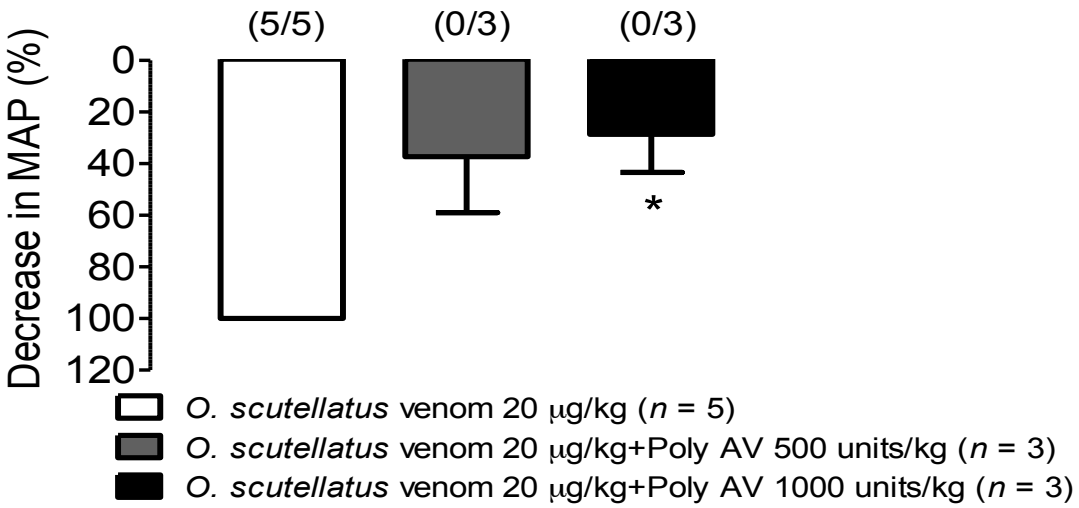

Figure 3: *The effect of O. scutellatus venom (20 μg/kg, i.v.) in the presence or absence of CSL snake polyvalent antivenom (500-1000 units/kg, i.v., bolus injection) on MAP, * P < 0.05, significantly different from venom alone (one-way ANOVA). (In brackets represents the number of animal presenting cardiovascular collapse/ number of total animal in each group).*

3.2.2 Effect of histamine and 5-HT receptor antagonists on sudden cardiovascular collapse

The administration of methysergide (n = 3), or the combination of cimetidine and mepyramine (n = 7), 15 min prior to the injection of *O. scutellatus* venom (20 μg/kg) failed to inhibit sudden cardiovascular collapse (Figure 4).

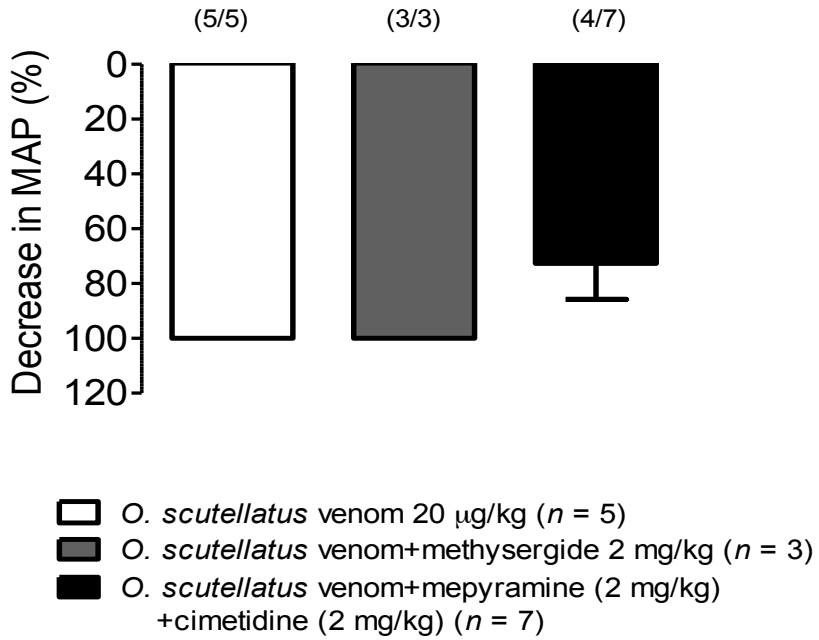

Figure 4: *The effect of O. scutellatus venom (20 μg/kg, i.v) on anaesthetized rats in the presence or absence of methysergide (2 mg/kg, i.v.) or a combination of cimetidine and mepyramine (2 mg/kg, i.v.) (In brackets represents the number of animal presenting cardiovascular collapse/ number of total animal in each group).*

3.3 Effect of H-89 on relaxant effect induced by O. scutellatus venom

The addition of the PKA inhibitor, H-89 significantly attenuated the relaxation produced by *O. scutellatus* venom on endothelium-denuded rat mesenteric artery rings ($P < 0.05$, Student's unpaired *t*-test, Figure 5).

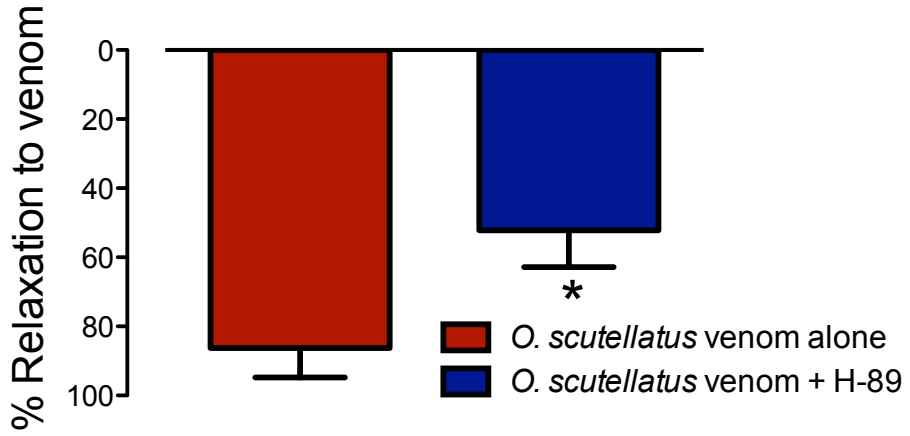

Figure 5: *The effect of O. scutellatus venom (10 µg/ml) on endothelium-denuded rat mesenteric artery rings in the absence or presence of H-89 (4 mM). * P < 0.05, Student's unpaired t-test, n = 6.*

4. Discussion (continued)

Further discussion concerning this additional data is included in the General Discussion (Chapter 9).

CHAPTER 6

VASCULAR RELAXATION EFFECTS OF PHOSPHOLIPASE A_2 TOXINS ISOLATED FROM PAPUAN TAIPAN VENOM

Monash University

Declaration for Thesis Chapter 6

This chapter is made up of the following publication and additional work undertaken but not included in the manuscript.

Declaration by candidate

In the case of Chapter 6, the nature and extent of my contribution to the work was the following:

Nature of Contribution	Extent of Contribution (%)
I declare that experiments and writing of this manuscript were solely undertaken by the myself, taking into consideration the advice and recommendations of co-authors	85%

The following co-authors contributed to the work.

Name	Nature of Contribution
Associate Professor Geoffrey K Isbister	Development of ideas, manuscript preparation
Dr Marianne Tare	Development of ideas, Manuscript preparation
Professor Helena C Parkington	Provision of expertise, development of ideas, manuscript preparation
Professor Wayne C Hodgson	Development of ideas, manuscript preparation

Candidate's signature: [redacted] Date: 07.06.2013

Declaration by co-authors

The undersigned hereby certify that:

(1) the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors;
(2) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(3) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(4) there are no other author of the publication according to these criteria;
(5) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journal or other publications and (c) the head of the responsible academic unit; and
(6) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location (s) Department of Physiology and Department of Pharmacology, Monash University, Clayton

		Date
Associate Professor Geoffrey K Isbister	[redacted]	07.06.2013
Dr Marianne Tare	[redacted]	06.06.2013
Professor Helena C Parkington	[redacted]	06.06.2013
Professor Wayne C Hodgson	[redacted]	07.06.2013

Hypotensive and vascular relaxant effects of phospholipase A_2 toxins from Papuan taipan (*Oxyuranus scutellatus*) venom

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**** The manuscript has been submitted to The European Journal of Pharmacology.**

Abstract

Phospholipase A_2 (PLA_2) toxins are common and abundant components of Australasian elapid venoms. These toxins are associated with a range of activities including neurotoxicity, myotoxicity and coagulation disturbances. We have recently reported that sudden cardiovascular collapse induced by Papuan taipan (*Oxyuranus scutellatus*) venom involves a combination of the release of dilator autacoids and a direct effect on the smooth muscle. In this study, we aimed to isolate PLA_2 components from Papuan taipan venom and investigate their contribution to the hypotensive action of this venom. *O. scutellatus* venom was fractionated using size-exclusion high performance liquid chromatography (HPLC). Venom fractions were screened for activity in anaesthetized rats. Fraction 3 from *O. scutellatus* venom (i.e. OSC3, 14.2 ± 1.0 % of whole venom) produced a 64 % decrease in mean arterial pressure. Reverse-phase HPLC indicated that OSC3 consisted of 2 major components (OSC3a and OSC3b). OSC3a and OSC3b produced a significant hypotensive response in anaesthetized rats which were attenuated by prior administration of indomethacin. N-terminal analysis indicated that OSC3a and b displayed sequence homology to PLA_2 toxins isolated from coastal taipan (*O. scutellatus scutellatus*) venom. These findings indicate that PLA_2 components may play an important role in the development of hypotension and vascular relaxation which may contribute to the effects observed after envenoming by Australasian elapids.

Keywords: vascular relaxation, hypotension, venom, anaesthetized rat, mesenteric artery

Abbreviations: ACh, acetylcholine; i.v., intra venous; i.p., intra peritoneal; MAP, mean arterial pressure; IbTX, iberiotoxin; 8-pCPT-cAMP-RPI, 8-(4-chlorophenylthio)adenosine-3', 5'-cyclic monophosphorothioate, Rp-isomer sodium salt; PLA_2, Phospholipase A_2

1. Introduction

Snake venoms are a complex mixture of biochemical compounds affecting vital physiological mechanisms that have evolved to assist in prey capture and digestion. Human systemic envenoming by Australasian elapids may result in a range of clinical outcomes including neurotoxicity, myotoxicity and coagulation disturbances (Isbister et al., 2008). Of the different classes of toxins that are responsible for the clinical effects observed after envenoming, toxins with a direct or indirect effect on the cardiovascular system are among the least well studied groups from Australasian elapid snake venoms.

Phospholipase A_2 (PLA_2) enzymes hydrolyze the *sn*-2 acyl bond of glycerophospholipids with the release of lysophospholipid and fatty acids (Kini, 2003). PLA_2 toxins are common components of snake venoms that can cause a range of different effects, including neurotoxicity (Chaisakul et al., 2010), myotoxicity (Wickramaratna et al., 2003) and hypotension in animal models (Cicala and Cirino, 1993).

Early hypotension following snakebite has been postulated to involve the formation of highly active mediators e.g. bradykinin (Ferreira and Habermehl, 1997), histamine (Fearn et al., 1964) and dilator autacoids (Huang, 1984b). Isolated PLA_2 toxins from the venom of *Vipera russelli* (Huang, 1984a) and *Naja mozambique mozambique* (Cicala and Cirino, 1993) cause marked hypotension in rats which involves the production of prostacyclin (Huang, 1984a) and the interaction of platelets and leucocytes (Cicala and Cirino, 1993), respectively. In addition, sudden cardiovascular collapse following envenoming by some Australasian elapids (*Pseudonaja textilis*, *Notechis scutatus*) has been suggested to be due to vascular occlusion in major arteries following the induction of a prothrombin activator (Tibballs, 1998; Tibballs et al., 1992).

We have previously documented the profound hypotensive effects of *O. scutellatus* (Papuan taipan) venom in rats. In addition, *O. scutellatus* venom reversed Bay K8644-induced aortic smooth muscle contraction indicating the likely involvement of L-type Ca^{2+} channels in this effect (Crachi et al., 1999). In a subsequent study, *O. scutellatus* venom failed to affect rate and force in

an isolated Langendorff heart preparation (Chaisakul et al., 2012). However, the venom caused relaxation of pre-contracted rat mesenteric arteries, an effect which was antagonized by indomethacin, IbTX and 8-pCPT cAMP-RPI. This suggests the involvement of dilator autacoids and a direct relaxing effect on vascular smooth muscle associated with the cAMP/protein kinase A (PKA) cascade (Chaisakul et al., 2012).

Cardiovascular toxicity following envenoming by Australasian elapids is a cause of mortality and morbidity in Papua New Guinea (Lalloo et al., 1997) and some areas of Australia (Johnston et al., 2002). However, there has been limited characterization of the cardiovascular components of these venoms. In the current study, we aimed to isolate and characterize the PLA_2 components of *O. scutellatus* venom that are responsible for, or contribute to, the cardiovascular effects of this venom. This work will provide insight into the mechanism behind the clinical effects of this venom.

2. Materials and methods

2.1 Venom preparation

Lyophilized Papuan taipan (*O. scutellatus*; Merauke, Irian Jaya) venom was purchased from Venom Supplies (Tanunda, South Australia). Venom was dissolved in Milli-Q H_2O. Insoluble material was removed by centrifugation at 10,000 x g at room temperature for 8 min. The supernatant was filter sterilized using a 0.22 µm membrane and applied to size-exclusion high performance liquid chromatography (HPLC). The protein content of isolated compound was determined using a BCA protein assay kit (Pierce biotechnology; Illinois, USA) as per manufacturer's instruction.

2.2 Chromatography

Both size-exclusion and reverse phase- (RP) HPLC were performed using a Shimadzu (Kyoto, Japan) HPLC system (LC-10ATvp pump and SPD-10AVP detector).

2.2.1 Size-exclusion HPLC

Size-exclusion HPLC was utilized to isolate component of the venom. Venom (3 mg) was applied to a Superdex G-75 column (13 µm; 10×300mm; GE Healthcare, Buckinghamshire, UK) equilibrated with ammonium acetate buffer (0.1 M; pH 6.8). The sample was eluted at a flow rate of 0.5 ml/min and the output was monitored at 280 nm. The fractions were collected and pooled, frozen at -80°C and then freeze-dried to remove the solvent. Screening for the hypotensive effects of each fraction was performed in anaesthetized rats as per section 2.5.

2.2.2 Reverse phase-HPLC

Following screening in the anaesthetized rat preparation, fractions of *O. scutellatus* obtained from size-exclusion HPLC as described in 2.2.1 were dissociated into their subunits using RP-HPLC. Samples (100 µg) were reconstituted in Milli-Q water and applied to a Phenomenex (Torrance, CA, USA) Jupiter analytical C18 column (150 mm × 2 mm; 5 µm; 300 Å) after equilibrating with solvent A (0.1% trifluoroacetic acid, TFA). The sample was eluted under the following gradient conditions: solvent B (90% acetonitrile in 0.09% TFA): 0 to 20% over 5 min, 20 to 60% for 5-40 min and then 60 to 80% for 40-45 min at a flow rate of 0.2 ml/min. The eluent was monitored at 214 nm. Fractions were collected, frozen at -80°C and then freeze-dried. Freeze-dried components were reconstituted with Milli-Q water and protein content determined using BCA protein assay kit. Fractions containing high protein concentration (≥ 0.1 mg/ml) were further investigated for molecular mass and N- terminal amino acid sequence.

2.3 Mass Spectrometry

Molecular masses were identified using MALDI-TOF as described previously (Chaisakul et al., 2010). Mass spectrometry analysis was performed using an Applied Biosystems (Foster City, CA, USA) 4700 TOF TOF Proteomics Analyser. The instrument was operated in positive polarity in linear mode using α-cyano-4-hydroxycinnamic acid (CHCA) matrix (Agilent Technologies, Palo Alto, CA, USA) for low-resolution protein analysis. Matrix (0.5 µl) was spotted on the sample

plate and allowed to air-dry; sample (0.5 µl) diluted in acetonitrile/water (1:1) containing 0.1% (v/v) formic acid was subsequently spotted on dried matrix and allowed to air-dry. Data from 2000 laser shots (337-nm nitrogen laser) were collected, and the signal was averaged and processed with the instrument manufacturer's 4000 series Data Explorer software.

2.4 N-Terminal Amino Acid Sequence Determination

Toxins obtained from RP-HPLC were loaded into the sequencing chamber of a Procise N-terminal amino acid sequencer (Applied Biosystems). Amino acid sequence was determined (Edman degradation, phenylthiohydantoin derivatization chemistry, and separation of derivatized amino acids by RP-HPLC) using the manufacturer's recommended methods and reagents.

2.5 Anaesthetized rat preparation

All procedures were approved by the MARP Animal Ethics Committee, Monash University. Male Sprague-Dawley rats (250-360 g) were anaesthetized with pentobarbitone (80-100 mg/kg, i.p.) which was supplemented as required. Cannulae were inserted into the trachea, jugular vein and carotid artery, for artificial respiration (if required), administration of toxins and measurement of blood pressure, respectively. Arterial blood pressure was recorded using a Gould Statham P23 pressure transducer connected to a PowerLab system. Mean arterial pressure (MAP) was defined as diastolic blood pressure plus one-third of pulse pressure (i.e. systolic minus diastolic blood pressures). At the conclusion of the experiment rats were killed by an overdose of pentobarbitone (i.v.).

As indicated, several antagonists have been administered 10 min prior the administration of PLA_2 compounds. Concentration of each antagonist was referred from the previous study (Chaisakul et al., 2012).

2.6 Rat mesenteric artery preparation

Rats (300-400 g) were killed by isoflurane and decapitated, the mesenteric bed removed and placed in ice-cold physiological saline solution (PSS). Lengths of mesenteric artery (1-2 mm) were studied in a wire myograph (Danish MyoTechnology) as described previously (Mazzuca et al., 2010). When required, the endothelium was removed by gently rubbing the intimal surface with thin roughened wire. Pre-heated PSS (5 ml) was added to the bath and bubbled with carbogen (95% O_2/5% CO_2) to maintain a pH of 7.4. The composition of PSS was as follows (mM): NaCl 120; KCl 5; $NaHCO_4$ 25; glucose 11; KH_2PO_4 1.0; $MgSO_4$; 1.2; $CaCl_2$ 2.5. The solution was maintained at 35°C. The solution was changed every 15 min. Incremental increases in tension were applied until the resting tension was equivalent to ~70 mmHg. The integrity of the endothelium was confirmed by a maximal relaxation to acetylcholine (ACh, 10 μM) in tissues precontracted with a sub-maximal concentration of phenylephrine (3 μM). Venom fractions were added discretely with only one concentration (10 μg/ml) examined in each tissue.

2.7 Determination of PLA_2 Activity

PLA_2 activities of the hypotensive components were determined using a secretory PLA_2 colorimetric assay kit (Cayman Chemical, USA, Cat No. 765001) as described previously (Chaisakul et al., 2010). In the assay, 1, 2-dithio analogue of diheptanoyl phosphatidylcholine was used as a substrate for PLA_2 enzymes. Free thiols generated following the hydrolysis of the thioester bond at the *sn*-2 position by PLA_2 are detected using 5, 5'-dithio-bis(2-nitrobenzoic acid (DTNB). Changes in color were monitored at 405 nm in a fusion α microplate reader (Perkin-Elmer, MA, USA), sampling every min for a 5 min period. PLA_2 activity was expressed as micromoles of phosphatidylcholine hydrolyzed per min per mg of enzyme.

2.8 Procoagulant assay

Frozen human disease-free plasma (Australian Red Cross Blood Bank) was thawed and warmed at 37°C. Tris-buffered saline (TBS) (100 μL/well) was used as control (TBS alone) as well as a buffer. Stock solutions of *O. scutellatus* venom and PLA_2 fractions (10-20 μg/ml; 100 μL)

were added to separate initial wells and serially diluted six times (1:2 dilution) yielding a venom/toxin concentration range of 0.3 to 20 µg/ml in a 96 well microtiter plate. $CaCl_2$; 0.2 M; 50 µL/mL plasma, was added to the plasma and was simultaneously added to each well (100 µL plasma/well) using a multichannel pipette. Kinetic absorbance was measured at 37°C every 30 s for 20 min at 340 nm using the BMG Fluostar Omega plate reader (BMG LABTECH, Australia). As previously described, a 0.02 increase in optical density (OD), from the average of the first two absorbance measurements, was used to indicate the commencement of clotting (Lane et al., 2011; O'Leary and Isbister, 2010).

2.9 Statistics

Student's unpaired *t-tests* were performed on responses to venom fractions in different animals and tissues, and paired *t*-tests were used to compare responses before and after the administration of toxin in the same animal. Multiple comparisons were made using a one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. Data were expressed as mean ± SEM. Statistical significance was accepted when $P < 0.05$. All statistical analysis was performed using Prism 5.0 software (GraphPad Software, San Diego, CA, USA).

2.10 Chemicals and drugs

The following drugs and solutions were used: ammonium acetate, ACh, indomethacin, phenylephrine HCl, all from Sigma Chemical Co. (St Louis, MO, USA), heparin sodium (Hospira, VIC, Australia), trifluoroacetic acid (Auspep, Melbourne, Australia), acetonitrile (Merck, Darmstadt, Germany), mepyramine maleate (May & Baker, Dagenham, UK).

3. Results

3.1 Effect of isolated fractions on anaesthetized rat

Size exclusion HPLC analysis of *O. scutellatus* venom indicated the presence of 8 distinct peaks (Figure 1a). These peaks were individually collected and tested for activity in the anaesthetized rat preparation. Peaks 3 and 4 induced a transient hypotensive response (i.e. 64% and 43% decrease in MAP for peaks 3 and 4, respectively) with the former producing the largest fall in MAP. As such, this peak (subsequently named OSC3) was chosen for further study. OSC3 represented 14.2 ± 1.0 % (n = 4) of the whole venom profile from size-exclusion HPLC analysis.

Administration of OSC3 (50 µg/kg, i.v.; Figure 1b) reduced MAP from 131 ± 10 mmHg to 60 ± 7 mmHg. This fraction did not significantly affect heart rate.

Further fractionation of OSC3 using a Phenomenex Jupiter analytical column indicated the presence of 2 major components, which we called OSC3a and OSC3b (Figure 2a), that were subsequently collected for molecular mass and N-terminal sequence determinations.

OSC3a and OSC3b (50 µg/kg, i.v.) reduced MAP by 50 ± 14 mmHg and 74 ± 5 mmHg (Figure 3a), respectively. Neither PLA_2 components produced a significant change in heart rate (Figure 3c). The hypotensive effect of OSC3b (50 µg/kg, i.v.) was significantly abolished upon second administration of the toxin in the same animal (n = 4, $P < 0.05$, Student's paired t-test; Figure 3b). Prior administration of indomethacin (30 mg/kg, i.v.) significantly attenuated the hypotensive effect of OSC3a (50 µg/kg, i.v., n = 4) and OSC3b (50 µg/kg, i.v., n = 4; $P < 0.05$, one-way ANOVA). However, although administration of mepyramine (2 mg/kg, i.v.) appeared to have some inhibitory effect on the hypotension produced by OSC3a (50 µg/kg, i.v., n = 4) or OSC3b (50 µg/kg, i.v., n = 4), but this effect was not statistically significant ($P > 0.05$, one-way ANOVA). Prior administration of a combination of mepyramine (2 mg/kg) and heparin (300 units/kg, i.v.) significantly attenuated the hypotensive effect of OSC3a but not OSC3b (Figures 4a and 4b).

3.2 Molecular mass determination

MALDI-TOF indicated OSC3a and OSC3b had molecular weights of 13,203 and 14,029 Da, respectively.

3.3 Amino acid sequence determination

The N-terminal sequence of 20 amino acid residues of OSC3a displayed 100% homology with a PLA_2 component of Taicatoxin, an oligomeric toxin isolated from the venom of Australian coastal taipan (*O. scutellatus scutellatus*) (Possani et al., 1992) and 95% homology with OS2 (Rouault et al., 2006), secretory PLA_2 from *O. scutellatus scutellatus* venom. The N-terminal sequence of 20 amino acid residues of OSC3b had 100% homology with OS1, a secretory PLA_2 isolated from *O. scutellatus scutellatus* venom (Lambeau et al., 1989) (Table 1).

3.4 Phospholipase A_2 activity

OSC3a and OSC3b had specific PLA_2 activities of 168 ± 4 and 1,294 ± 7 μmol/min/mg (n = 4), respectively. The positive control, bee venom PLA_2, had a specific activity of 320 ± 13 μmol/min/mg (n = 4).

3.5 Isolated rat mesenteric artery

OSC3a and OSC3b (10 μg/ml) induced vasorelaxation in endothelium-intact mesenteric arteries of 83 ± 9% and 70 ± 10% (n = 4; Figure 5), respectively. Removal of the endothelium significantly reduced the relaxation induced by OSC3b (13 ± 8%, $P < 0.05$, Student's unpaired t-test, n = 5; Figure 5). However, the relaxation evoked by OSC3a (57 ± 13%, n = 5) was not significantly affected by endothelial denudation ($P > 0.05$ Student's unpaired t-test).

3.6 Procoagulant assay

In the coagulation test, fractions OSC3a and OSC3b (0.6 - 20 μg/ml) of *O. scutellatus* venom, failed to clot human plasma compared with *O. scutellatus* venom (2.5 - 10 μg/ml; Figure 6).

4. Discussion

In this study, we isolated two vasoactive PLA_2 components from the venom of *O. scutellatus*. Both toxins produced *in vivo* hypotension and relaxed *in vitro* vascular tissue. We have previously reported that *O. scutellatus* venom, 20 µg/kg, i.v., induces cardiovascular collapse in anaesthetized rats (Chaisakul et al., 2012). In the current study, a considerably higher dose (i.e. 50 µg/kg, i.v.) of a combination of the two toxins (i.e. OSC3) only induced a moderate hypotensive response without cardiovascular collapse. Given that the toxins represent 14.2% of the whole venom, this contributes approximately 2.8 µg/kg to the dose of whole venom that was previously reported to cause cardiovascular collapse. However, OSC3a and/or OSC3b failed to induce complete early cardiovascular collapse. This indicates that the mechanism behind the cardiovascular collapse following envenoming by *O. scutellatus* may be either due to the effect of PLA_2 synergizing with other venom components or that other venom components are responsible for this outcome.

Interestingly, the hypotensive effects of OSC3a and OSC3b were attenuated upon second administration of the toxin in the same animal, suggesting that this effect was mediated by depletable endogenous mediators or tachyphylaxis. Prior administration of indomethacin reduced the hypotensive effect of both OSC3a and OSC3b suggesting that cyclooxygenase metabolites (e.g. dilator prostaglandins or prostacyclin) may be involved.

Previously, purified PLA_2s from snake venoms have been reported to induce histamine release from human colon, lung and tonsil mast cells (Wei et al., 2006; Wei et al., 2010). In the present study, pre-administration of mepyramine, an H_1-receptor antagonist, markedly reduced the hypotensive effect following the administration of isolated PLA_2s from *O. scutellatus* venom. However, significant attenuation of hypotension was not observed. This might be due to the small number of *n* value in this study. Heparin has been reported to inhibit histamine release (Inase et al., 1993). Thus, the combination of mepyramine and heparin was administered prior to the injection of snake PLA_2 components. Interestingly, the hypotensive effect of OSC3a was significantly inhibited by this combination.

PLA_2 enzymes are abundant components of scorpion, bee as well as snake venoms (Lambeau et al., 1990). Snake PLA_2 contributes a number of pharmacological effects e.g. pre-synaptic neurotoxicity (Cull-Candy et al., 1976; Fohlman et al., 1976), anticoagulation (Kini, 2005), hypotension (Huang, 1984a), myotoxicity (Harris and MacDonell, 1981), and recently, isolated PLA_2 toxins from coastal taipan have been reported to affect sperm mobility (Escoffier et al., 2010). Snake PLA_2 toxin-induced relaxation may contribute to the hypotensive response following envenoming. Indeed, OSC3a induced relaxation in endothelium-intact and -denuded mesenteric arteries while OSC3b only induced endothelium-dependent relaxation suggesting that OSC3a also may have a direct action on vascular smooth muscle. OSC3a and OSC3b had a molecular mass between 13 and 14 kDa, which is within the range for elapid venom PLA_2 components (Chaisakul et al., 2010; Kuruppu et al., 2005). The N-terminal amino acid sequences of OSC3a and OSC3b showed 100% sequence homology with the PLA_2 component of taicatoxin (Possani et al., 1992) and OS1; a secretory PLA_2 isolated from *O. scutellatus scutellatus* venom, respectively (Lambeau et al., 1989). Taicatoxin is an oligomeric toxin comprising three components, α-neurotoxin-like peptide, a neurotoxic phospholipase peptide and a serine protease inhibitor. Taicatoxin blocks the high threshold calcium channel current of excitable membranes in guinea-pig ventricular heart cells, even in the absence of PLA_2 activity (Possani et al., 1992). The secretory PLA_2, OS1, has been previously reported to induce vascular endothelial cell migration (Rizzo et al., 2000).

It has been postulated that snake venom prothrombin activators mediate the severe depression of systemic blood pressure and cardiac output observed in anaesthetized mechanically ventilated dogs (Tibballs, 1998; Tibballs et al., 1992). We therefore investigated the coagulation effects of OSC3a and OSC3b, and compared these to *O. scutellatus* venom. However, relatively high concentrations of OSC3a and OSC3b failed to induce clotting, in contrast to the effects of the whole venom. This suggests that the hypotensive effect induced by OSC3a and 3b is unlikely to be due to the same toxin that causes venom-induced consumptive coagulopathy (VICC) or be a result of VICC.

In conclusion, the isolated PLA_2 fractions from *O. scutellatus* venom appear to play a significant role in the hypotensive and vasorelaxant responses *in vivo* and *in vitro* studies. The release of dilator autacoids is a significant pharmacological effect of PLA_2 to generate vascular relaxation.

Conflict of interest

None.

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References

Chaisakul, J., Isbister, G.K., Konstantakopoulos, N., Tare, M., Parkington, H.C., Hodgson, W.C., 2012. In vivo and in vitro cardiovascular effects of Papuan taipan (*Oxyuranus scutellatus*) venom: Exploring "sudden collapse". Toxicol Lett 213, 243-248.

Chaisakul, J., Konstantakopoulos, N., Smith, A.I., Hodgson, W.C., 2010. Isolation and characterisation of P-EPTX-Ap1a and P-EPTX-Ar1a: pre-synaptic neurotoxins from the venom of the northern (*Acanthophis praelongus*) and Irian Jayan (*Acanthophis rugosus*) death adders. Biochem Pharmacol 80, 895-902.

Cicala, C., Cirino, G., 1993. Phospholipase A_2-induced hypotension in the rat and its pharmacological modulation. Gen Pharmacol 24, 1197-1202.

Crachi, M.T., Hammer, L.W., Hodgson, W.C., 1999. A pharmacological examination of venom from the Papuan taipan: (*Oxyuranus scutellatus canni*). Toxicon 37, 1721-1734.

Cull-Candy, S.G., Fohlman, J., Gustavsson, D., Lullmann-Rauch, R., Thesleff, S., 1976. The effects of taipoxin and notexin on the function and fine structure of the murine neuromuscular junction. Neuroscience 1, 175-180.

Escoffier, J., Couvet, M., de Pomyers, H., Ray, P.F., Seve, M., Lambeau, G., De Waard, M., Arnoult, C., 2010. Snake venoms as a source of compounds modulating sperm physiology: Secreted phospholipases A_2 from *Oxyuranus scutellatus scutellatus* impact sperm motility, acrosome reaction and in vitro fertilization in mice. Biochimie 92, 826-836.

Fearn, H.J., Smith, C., West, G.B., 1964. Capillary Permeability Responses to Snake Venoms. J Pharm Pharmacol 16, 79-84.

Ferreira, L.A.F., Habermehl, G.G., 1997. Bradykinin potentiating peptides from snake venoms and their spatial structures. Toxicon 35, 807.

Fohlman, J., Eaker, D., Karlsoon, E., Thesleff, S., 1976. Taipoxin, an extremely potent presynaptic neurotoxin from the venom of the australian snake taipan (*Oxyuranus s. scutellatus*). Isolation, characterization, quaternary structure and pharmacological properties. Eur J Biochem 68, 457-469.

Harris, J.B., MacDonell, C.A., 1981. Phospholipase A_2 activity of notexin and its role in muscle damage. Toxicon 19, 419-430.

Huang, H.C., 1984a. Effects of phospholipases A_2 from *Vipera russelli* snake venom on blood pressure, plasma prostacyclin level and renin activity in rats. Toxicon 22, 253-264.

Huang, H.C., 1984b. Release of slow reacting substance from the guinea-pig lung by phospholipases A_2 of *Vipera russelli* snake venom. Toxicon 22, 359-372.

Inase, N., Schreck, R.E., Lazarus, S.C., 1993. Heparin inhibits histamine release from canine mast cells. Am J Physiol 264, L387-390.

Isbister, G.K., Brown, S.G., MacDonald, E., White, J., Currie, B.J., 2008. Current use of Australian snake antivenoms and frequency of immediate-type hypersensitivity reactions and anaphylaxis. Med J Aust 188, 473-476.

Johnston, M.A., Fatovich, D.M., Haig, A.D., Daly, F.F., 2002. Successful resuscitation after cardiac arrest following massive brown snake envenomation. Med J Aust 177, 646-649.

Kini, R.M., 2003. Excitement ahead: structure, function and mechanism of snake venom phospholipase A_2 enzymes. Toxicon 42, 827-840.

Kini, R.M., 2005. Structure-function relationships and mechanism of anticoagulant phospholipase A_2 enzymes from snake venoms. Toxicon 45, 1147-1161.

Kuruppu, S., Reeve, S., Banerjee, Y., Kini, R.M., Smith, A.I., Hodgson, W.C., 2005. Isolation and pharmacological characterization of cannitoxin, a presynaptic neurotoxin from the venom of the Papuan Taipan (*Oxyuranus scutellatus canni*). J Pharmacol Exp Ther 315, 1196-1202.

Lalloo, D.G., Trevett, A.J., Nwokolo, N., Laurenson, I.F., Naraqi, S., Kevau, I., Kemp, M.W., James, R., Hooper, L., David, R., Theakston, G., Warrell, D., 1997. Electrocardiographic abnormalities in patients bitten by taipans (*Oxyuranus scutellatus canni*) and other elapid snakes in Papua New Guinea. Trans R Soc Trop Med Hyg 91, 53-56.

Lambeau, G., Barhanin, J., Schweitz, H., Qar, J., Lazdunski, M., 1989. Identification and properties of very high affinity brain membrane-binding sites for a neurotoxic phospholipase from the taipan venom. J Biol Chem 264, 11503-11510.

Lambeau, G., Schmid-Alliana, A., Lazdunski, M., Barhanin, J., 1990. Identification and purification of a very high affinity binding protein for toxic phospholipases A_2 in skeletal muscle. J Biol Chem 265, 9526-9532.

Lane, J., O'Leary, M.A., Isbister, G.K., 2011. Coagulant effects of black snake (*Pseudechis* spp.) venoms and in vitro efficacy of commercial antivenom. Toxicon 58, 239-246.

Mazzuca, M.Q., Wlodek, M.E., Dragomir, N.M., Parkington, H.C., Tare, M., 2010. Uteroplacental insufficiency programs regional vascular dysfunction and alters arterial stiffness in female offspring. J Physiol 588, 1997-2010.

O'Leary, M.A., Isbister, G.K., 2010. A turbidimetric assay for the measurement of clotting times of procoagulant venoms in plasma. J Pharmacol Toxicol Methods 61, 27-31.

Possani, L.D., Martin, B.M., Yatani, A., Mochca-Morales, J., Zamudio, F.Z., Gurrola, G.B., Brown, A.M., 1992. Isolation and physiological characterization of taicatoxin, a complex toxin with specific effects on calcium channels. Toxicon 30, 1343-1364.

Rizzo, M.T., Nguyen, E., Aldo-Benson, M., Lambeau, G., 2000. Secreted phospholipase A_2 induces vascular endothelial cell migration. Blood 96, 3809-3815.

Rouault, M., Rash, L.D., Escoubas, P., Boilard, E., Bollinger, J., Lomonte, B., Maurin, T., Guillaume, C., Canaan, S., Deregnaucourt, C., Schrevel, J., Doglio, A., Gutierrez, J.M., Lazdunski, M., Gelb, M.H., Lambeau, G., 2006. Neurotoxicity and other pharmacological activities of the snake venom phospholipase A_2 OS2: the N-terminal region is more important than enzymatic activity. Biochemistry 45, 5800-5816.

Tibballs, J., 1998. The cardiovascular, coagulation and haematological effects of tiger snake (*Notechis scutatus*) prothrombin activator and investigation of release of vasoactive substances. Anaesth Intensive Care 26, 536-547.

Tibballs, J., Sutherland, S.K., Rivera, R.A., Masci, P.P., 1992. The cardiovascular and haematological effects of purified prothrombin activator from the common brown snake (*Pseudonaja textilis*) and their antagonism with heparin. Anaesth Intensive Care 20, 28-32.

Wei, J.F., Mo, Y.Z., Qiao, L.Y., Wei, X.L., Chen, H.Q., Xie, H., Fu, Y.L., Wang, W.Y., Xiong, Y.L., He, S.H., 2006. Potent histamine-releasing activity of atrahagin, a novel snake venom metalloproteinase. Int J Biochem Cell Biol 38, 510-520.

Wei, J.F., Wei, X.L., Chen, Q.Y., He, S.H., 2010. Induction of inflammatory cell accumulation by TM-N49 and promutoxin, two novel phospholipase A_2. Toxicon 56, 580-588.

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.

Figures

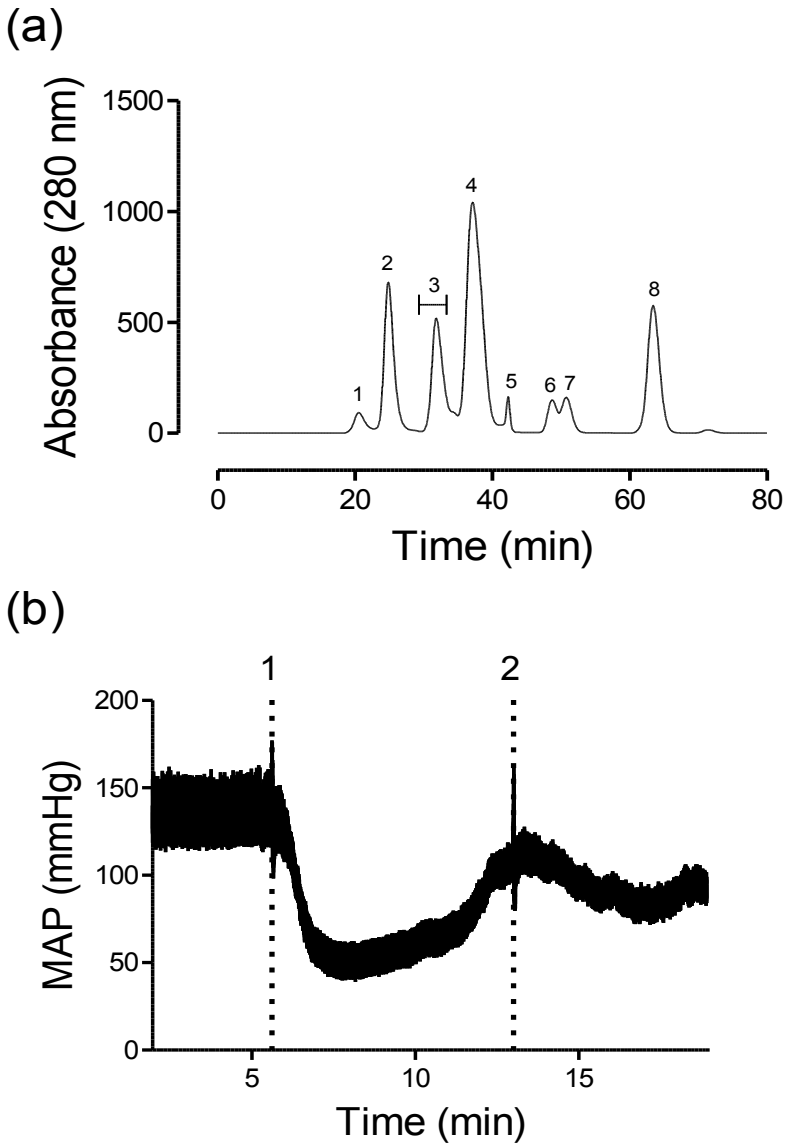

Figure 1: *(a) Size-exclusion chromatograph of O. scutellatus venom run on Superdex G-75 column equilibrated with ammonium acetate (0.1 M, pH 6.8) at a flow rate of 0.5 ml/min, highlighted section refer to fraction with hypotensive effect (OSC3). (b) Effect of OSC3 (50 µg/kg, i.v., at time point 1 and 2) on MAP in an anaesthetized rat.*

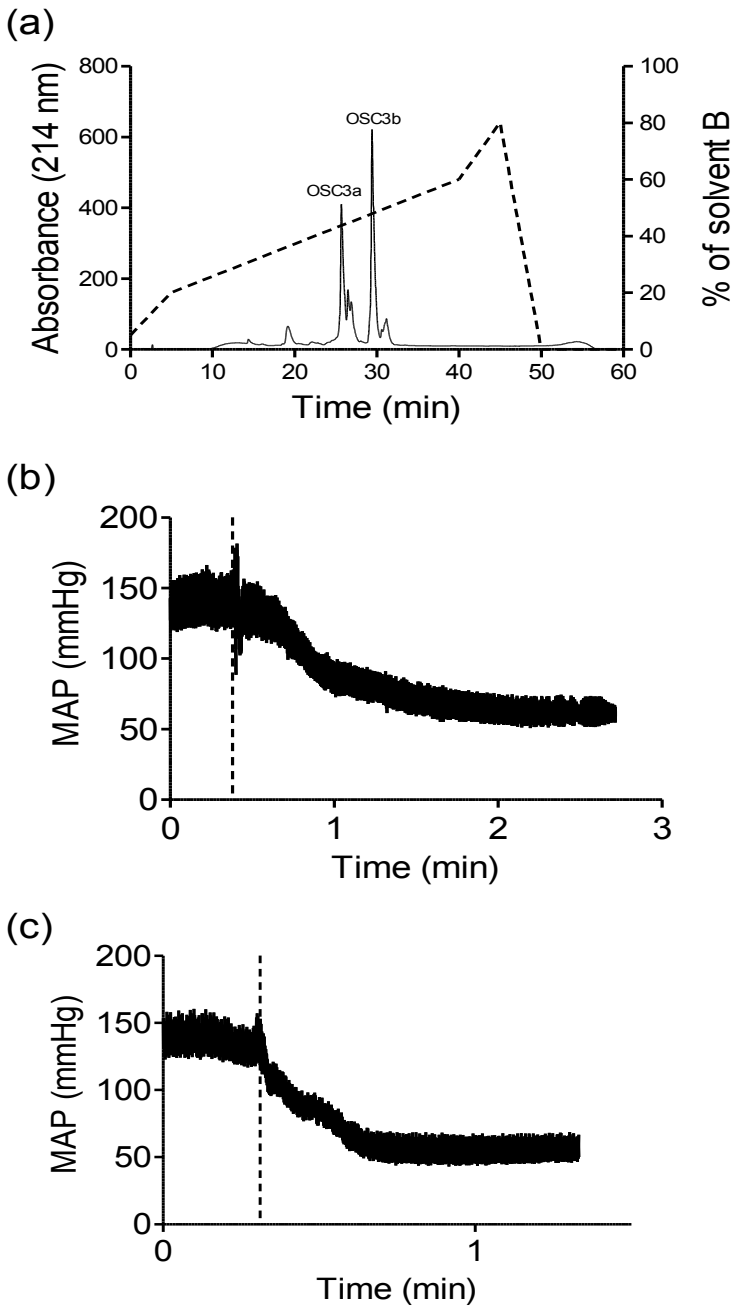

Figure 2: *RP-HPLC chromatograph of OSC3 on a Jupiter analytical C18 column, equilibrated with 0.1%, TFA (solvent A) and eluted with varied gradient condition of 90% acetonitrile in 0.09% TFA (solvent B) at a flow rate of 0.2 ml/min. The presence of OSC3a and OSC3b is indicated (a). The effect of (b) OSC3a (50 µg/kg, i.v.) or (c) OSC3b (50 µg/kg, i.v.) on MAP of anaesthetized rats.*

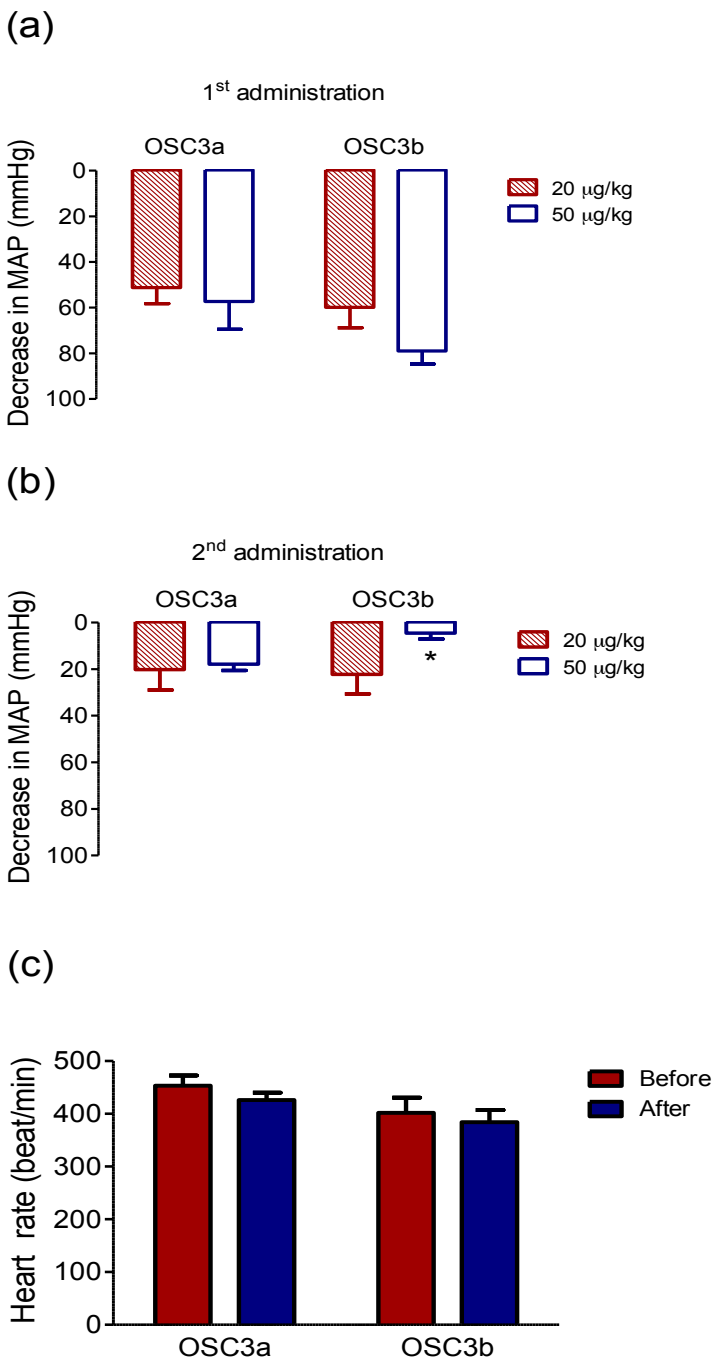

Figure 3: *MAP following 1st (a) and 2nd (b) administration of OSC3a and OSC3b (20-50 µg/kg, i.v.; n = 4). *significant difference in MAP compared to 1st administration, P < 0.05, Student's paired t-test. (c) A comparison of rat heart rate before and after the administration of OSC3a or OSC3b (50 µg/kg, i.v.).*

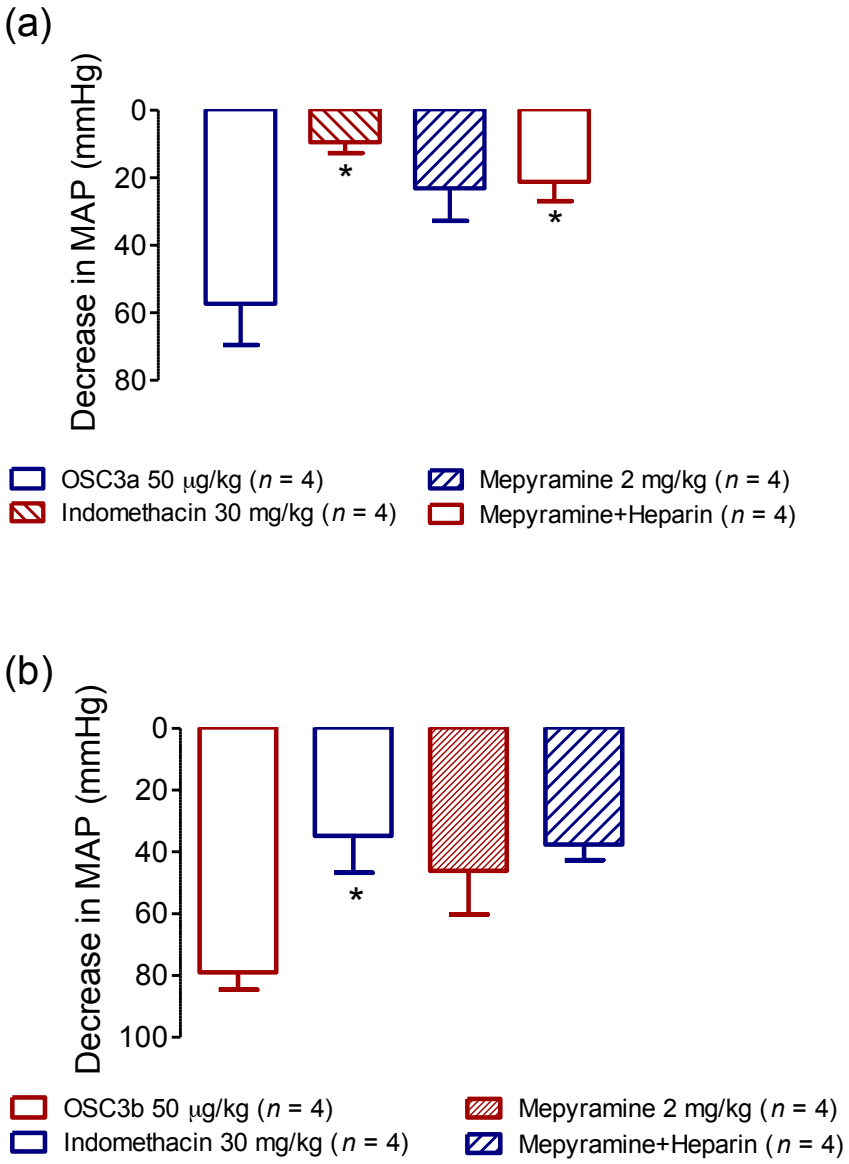

Figure 4: *Effect of (a) OSC3a (50 μg/kg, i.v.) or (b) OSC3b (50 μg/kg, i.v.) in the absence or presence of mepyramine (2 mg/kg, i.v.), indomethacin (30 mg/kg, i.v.) or the combination of mepyramine (2 mg/kg, i.v.) and heparin (300 units/kg, i.v.) on MAP of anaesthetized rats. * P < 0.05, significantly different from OSC3a or OSC3b alone, one-way ANOVA.*

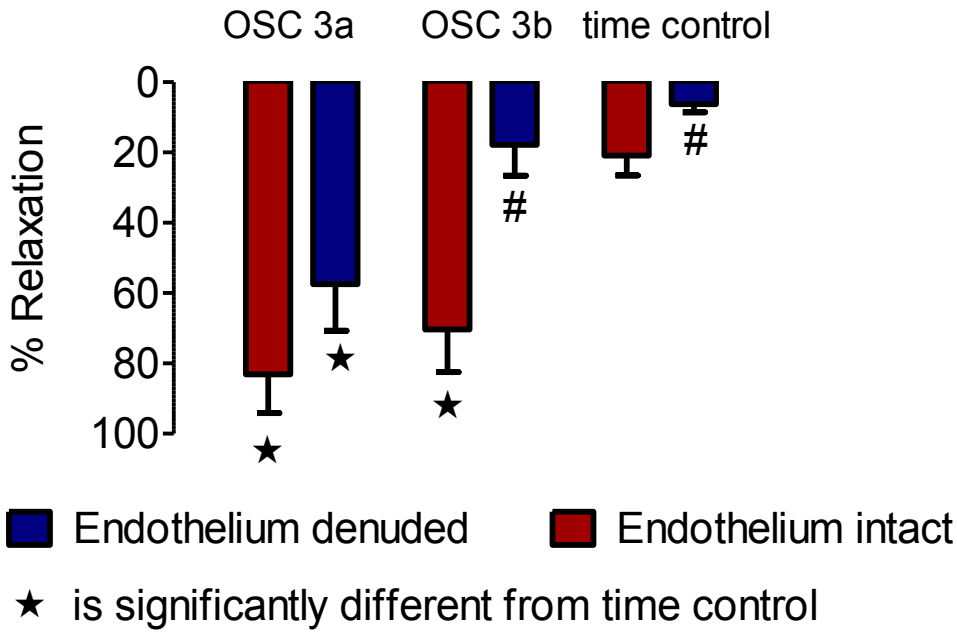

Figure 5: *Relaxation effects of OSC3a and OSC3b (10 µg/ml) on endothelium-intact (n = 4) and endothelium-denuded rat mesenteric arteries (n = 5). # P < 0.05, is significantly different from endothelium-intact, Student's unpaired t-test.*

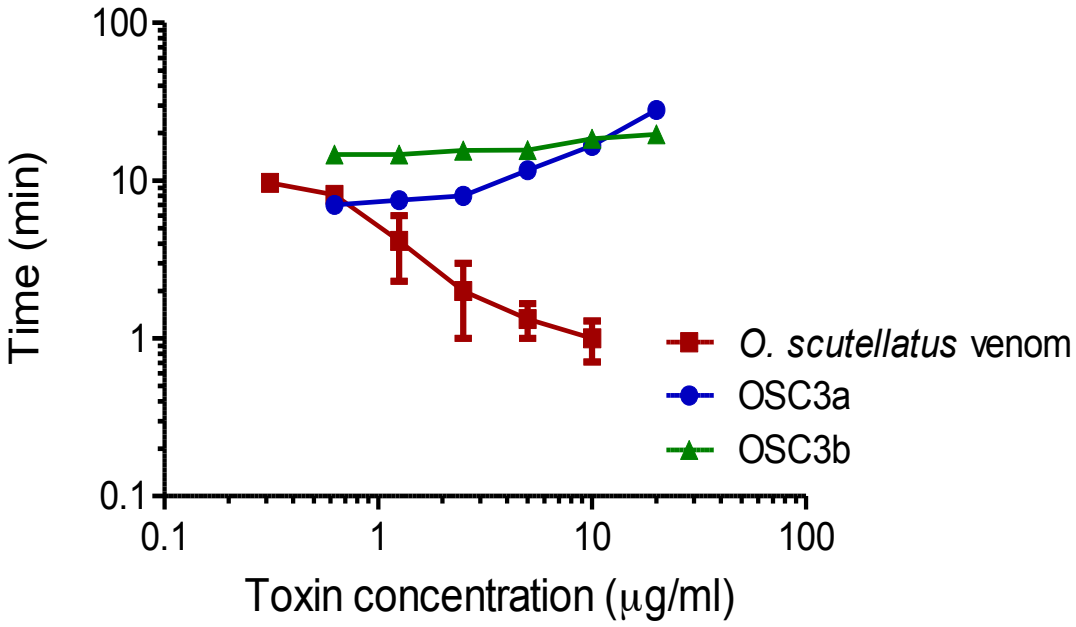

Figure 6: *Plot of clotting time versus concentration of O. scutellatus venom and OSC3a or 3b (3 duplications).*

Table 1: N-terminal sequences of OSC3a and OSC3b compared with other closely related toxins

Species	Toxins	N-terminal sequence
O. scutellatus	OSC3a (13,203 Da)	NLAQFGFMIR CANGGSRSAL
O. s. scutellatus	Taicatoxin[a]; PLA_2 component (16,000 Da)	NLAQFGFMIR CANGGSRSAL
	OS2[c] (16,104 Da)	NLAQFGFMIR CANGGSRS**P**L
O. scutellatus	OSC3b (14,029 Da)	SLLNFANLIE CANHGTRSAL
O. s. scutellatus	OS1[b, c]; secretory PLA_2 (16,898 Da)	SLLNFANLIE CANHGTRSAL

Bold letter indicates different amino acid present at the respective position in the toxin.

[a] Possani et al. (1992); [b] Lambeau et al. (1989); [c]Rouault et al. (2006)

1. Introduction

The supplementary data have been obtained in order to demonstrate: (1) the effect of the individual venom fractions on blood pressure and (2) the relaxant effects of fractions on isolated arteries in the presence of inhibitors. This additional work was not included in the manuscript.

2. Materials and Methods (continued)

2.1 Anaesthetized rat preparation

As per manuscript.

2.2 isolated rat mesenteric artery

As per manuscript.

2.3 Chick biventer cervicis nerve-muscle preparation

As per manuscript on Chapter 2.

2.4 Data analysis

As per manuscript.

3. Results

3.1 Screening fractions of O. scutellatus venom in anaesthetized rats

Fractions of *O. scutellatus* (Peaks 1-8) were screened in anaesthetized rats. Peak 3 (as per manuscript) and peak 4 (Figure 1c) displayed a hypotensive effect in anaesthetized rats. A combination of fractions 1-8 (50 µg/kg, i.v.) produced a hypotensive effect without sudden cardiovascular collapse (Figure 2).

NB. The size-exclusion column used for the isolation of toxins has a separation range of 3,000-70,000 Da thus non-detectable peaks of *O. scutellatus* venom or higher molecular mass toxins, which were not studied, may contribute to complete sudden collapse.

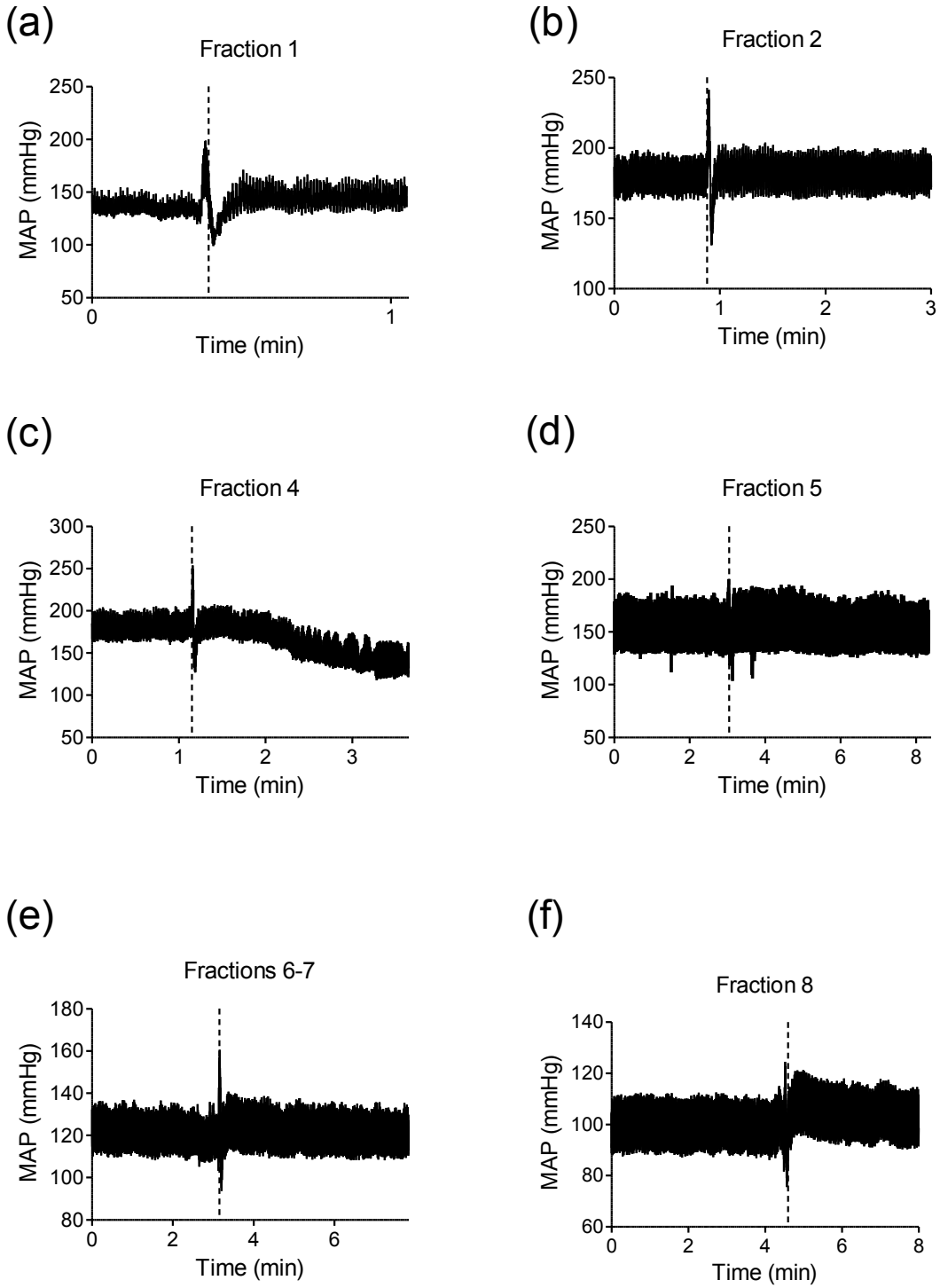

Figure 1: *Screening of O. scutellatus peaks (50 µg/kg, i.v.) on the MAP of anaesthetized rats (n = 2-4).*

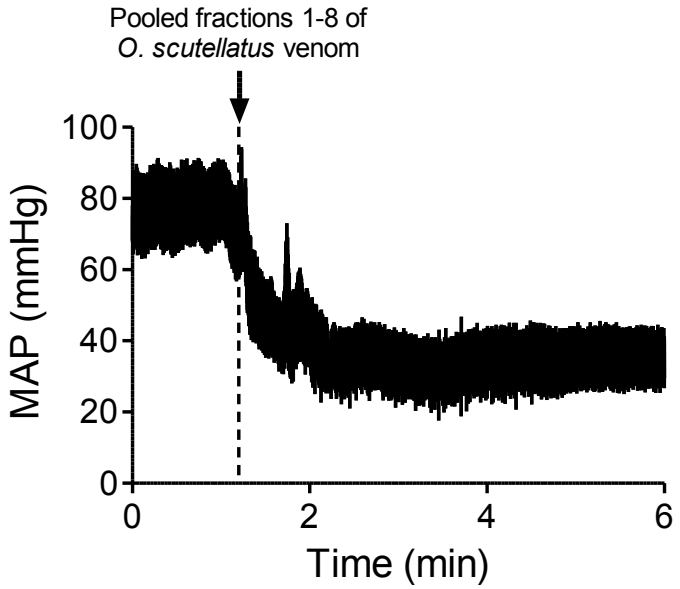

Figure 2: *Effect of pooled fractions 1-8 of O. scutellatus venom (50 µg/kg, i.v.) on the MAP of anaesthetized rats (n = 3).*

3.2 Relaxation effect of OSC3 on isolated rat mesenteric artery rings

OSC3 (3 and 10 µg/ml) induced relaxation responses in endothelium-intact mesenteric arteries (79 ± 9 and 98 ± 2% of PE precontraction, n = 9; Figures 3a and 4a). Removal of the endothelium did not significantly affect vascular relaxation induced by OSC3 (n = 8, $P > 0.05$, Student's unpaired t-test; Figures 3b and 4a). However, the relaxation produced by OSC3 (10 µg/ml) was significantly inhibited in both endothelium-intact and endothelium-denuded mesenteric arteries upon second addition of toxin in the same preparation (Figures 4b and 4c, n = 4-9, $P < 0.05$, Student's paired t-tests).

In separate experiments, neither L-NAME (0.2 mM) nor indomethacin (10 µM) inhibited relaxation induced by OSC3 (3 µg/ml) in endothelium-intact mesenteric arteries ($P > 0.05$, one-way ANOVA, n = 7-8; Figure 5a). Indomethacin (10 µM) and 8-pCPT-cAMP (10 µM) significantly abolished relaxation induced by OSC3 on endothelium-denuded mesenteric arteries (3 µg/ml, $P < 0.05$, one-way ANOVA, n = 6; Figure 5b).

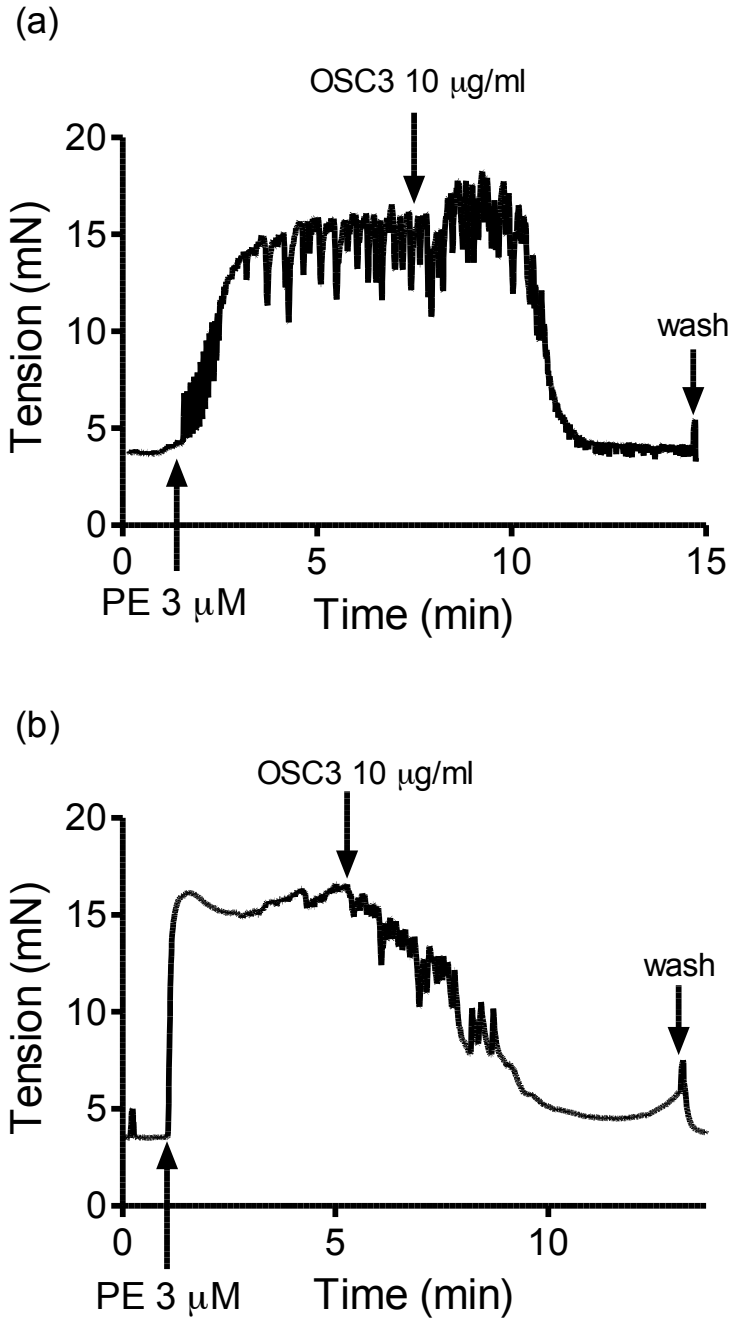

Figure 3: *Traces showing vascular relaxation produced by OSC3 (10 μg/ml) on (a) endothelium-intact or (b) endothelium-denuded rat mesenteric artery.*

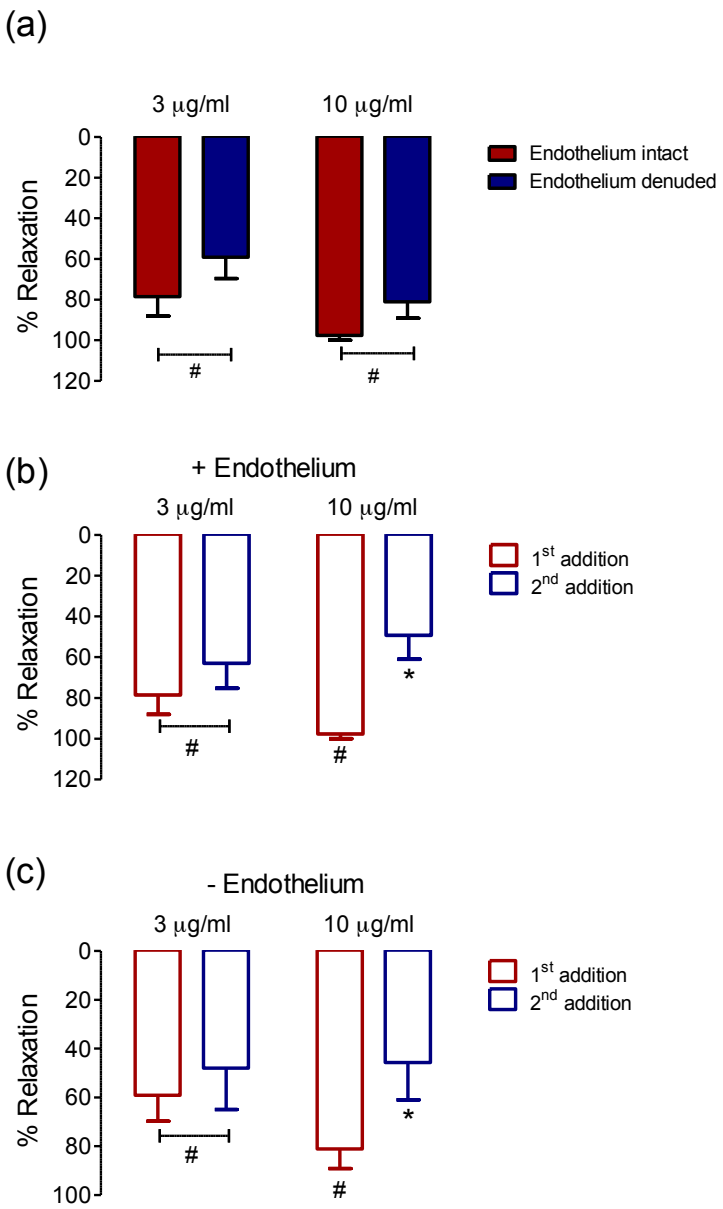

Figure 4: *(a) Relaxation effects of OSC3 (3-10 μg/ml) on pre-constricted mesenteric artery rings in the presence and absence of the endothelium. # P < 0.05, significantly different from time control (Figure 5 as per manuscript), Student's unpaired t-test (n = 8-9). Relaxation effects of 2nd addition of OSC3 on (b) endothelium-intact or (c) endothelium-denuded rat mesenteric arteries. * P < 0.05, significantly different from the 1st addition, Student's paired t-test (n = 4-9).*

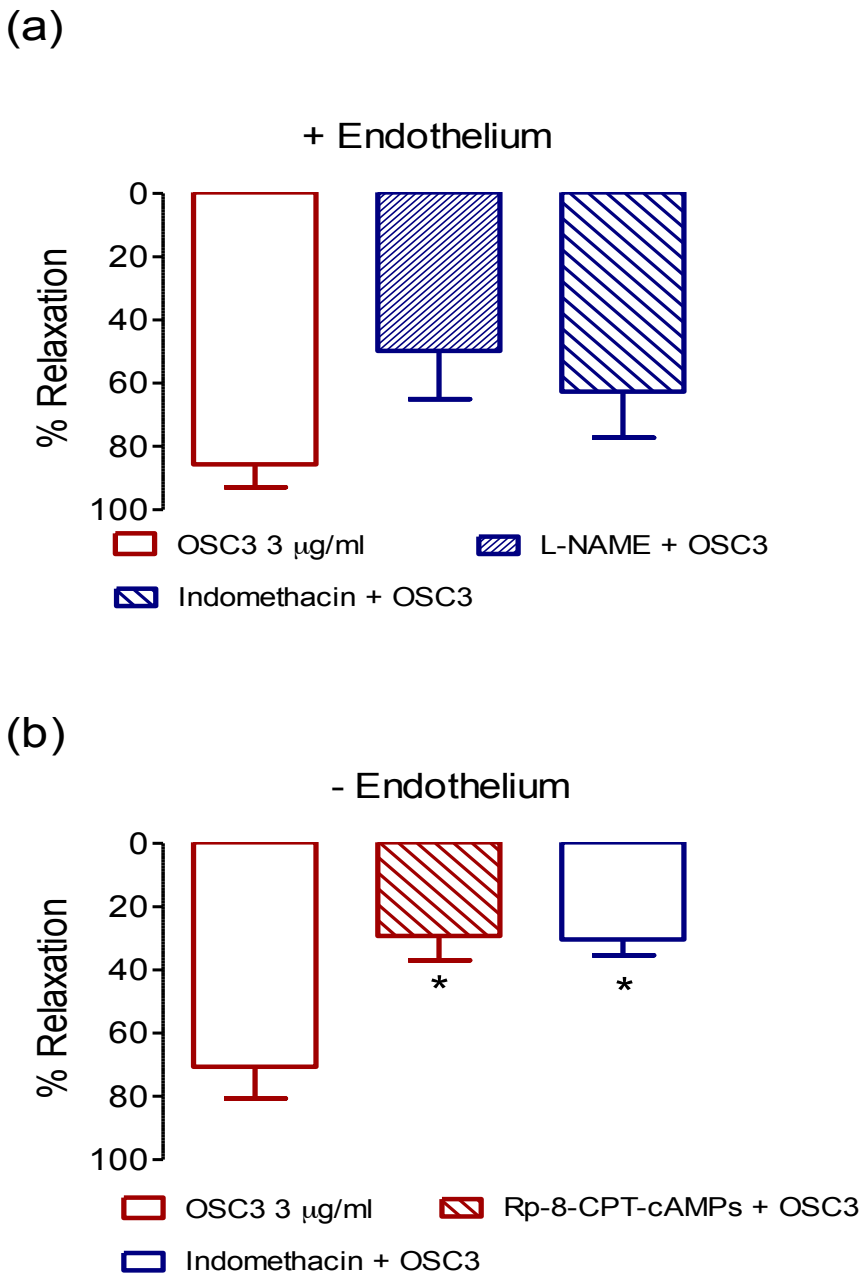

Figure 5: *(a) Relaxation effect of OSC3 (3 μg/ml, n = 8) on endothelium-intact rat mesenteric arteries in the absence and presence of L-NAME (0.2 mM, n = 7) or indomethacin (10 μM, n = 7). (b) Relaxation effect of OSC3 (3 μg/ml, n = 6) on endothelium-denuded rat mesenteric arteries in the absence and presence of Rp-8-CPT-cAMPs (10 μM, n = 6) or indomethacin (10 μM, n = 6).*
** P < 0.05, significantly different, one-way ANOVA.*

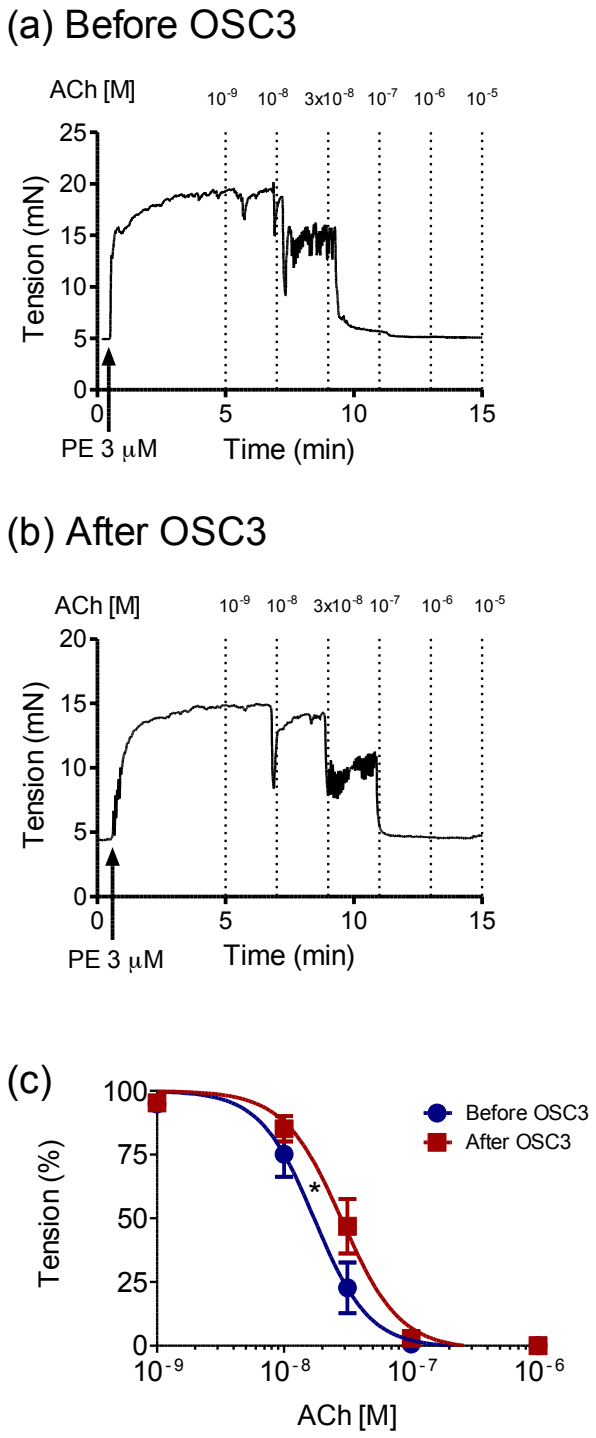

Figure 6: *Traces showing concentration relaxation effect-produced by ACh (10^{-5}-10^{-9} M) (a) prior or (b) after the addition of OSC3. (c) Concentration relaxation curves to ACh obtained before and after addition of OSC3 10 µg/ml (n = 7). * P < 0.05, significantly different, two-way ANOVA.*

3.3 Effect of OSC3 on concentration relaxation curves to ACh in isolated rat mesenteric arteries

OSC3 (10 µg/ml) significantly shifted the concentration-response curve to ACh to the right ($n = 7$, $P < 0.05$, two-way ANOVA) suggesting a reduction in endothelial function (Figure 6).

3.4 Chick biventer cervicis nerve-muscle preparation

OSC3 (10 µg/ml) failed to produce a complete inhibition of twitch height in the indirectly stimulated CBCNM preparation (Figure 7) after the addition of isolated toxin of 120 min. This indicated an absence of neurotoxicity for OSC3.

A significant difference in twitch height between vehicle and OSC3 was observed at the 120 min time point ($P < 0.0001$, Student's unpaired *t*-test).

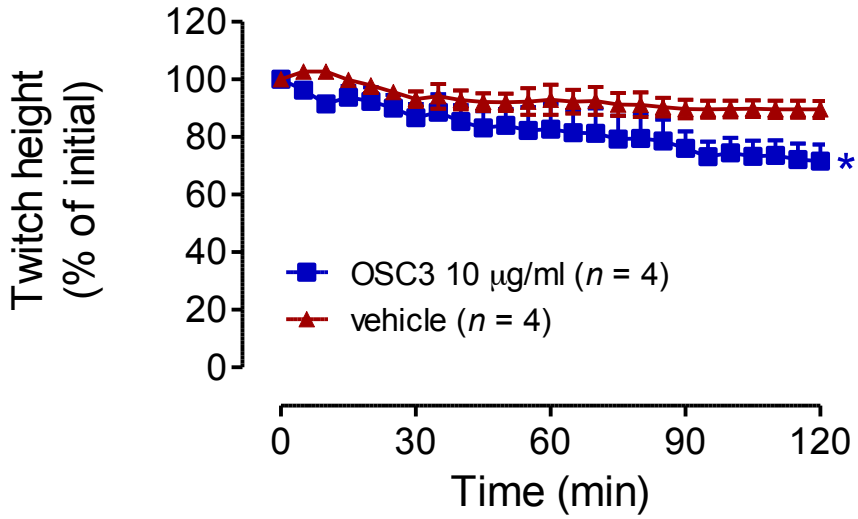

Figure 7: *Effect of OSC3 (10 µg/ml) on indirectly stimulated CBCNM preparation, *P < 0.001, Student's unpaired t-test.*

4. Discussion (continued)

Further discussion concerning the additional data is included in the General Discussion (Chapter 9).

Chapter 7

An examination of cardiovascular collapse induced by eastern brown snake (*Pseudonaja textilis*) venom

Monash University

Declaration for Thesis Chapter 7

This chapter is made up of the following publication.

Declaration by candidate

In the case of Chapter 7, the nature and extent of my contribution to the work was the following:

Nature of Contribution	Extent of Contribution (%)
I declare that experiments and writing of this manuscript were solely undertaken by the myself, taking into consideration the advice and recommendations of co-authors	90 %

The following co-authors contributed to the work.

Name	Nature of Contribution
Associate Professor Geoffrey K Isbister	Development of ideas, manuscript preparation
Dr Sanjaya Kuruppu	Provision of expertise and facilities
Dr Nicki Konstantakopoulos	Development of ideas, manuscript preparation
Professor Wayne C Hodgson	Development of ideas, manuscript preparation

Candidate's signature: [redacted] Date: 07.06.2013

Declaration by co-authors

The undersigned hereby certify that:

(1) the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors;
(2) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(3) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(4) there are no other author of the publication according to these criteria;
(5) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journal or other publications and (c) the head of the responsible academic unit; and
(6) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location (s) Department of Pharmacology and Department of biochemistry and molecular biology Monash University, Clayton

		Date
Associate Professor Geoffrey K Isbister	[redacted]	07.06.2013
Dr Sanjaya Kuruppu	[redacted]	04.06.2013
Dr Nicki Konstantakopoulos	[redacted]	04.06.2013
Professor Wayne C Hodgson	[redacted]	07.06.2013

An examination of cardiovascular collapse induced by eastern brown snake (*Pseudonaja textilis*) venom

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****This manuscript has been accepted to publish in Toxicology Letters.**

Abstract

The *Pseudonaja* genus (Brown snakes) is widely distributed across Australia and bites account for significant mortality. Venom-induced consumption coagulopathy (VICC) and, less often, early cardiovascular collapse occur following envenoming by these snakes. We have previously examined possible mechanism(s) behind the early cardiovascular collapse following Papuan taipan (*Oxyuranus scutellatus*) envenoming. In the present study, we investigate early cardiovascular collapse in anaesthetized rats following administration of eastern brown snake (*Pseudonaja textilis*) venom, and prevention of this effect with prior administration of 'priming' doses (i.e. doses of venom which caused a transient hypotensive response) of venom. *P. textilis* venom (5-10 µg/kg, i.v.) induced cardiovascular collapse in anaesthetized rats, characterized by a rapid decrease in systolic blood pressure until non recordable. Prior administration of 'priming' doses of *P. textilis* venom (2 and 3 µg/kg) or, at least, 4-5 doses of *O. scutellatus* (2 µg/kg, i.v.) or *Daboia russelii limitis* (20 µg/kg, i.v.) venoms prevented cardiovascular collapse induced by *P. textilis* venom. Moreover, early collapse was also inhibited by prior administrations of 2 discrete doses of *Acanthophis rugosus* venom. Prior administration of commercial polyvalent snake antivenom (500-3000 units/kg, i.v.) or heparin (300 units/kg, i.v.) also inhibited *P. textilis* venom-induced cardiovascular collapse. Our results indicate that *P. textilis* venom-induced cardiovascular collapse can be prevented by prior administration of sub lethal doses of venom from *P. textilis*, *O. scutellatus*, *A. rugosus* and *D. russelii limitis*. This suggests that sudden cardiovascular collapse following envenoming is likely to involve a common mechanism/pathway activated by different snake venoms.

Keywords: anaesthetized rat, antivenom, brown snake, cardiovascular collapse, venom

Abbreviations: ANOVA, Analysis of variance; i.v., intra venous administration; i.p., intra peritoneal administration; MAP, mean arterial pressure; PVDF, polyvinylidene difluoride; HRP, horseradish peroxidase; TBST, Tris-Buffered Saline and Tween 20.

Introduction

Envenoming by snakes of genus *Pseudonaja* (brown snakes) is a significant cause of morbidity and mortality in Australia (Allen et al., 2012). Clinical symptoms following envenoming by *Pseudonaja* spp. include venom-induced consumption coagulopathy (VICC) and sudden cardiovascular collapse, with neurotoxicity being rare (Allen et al., 2012; Barber et al., 2012) despite the presence of a pre-synaptic neurotoxin, textilotoxin (Barber et al., 2012). This peculiarity of brown snake envenoming, often referred to as the 'Brown snake paradox', appears to be due to the small amount of pre-synaptic neurotoxin in the whole venom and the relative low potency of this toxin (Barber et al., 2012).

A recent study has shown that about one quarter of patients with brown snake envenoming have an early collapse or hypotension, with cardiac arrest and death occurring in 6% of cases (Allen et al., 2012). The early cardiovascular collapse often occurs in the pre-hospital setting and appears to respond to basic and advanced life supported (Allen et al., 2012; Johnston et al., 2002).

A number of toxins have been isolated and characterized from *Pseudonaja* spp. venoms. These include pre- (Su et al., 1983) and post- (Tyler et al., 1987) synaptic neurotoxins, phospholipase A_2 (PLA_2) components, and prothrombin activators (Rao and Kini, 2002; Stocker et al., 1994). Prothrombin activators promote the conversion of prothrombin to thrombin leading to consumption of clotting factors (fibrinogen, factor V and factor VIII) which can result in serious hemorrhage including life-threatening intracranial hemorrhage (Isbister et al., 2010).

Administration of brown snake or tiger snake (*Notechis* sp.) prothrombin activators, to anaesthetized dogs, caused severe depression of systolic blood pressure and cardiac output which was suggested to be due to thrombotic obstruction of the pulmonary vasculature (Tibballs, 1998; Tibballs et al., 1992). Recently, we have shown that administration of Papuan taipan (*O. scutellatus*) venom caused cardiovascular collapse and prior administration of small priming (i.e. hypotensive) doses of venom 'protected' rats from the effects of a subsequent larger toxic doses which would otherwise cause cardiovascular collapse if given alone (Chaisakul et al., 2012).

Antivenom is the major treatment for systemically envenomed patients although studies have questioned *in vivo* effectiveness (Isbister et al., 2009) and *in vitro* (Judge et al., 2006) efficacy of brown snake antivenom. Large quantities and multiple administrations of antivenom have been recommended to neutralize cardiovascular depression and coagulopathy following brown snake envenoming (Johnston et al., 2002; Tibballs and Sutherland, 1991). However, recent studies indicate that much lower quantities of antivenom (i.e. one vial) can be used in all cases (Allen et al., 2012; Kulawickrama et al. 2010).

Although sudden cardiovascular collapse following envenoming by brown snakes has been studied (Tibballs and Sutherland, 1991; Tibballs et al., 1989), the mechanisms of the collapse and potential therapeutic agents to prevent this collapse have not been defined. In this study, we determined whether administration of small priming doses of *P. textilis* venom could confer protection against sudden cardiovascular collapse. We also examined cross genus and cross family neutralization, as well as the efficacy of the commercially available snake antivenom to prevent sudden cardiovascular collapse in brown snake (*Pseudonaja* spp.) envenoming.

2. Materials and methods

2.1 Venom preparation and storage

Freeze-dried eastern brown snake (*Pseudonaja textilis*), Papuan taipan (*Oxyuranus scutellatus*), Irian Jayan death adder (*Acanthophis rugosus*) and Russell's viper (*Daboia russelii limitis*) venoms were purchased from Venom Supplies (Tanunda, South Australia). Venom was dissolved in Milli-Q water and stored at -20°C until required. Thawed solutions were kept on ice during experiments. Venom protein content was determined via a BCA Protein Assay Kit (Pierce biotechnology; Illinois, USA) as per manufacturer's instructions.

2.2 Anaesthetized rat preparation

All procedures were approved by the School of Biomedical Science (SOBS)-B Animal Ethics Committee, Monash University. Male Sprague-Dawley rats (250-350 g) were anaesthetized

with pentobarbitone sodium (60-100 mg/kg, i.p., supplemented as required). Cannulae were inserted into the trachea, jugular vein and carotid artery, for artificial respiration (if required), administration of drugs/venom and measurement of blood pressure, respectively. Arterial blood pressure was recorded using a Gould Statham P23 pressure transducer connected to a PowerLab system. Venoms were administered via the jugular vein. At the conclusion of the experiment animals were killed by an overdose of pentobarbitone (i.v.).

As indicated, the CSL polyvalent antivenom or inhibitors have been administered 10 min prior the administration of the lethal dose of *P. textilis* venom. Priming doses of snake venoms were administered 5-10 min interval.

2.3 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS – PAGE)

SDS–PAGE was performed according to the method described by Laemmli (Laemmli, 1970). *P. textilis* venom (20 µg) was resolved on a 12.5% SDS-PAGE under reducing condition (5% β-mercaptoethanol in Laemmli's sample buffer (62.5 mM Tris-hydrochloride, 25% glycerol, 2% SDS, 0.01% bromophenol blue). Venom was heated for 5 min at 95˚C prior to resolving on SDS-PAGE. Protein bands were visualized by staining with BioSafe Coomassie G-250 solution (Bio-Rad Laboratories; Hercules, CA USA), followed by de-staining in distilled water. Gel image was captured utilizing Typhoon Trio scanner (GE Healthcare; Uppsala, Sweden).

2.4 Western blot

P. textilis venom (20 µg) was resolved on a 12.5% SDS-PAGE and transferred onto a PVDF (polyvinylidene difluoride) membrane. The membrane was incubated with 5% skim milk in TBST (20 mM Tris, 0.5 M NaCl, 0.5 % Tween-20) to prevent non-specific binding and then incubated with primary antibody (CSL Ltd. polyvalent snake antivenom diluted 1:500-fold in TBST with 5% skim milk) overnight at 4˚C. Immunoreactive bands were visualized using appropriate secondary antibodies (goat-anti-horse-IgG-HRP, Santa Cruz biotechnology, Dallas, TX, USA) and ECL™ Chemiluminescence detection reagent (GE Healthcare; Uppsala, Sweden).

2.5 Chemicals and drugs

The following drugs were used: mepyramine maleate (May & Baker, Dagenham, UK); heparin sodium (Hospira, VIC, Australia); Polyvalent snake antivenom (CSL Ltd., Melbourne, Australia).

2.6 Data analysis and Statistics

In the anaesthetized rat preparation, pulse pressure is defined as the difference between systolic and diastolic blood pressures. Mean arterial pressure (MAP) was calculated as diastolic blood pressure plus one-third of pulse pressure. The decrease in MAP following the administration of venoms was measured and is presented as mean ± SEM.

Statistical analysis was performed using Prism 5.0 software (GraphPad Software, San Diego, CA, USA). Student's unpaired *t-tests* were performed on responses to venom in different animals, and paired *t*-tests were used to compare responses before and after the administration of venom in the same animal. Multiple comparisons were made using a one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. Values of $P < 0.05$ were accepted as significant. Data are expressed as mean ± SEM.

3. Results

3.1 Effect of single dose of P. textilis venom

P. textilis venom (5 or 10 μg/kg, i.v.) caused rapid cardiovascular collapse, as defined by the absence of detectable blood pressure (Figures 1a and 1b). Lower doses of *P. textilis* venom (i.e. 2 and 3 μg/kg, i.v.) produced transient decreases in MAP (i.e. 19 ± 13 and 65 ± 28 %, respectively), without cardiovascular collapse (Figure 1b). No significant change in heart rate was observed at the point when MAP had decreased by 50% following the administration of venom (Figure 1c).

3.2 Effect of multiple doses of P. textilis venom

In separate experiments, prior administration of 2 µg/kg (i.v.), and 3 µg/kg (i.v.) of *P. textilis* venom prevented cardiovascular collapse induced by the subsequent administration of 5 µg/kg (i.v.) and 10 µg/kg (i.v.) of *P. textilis* venom (Figure 1b). Instead, these doses of venom caused moderate transient hypotensive responses. Under these conditions, the rat was able to tolerate a total venom 'load' of 20 µg/kg, which was four times the dose which produced cardiovascular collapse when given alone.

To determine the 'threshold' priming dose required to prevent cardiovascular collapse and transient hypotension, repeated amounts of 2 µg/kg (i.v.) of *P. textilis* venom were given prior to the administration of 10 µg/kg of *P. textilis* venom i.e. a dose which, when injected alone, caused collapse in 100% of rats. The administration of a single priming dose of 2 µg/kg of *P. textilis* venom prevented collapse when 10 µg/kg of *P. textilis* venom was given, but there was still a transient hypotensive response (Figure 2a). Two and three priming doses of 2 µg/kg (i.v.) of *P. textilis* venom prevented collapse and also decreased the hypotensive response, following the administration of 10 µg/kg of *P. textilis* venom (Figure 2a).

3.3 Effect of prior administration of mepyramine or heparin

Prior administration of heparin (300 units/kg, i.v.) significantly prevented cardiovascular collapse from the subsequent administration of *P. textilis* venom (5 µg/kg, i.v.), but did not prevent the transient hypotensive response (Figure 2b).

Administration of mepyramine (2 mg/kg, i.v.) prior to *P. textilis* venom (5 µg/kg, i.v.) prevented cardiovascular collapse in 4 of 7 rats tested. However, venom still produced a marked hypotensive effect in these rats (Figure 2b).

3.4 Protective effect of O. scutellatus, D. russelii limitis and A. rugosus venoms

Previously, we have shown that administration of five discrete doses of *O. scutellatus* venom (5 µg/kg, i.v.) protects against *O. scutellatus*-induced sudden cardiovascular collapse. In the

present study, *O. scutellatus* venom (5 µg/kg, i.v.) produced a small hypotensive response in anaesthetized rats (Figure 3a). The administration of five priming doses of *O. scutellatus* venom (5 µg/kg, i.v.) in the same rat, prevented cardiovascular collapse produced by subsequent administration of *P. textilis* venom (10 µg/kg, i.v.), but not the transient hypotensive response (Figures 3a and 3b).

Administration of Russell's viper (*D. r. limitis*) venom (50 µg/kg, i.v.) caused a rapid hypotensive effect followed by cardiovascular collapse within 15 min in anaesthetized rats (Figure 4a). A lower dose of *D. r. limitis* venom (20 µg/kg, i.v.) produced a 29 ± 3 % decrease in MAP without cardiovascular collapse (Figure 4b). Administration of 4, but not 2 or 3, priming doses of *D. r. limitis* venom (20 µg/kg, i.v.) prevented *P. textilis* venom-induced cardiovascular collapse ($P < 0.05$, one-way ANOVA, Figure 4c). After prior administration of four doses of *D. r. limitis* venom (20 µg/kg, i.v.), *P. textilis* venom (10 µg/kg, i.v.) only induced a small hypotensive response with the MAP of 36 ± 8 mmHg (Figure 4d).

The administration of *A. rugosus* venom (50 µg/kg, i.v.) produced a decrease in MAP of anaesthetized rats to 55 ± 3% (Figure 5b). Prior administration of 2 priming doses of *A. rugosus* venom (50 µg/kg, i.v.) significantly prevented *P. textilis* venom-induced cardiovascular collapse ($P < 0.05$, one-way ANOVA) with the reduction in MAP of 49 ± 7% (Figures 5a and b).

3.5 Effect of CSL polyvalent snake antivenom on cardiovascular collapse

Prior administration (10 min) of a bolus dose of CSL polyvalent snake antivenom (500-3,000 units/kg, i.v.) significantly prevented cardiovascular collapse from subsequent administration of *P. textilis* venom (10 µg/kg, i.v.) and caused a dose-dependent reduction in the hypotensive effects of venom ($P < 0.05$, one-way ANOVA; Figure 6c). A plot of log antivenom concentration versus magnitude of MAP to venom indicated an IC_{50} value of 437 units/kg (Figure 6d).

Administration of polyvalent antivenom (1000 units/kg, i.v. bolus) at 50 % decrease in MAP prevented complete collapse. However, a marked decrease in MAP was observed with gradual recovery within 1-2 min following the administration of antivenom (Figure 6b).

3.6 SDS-PAGE and Western blot

SDS-PAGE analysis of *P. textilis* venom indicates the presence of multiple protein bands with molecular weights in the range of 15-200 kDa (Figure 7, lane A). Western blot analysis indicated that CSL polyvalent snake antivenom detected the majority of the proteins in the venom (Figure 7, lane B).

Discussion

The present study demonstrated that relatively low doses of brown snake (*P. textilis*) venom can induce cardiovascular collapse in anaesthetized rats, an effect similar to the hypotensive collapse seen in human envenoming. The potency of *P. textilis* venom in causing cardiovascular collapse is consistent with collapse being reported most commonly in human brown snake envenoming (Allen et al., 2012) compared to other Australian elapids (Isbister et al., 2012; Kulawickrama et al., 2010) or other snakes worldwide (Kularatne, 2003). In our animal model, cardiovascular collapse was prevented by prior administration of priming doses of the same venom (*P. textilis* venom), priming doses of different venoms, heparin and commercial polyvalent antivenom. This is similar to Papuan taipan venom, but additionally cross-species and cross-family effect of priming doses suggests a common mechanism of the cardiovascular collapse. The protective effect of CSL polyvalent snake antivenom indicates the involvement of antigenic venom components in this cardiovascular collapse.

The administration of small 'priming' doses of *P. textilis* venom prevented cardiovascular collapse and hypotension following the addition of larger doses of *P. textilis* venom. This observation supports our previous study examining the cardiovascular effects of Papuan taipan (*O. scutellatus*) venom, and our hypothesis that cardiovascular collapse following envenoming may be due to the release of depletable mediators (Chaisakul et al., 2012). Interestingly, prior administration of small priming doses of venom from a different genus (i.e. *O. scutellatus* and *A. rugosus*) but same family (i.e. Elapidae) also 'protected' against collapse, as did Russell's viper venom, which is from a different family of snakes (i.e. Viperidae). This indicates that

cardiovascular collapse induced by different species of snakes is likely to involve a common mechanism or pathway for all snake venoms causing this effect.

It has been suggested that the early cardiovascular collapse is due to the prothrombin activators in brown snake venom or procoagulant toxins in other venoms (Tibballs, 1998; Tibballs et al., 1992). However, in this study we have shown that death adder venom (*A. rugosus*) can produce early cardiovascular collapse, albeit at a much higher dose than brown snake or taipan venom. In addition, priming doses of death adder venom prevented cardiovascular collapse by subsequent addition of brown snake venom. A previous study also found that death adder venom (*A. antarcticus*) caused hypotension (Venning et al, 1995). This suggests that procoagulant activity in snake venoms is not necessary for the venom to cause early cardiovascular collapse. In addition, we have previously shown that the protective effect of priming with low doses of venom only lasts 1 hour. This suggests that the depletable mediators responsible for the hypotensive collapse are replenished in this short period time. This is not consistent with depletion of clotting factors that take 12 to 48 hours to replenish depending on specific clotting factors (8 to 12 hours for factors V and VIII and 24 to 48 hours for fibrinogen) (Isbister et al., 2010).

Early hypotensive collapse in humans, which often resolves within 30 minutes, suggests vasodilation as the underlying pathophysiology rather than a myocardial cause. In some ways this is similar to anaphylactic shock which is due to release of endogenous immune mediators (Stone et al., 2009). We investigated the effect of the histamine receptor antagonist, mepyramine (H_1-receptor antagonist) and heparin on cardiovascular collapse. Cardiovascular collapse was only partially prevented by mepyramine with 4 of 7 rats still displaying collapse. It appears that multiple mediators may be involved consistent with our previous study (Chaisakul et al., 2012). However, heparin prevented collapse in all animals tested. The mechanism behind this 'protective' effect may be that heparin inhibits histamine release from mast cells (Inase *et al.*, 1993). Isolated PLA_2 and metalloproteinase from snake venoms have been reported to activate human mast cells resulting in histamine release which was inhibited by heparin incubation (Wei *et al.*, 2006; Wei *et al.*, 2010).

In conclusion, cardiovascular collapse can be abolished by prior administration of sub-lethal doses of venoms indicating the likely release of depletable substances. It is likely that immune mediators which can cause hypotension and vasodilatation (e.g. histamine, bradykinin or 5-HT) may contribute to the sudden collapse.

Acknowledgement

Janeyuth Chaisakul is kindly supported by a scholarship from the National Science and Technology Development Agency of the Royal Thai Government. Geoff Isbister was funded by an NHMRC Clinical Career Development Award (ID605817).

References

Allen, G.E., Brown, S.G., Buckley, N.A., O'Leary, M.A., Page, C.B., Currie, B.J., White, J., Isbister, G.K., 2012. Clinical Effects and Antivenom Dosing in Brown Snake (*Pseudonaja* spp.) Envenoming - Australian Snakebite Project (ASP-14). PLoS One 7, e53188.

Barber, C.M., Isbister, G.K., Hodgson, W.C., 2012. Solving the 'Brown snake paradox': in vitro characterisation of Australasian snake presynaptic neurotoxin activity. Toxicol Lett 210, 318-323.

Chaisakul, J., Isbister, G.K., Konstantakopoulos, N., Tare, M., Parkington, H.C., Hodgson, W.C., 2012. In vivo and in vitro cardiovascular effects of Papuan taipan (*Oxyuranus scutellatus*) venom: Exploring "sudden collapse". Toxicol Lett 213, 243-248.

Inase, N., Schreck, R.E., Lazarus, S.C., 1993. Heparin inhibits histamine release from canine mast cells. Am J Physiol 264, L387-390.

Isbister, G.K., Duffull, S.B., Brown, S.G., 2009. Failure of antivenom to improve recovery in Australian snakebite coagulopathy. QJM 102, 563-568.

Isbister, G.K., O'Leary, M.A., Elliott, M., Brown, S.G., 2012. Tiger snake (*Notechis* spp) envenoming: Australian Snakebite Project (ASP-13). Med J Aust 197, 173-177.

Isbister, G.K., Scorgie, F.E., O'Leary, M.A., Seldon, M., Brown, S.G., Lincz, L.F., 2010. Factor deficiencies in venom-induced consumption coagulopathy resulting from Australian elapid envenomation: Australian Snakebite Project (ASP-10). J Thromb Haemost 8, 2504-2513.

Johnston, M.A., Fatovich, D.M., Haig, A.D., Daly, F.F., 2002. Successful resuscitation after cardiac arrest following massive brown snake envenomation. Med J Aust 177, 646-649.

Judge, R.K., Henry, P.J., Mirtschin, P., Jelinek, G., Wilce, J.A., 2006. Toxins not neutralized by brown snake antivenom. Toxicol Appl Pharmacol 213, 117-125.

Kularatne, S.A., 2003. Epidemiology and clinical picture of the Russell's viper (*Daboia russelii russelii*) bite in Anuradhapura, Sri Lanka: a prospective study of 336 patients. Southeast Asian J Trop Med Public Health 34, 855-862.

Kulawickrama, S., O'Leary, M.A., Hodgson, W.C., Brown, S.G., Jacoby, T., Davern, K., Isbister, G.K., 2010. Development of a sensitive enzyme immunoassay for measuring taipan venom in serum. Toxicon 55, 1510-1518.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-685.

Rao, V.S., Kini, R.M., 2002. Pseutarin C, a prothrombin activator from *Pseudonaja textilis* venom: its structural and functional similarity to mammalian coagulation factor Xa-Va complex. Thromb Haemost 88, 611-619.

Stocker, K., Hauer, H., Muller, C., Triplett, D.A., 1994. Isolation and characterization of TextarinR, a prothrombin activator from eastern brown snake (*Pseudonaja textilis*) venom. Toxicon 32, 1227-1236.

Stone, S.F., Cotterell, C., Isbister, G.K., Holdgate, A., Brown, S.G., 2009. Elevated serum cytokines during human anaphylaxis: Identification of potential mediators of acute allergic reactions. J Allergy Clin Immunol 124, 786-792 e784.

Su, M.J., Coulter, A.R., Sutherland, S.K., Chang, C.C., 1983. The presynaptic neuromuscular blocking effect and phospholipase A_2 activity of textilotoxin, a potent toxin isolated from the venom of the Australian brown snake, Pseudonaja textilis. Toxicon 21, 143-151.

Tibballs, J., 1998. The cardiovascular, coagulation and haematological effects of tiger snake (*Notechis scutatus*) prothrombin activator and investigation of release of vasoactive substances. Anaesth Intensive Care 26, 536-547.

Tibballs, J., Sutherland, S., 1991. The efficacy of antivenom in prevention of cardiovascular depression and coagulopathy induced by brown snake (*Pseudonaja*) species venom. Anaesth Intensive Care 19, 530-534.

Tibballs, J., Sutherland, S., Kerr, S., 1989. Studies on Australian snake venoms. Part 1: The haemodynamic effects of brown snake (*Pseudonaja*) species in the dog. Anaesth Intensive Care 17, 466-469.

Tibballs, J., Sutherland, S.K., Rivera, R.A., Masci, P.P., 1992. The cardiovascular and haematological effects of purified prothrombin activator from the common brown snake (*Pseudonaja textilis*) and their antagonism with heparin. Anaesth Intensive Care 20, 28-32.

Tyler, M.I., Spence, I., Barnett, D., Howden, M.E., 1987. Pseudonajatoxin b: unusual amino acid sequence of a lethal neurotoxin from the venom of the Australian common brown snake, *Pseudonaja textilis*. Eur J Biochem 166, 139-143.

Venning, M.G., Shatursky, O.Y., Bailey, E., 1995. Vasodilator action of death adder (*Acanthophis antarcticus*) venom. Proc Aust Soc Clin Exp Pharmacol Toxicol 2, 132.

Wei, J.F., Mo, Y.Z., Qiao, L.Y., Wei, X.L., Chen, H.Q., Xie, H., Fu, Y.L., Wang, W.Y., Xiong, Y.L., He, S.H., 2006. Potent histamine-releasing activity of atrahagin, a novel snake venom metalloproteinase. Int J Biochem Cell Biol 38, 510-520.

Wei, J.F., Wei, X.L., Chen, Q.Y., He, S.H., 2010. Induction of inflammatory cell accumulation by TM-N49 and promutoxin, two novel phospholipase A_2. Toxicon 56, 580-588.

Figures

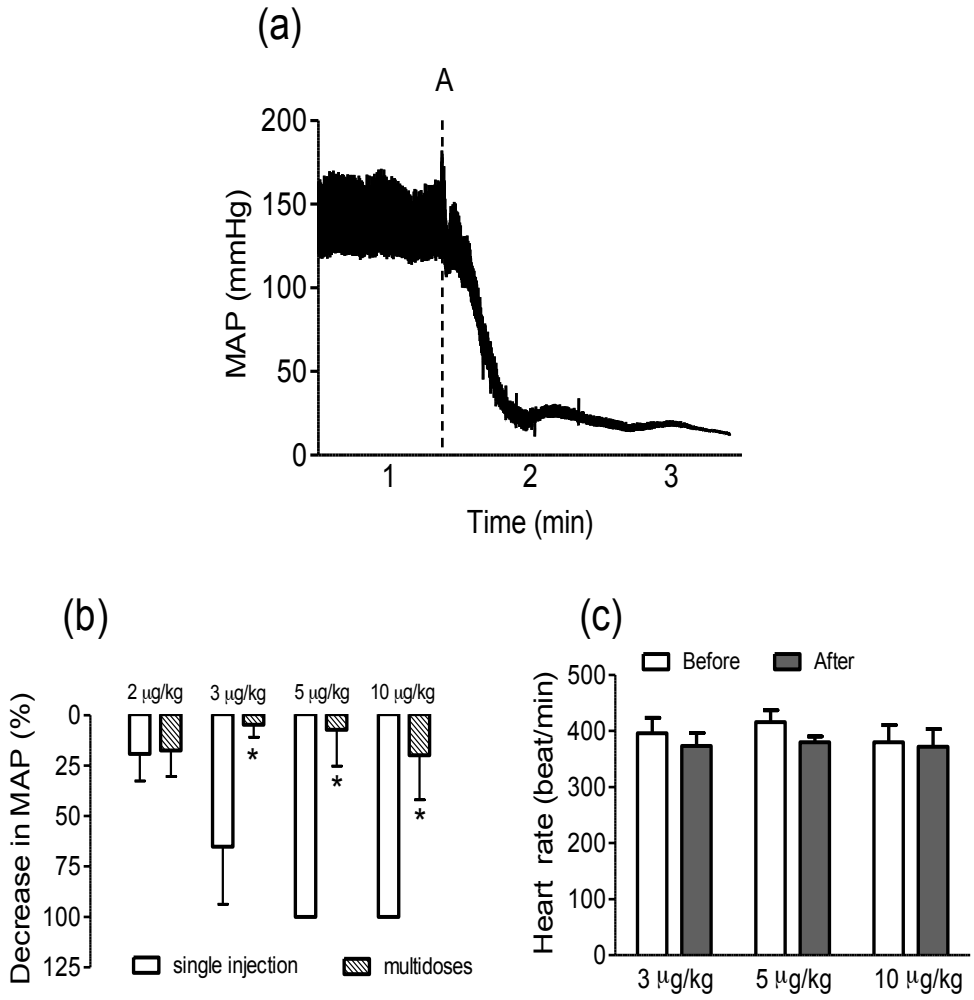

Figure 1: *(a) Effect of P. textilis venom (10 μg/kg, i.v.) on MAP in an anaesthetized rat. (b) Effect of P. textilis venom after a single injection (2, 3, 5 or 10 μg/kg, i.v., n = 5) in different rats or sequential addition of venom (2, 3, 5 and 10 μg/kg, i.v., n = 7) in the same rat on MAP. *P < 0.05, significantly different from single injection, Student's unpaired t-test. (c) A comparison of heart rate before and after single injection of venom (3, 5 and 10 μg/kg, i.v., n = 3) (measured at the point when MAP had decreased by 50%).*

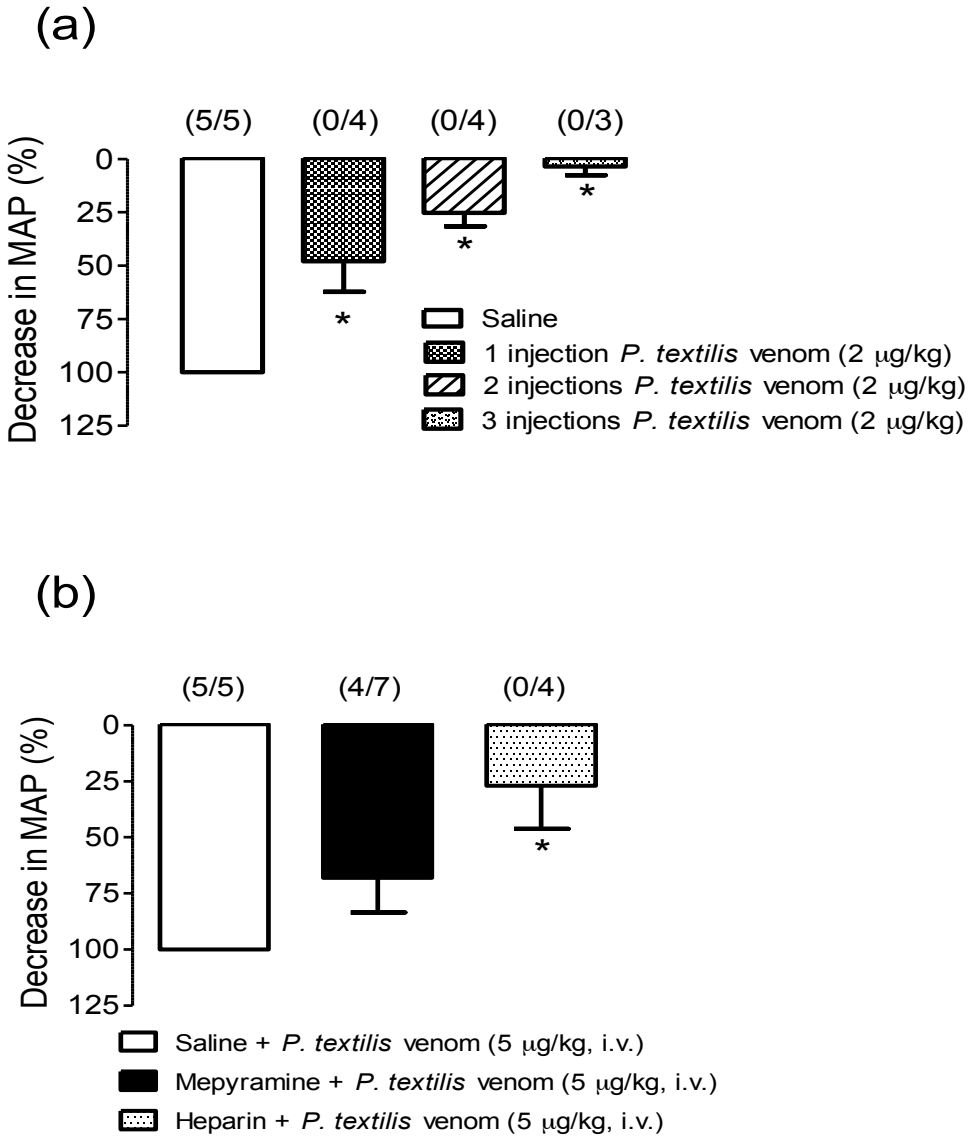

Figure 2: *(a) Effect of P. textilis venom (10 μg/kg, i.v.) on MAP in anaesthetized rats following the prior administration of saline or 2, 3 or 4 discrete doses of P. textilis venom (2 μg/kg, i.v., n = 3-4), (b) Effect of P. textilis venom (5 μg/kg, i.v.) on MAP in anaesthetized rats in the presence of saline, mepyramine 2 mg/kg or heparin 300 units/kg (n = 4-7), * P < 0.05, significantly different from prior administration of saline, one-way ANOVA; (In brackets represent the number of animals with complete sudden collapse/ number of animals in each group).*

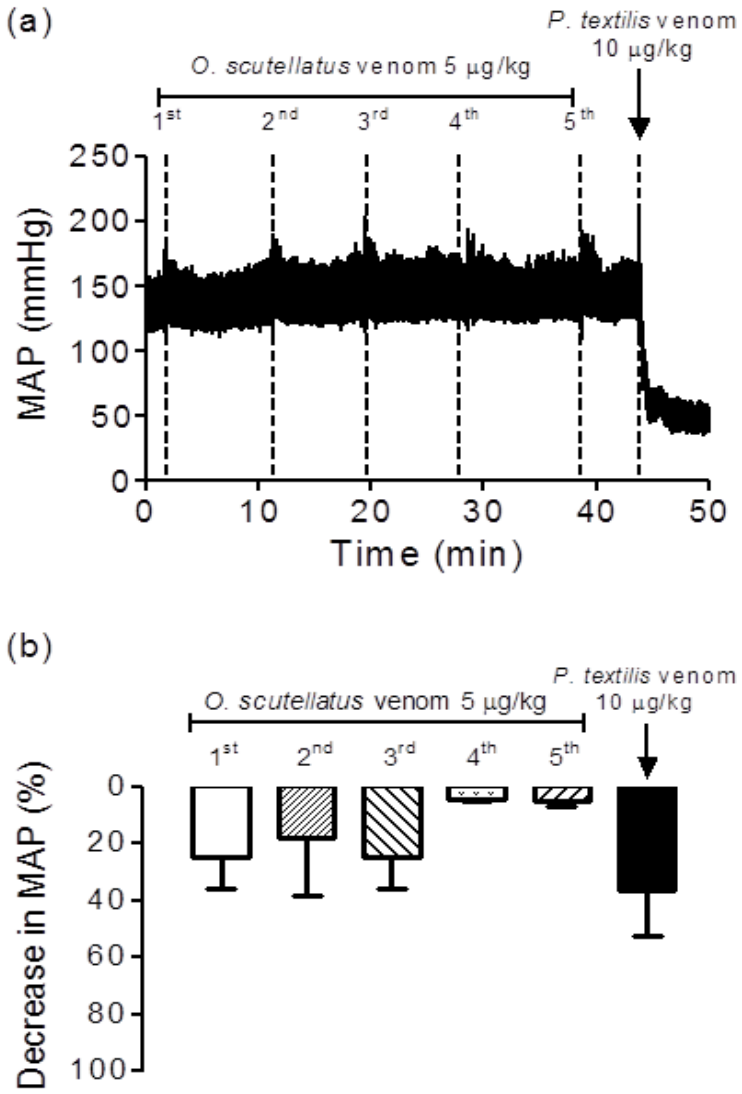

Figure 3: *(a) Five sequential additions of O. scutellatus venom (5 µg/kg, i.v.) prior to the administration of P. textilis venom (10 µg/kg, i.v.) in the same anaesthetized rat. (b) Protective effect of prior administration of 5 discrete doses of O. scutellatus venom (5 µg/kg, i.v.) on P. textilis venom-induced cardiovascular collapse in anaesthetized rats (n = 3).*

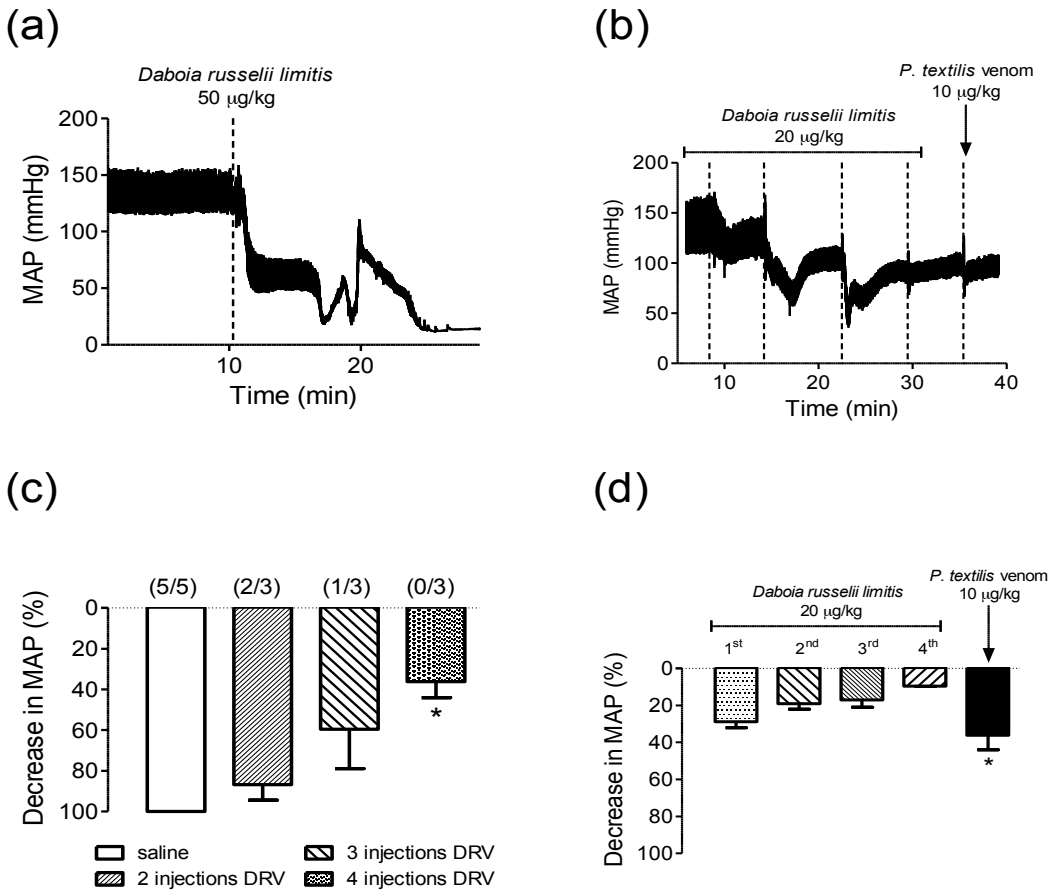

Figure 4: *(a) Effect of D. russelii limitis venom (50 μg/kg, i.v.) on MAP in an anaesthetized rat. (b) Effect of P. textilis venom (10 μg/kg, i.v.) following sequential addition of D. russelii limitis venom in the same anaesthetized rat (20 μg/kg, i.v., at individual time points) on MAP. (c) Effect of P. textilis venom (10 μg/kg, i.v.) on MAP in anaesthetized rats following the prior administration of 2, 3 or 4 discrete doses of D. russelii limitis venom (DRV) (20 μg/kg, i.v., n = 3-5) or saline, (d) Effect of P. textilis venom (10 μg/kg, i.v.) following sequential addition of D. russelii limitis venom in the same anaesthetized rat (4 injections of 20 μg/kg venom, i.v., n = 3) on MAP, *P < 0.05, significantly different from prior administration of saline, one-way ANOVA; (In brackets represent the number of animals with complete sudden collapse/number of animals in each group).*

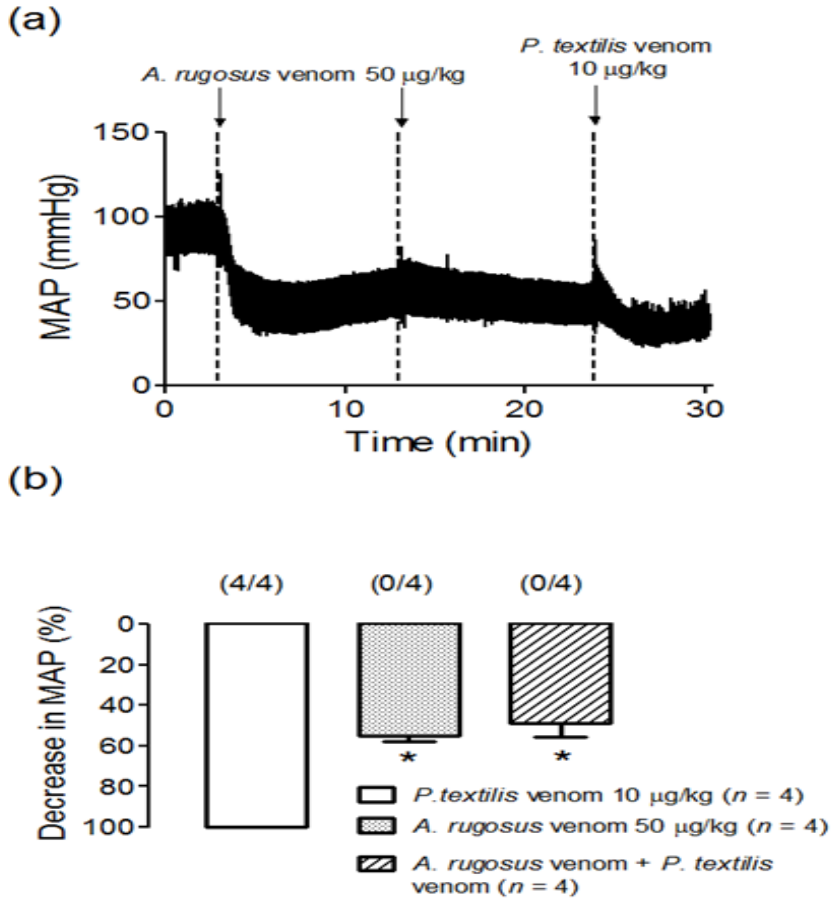

Figure 5 *(a) Trace showing the effect of 2 discrete doses of A. rugosus venom (50 µg/kg, i.v.) prior to the administration of P. textilis venom (10 µg/kg, i.v.) in an anaesthetized rat, arrows indicate time of injection. (b) Decrease in MAP of anaesthetized rats following the administration of 2 discrete doses of A. rugosus venom (50 µg/kg, i.v.) prior to P. textilis venom compared with rat MAP following the administration of P. textilis or A. rugosus venoms alone. *P < 0.05, significantly different from the administration of P. textilis venom alone, one-way ANOVA; (In brackets represent the number of animals with complete sudden collapse/number of animals in each group).*

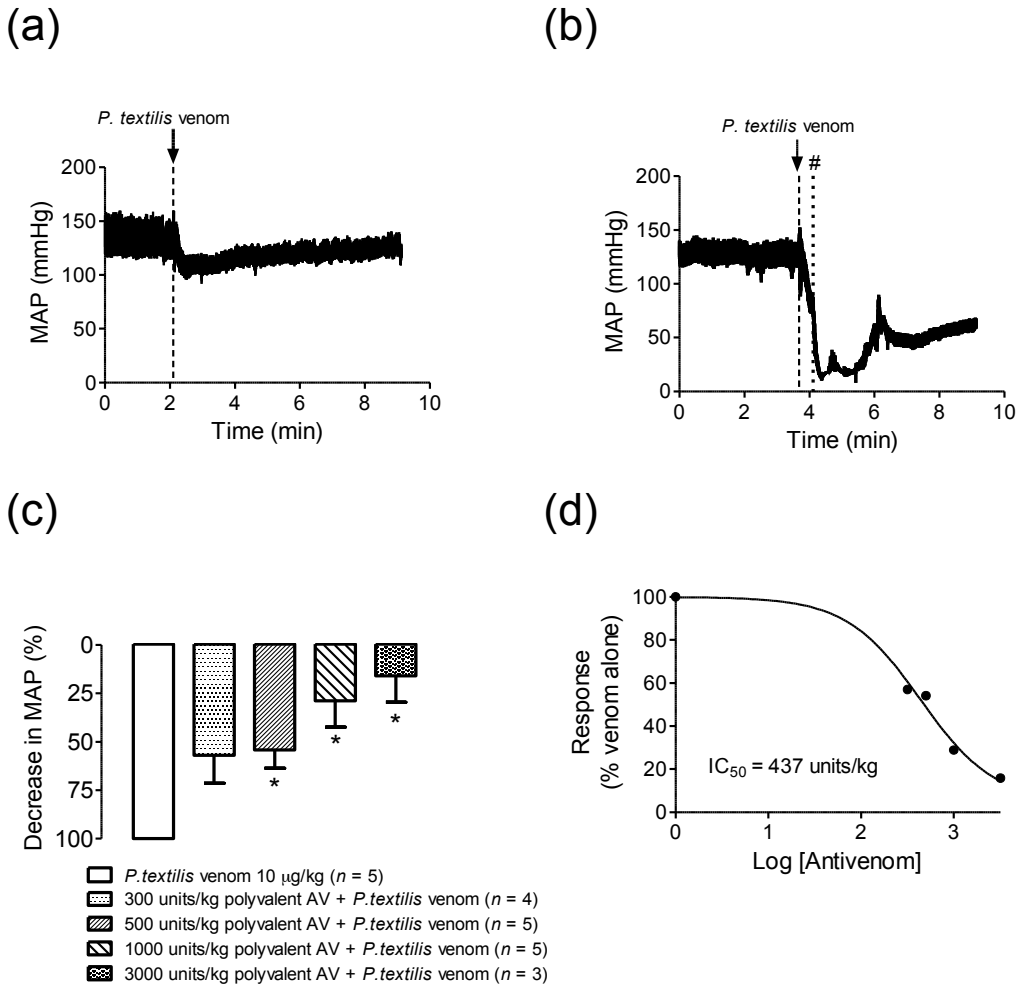

Figure 6: *(a) Effect of P. textilis venom (10 µg/kg) on MAP in the presence of prior administration of polyvalent snake antivenom (3,000 units/kg) for 10 min in an anaesthetized rat. (b) Trace showing the protective effect of polyvalent snake antivenom (1000 units/kg, i.v. bolus) administered at the time point of a 50 % decrease in MAP induced by P. textilis venom (10 µg/kg, i.v.) on anaesthetized rat (n = 3). # antivenom administration time point. (c) Effect of CSL snake polyvalent antivenom (300-3000 units/kg, i.v. bolus, n = 3-5) on a decrease in MAP induced by P. textilis venom (10 µg/kg, i.v.). *P < 0.05, significantly different from P. textilis venom (10 µg/kg, i.v.) alone, one-way ANOVA. (d) Logarithm inhibitor (antivenom) concentration-response curve to estimate the IC50 of CSL polyvalent snake antivenom.*

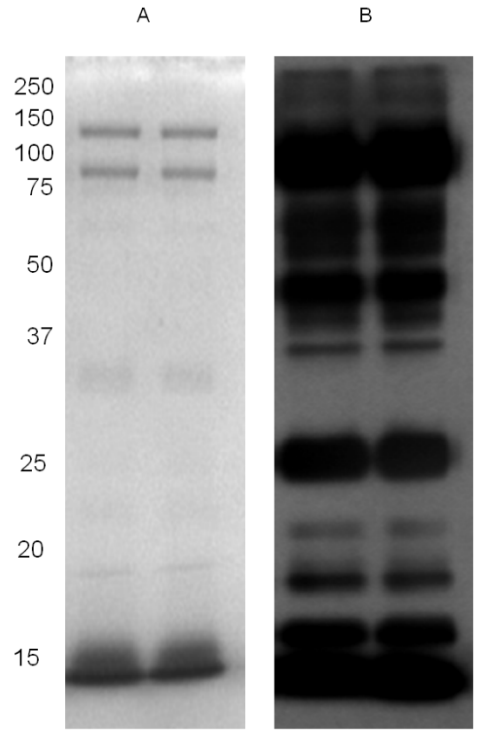

Figure 7: *SDS-PAGE of P. textilis venom (lane A) and Western blot of P. textilis venom with CSL polyvalent snake antivenom (lane B).*

CHAPTER 8

PROTHROMBIN ACTIVATOR-LIKE COMPOUND FROM *PSEUDONAJA TEXTILIS* VENOM INDUCES EARLY CARDIOVASCULAR COLLAPSE

Monash University

Declaration for Thesis Chapter 8

This chapter is made up of the following publication.

Declaration by candidate

In the case of Chapter 8, the nature and extent of my contribution to the work was the following:

Nature of Contribution	Extent of Contribution (%)
I declare that experiments and writing of this manuscript were solely undertaken by the myself, taking into consideration the advice and recommendations of co-authors	80 %

The following co-authors contributed to the work.

Name	Nature of Contribution
Associate Professor Geoffrey K Isbister	Development of ideas, manuscript preparation
Dr Margaret A O'Leary	Fractionation of toxin
Dr Sanjaya Kuruppu	Provision of expertise and facilities
Professor Helena C Parkington	Provision of expertise and facilities
Professor Wayne C Hodgson	Development of ideas, manuscript preparation

Candidate's signature: [redacted] Date: 07.06.2013

Declaration by co-authors

The undersigned hereby certify that:

(1) the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors;
(2) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(3) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(4) there are no other author of the publication according to these criteria;
(5) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journal or other publications and (c) the head of the responsible academic unit; and
(6) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location (s) Department of Pharmacology, Monash University

		Date
Associate Professor Geoffrey K Isbister	[redacted]	07.06.2013
Dr Margaret A O'Leary	[redacted]	07.06.2013
Dr Sanjaya Kuruppu	[redacted]	04.06.2013
Professor Helena C Parkington	[redacted]	06.06.2013
Professor Wayne C Hodgson	[redacted]	07.06.2013

Prothrombin activator-like compound from *Pseudonaja textilis* venom induces early cardiovascular collapse

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Summary

Brown snake (*Pseudonaja* spp.)-induced early cardiovascular collapse is a significant cause of morbidity and mortality in Australia. It has been suggested that prothrombin activators cause cardiovascular collapse in anaesthetized animals due to severe cardiovascular depression. We have recently reported that collapse induced by Australasian elapid venoms involves the release of endogenous mediators. In this study we identify a toxin from *Pseudonaja textilis* venom that causes early collapse. *P. textilis* venom was fractionated using a column of hydroxyapatite. Three fractions were obtained and tested for early collapse in anaesthetized rats. Only fraction 3 (PTV3; 10 or 20 μg/kg, i.v.) produced a rapid decrease in mean arterial pressure (MAP) followed by cardiovascular collapse. PTV3-induced early collapse was abolished by prior administration of a smaller priming dose of PTV3 (i.e. 2 and 5 μg/kg, i.v.) or heparin (300 units/kg, i.v.). *P. textilis* venom (1 and 3 μg/ml), but not PTV3 (1 or 3 μg/ml), induced endothelium-dependent relaxation in isolated rat mesenteric arteries. PTV3 showed 9-10 bands on SDS-PAGE gel. N-terminal sequencing identified some protein bands of PTV3 as subunits of venom prothrombin activator pseutarin C of *P. textilis* venom. Our results indicate that prothrombin activator-like toxin is likely to be a contributor to the rapid collapse induced by *P. textilis* venom.

Keywords: collapse, snake venom, anaesthetized rat, prothrombin activator, brown snake

1. Introduction

Envenoming by snakes of genus *Pseudonaja* (brown snake) may result in life-threatening outcomes including venom-induced consumption coagulopathy (VICC), progressive hemorrhage and sudden cardiovascular collapse. Indeed, death due to 'brown snake-induced early cardiovascular collapse' is the most common cause of death from snake envenoming in Australia (Allen *et al.*, 2012; Currie, 2004). Although rapid cardiovascular collapse following the injection of snake venom has been shown in a number of *in vivo* studies, the toxins responsible for this effect have not been identified. A number of different mechanisms and toxins have been suggested to be responsible. It has been hypothesized that temporary occlusion of major vessels induced by prothrombin activator is a cause of hypotension, collapse and cardiac arrest (Tibballs *et al.*, 1991; Tibballs *et al.*, 1992). It has also been suggested that the release of depletable endogenous vasoactive mediators (e.g. histamine, bradykinin) results in hypotension (Myint *et al.*, 1985). We have recently reported that sudden cardiovascular collapse induced *in vivo* by taipan (*Oxyuranus scutellatus*) venom involves the release of eicosanoids and activation of protein kinase A (PKA), without a direct effect on the heart (Chaisakul *et al.*, 2012). Prior administration of small priming doses of venom prevented collapse suggesting the involvement of endogenous mediators.

A number of components which affect the cardiovascular system have been isolated from animal venoms. These include bradykinin potentiating peptides (Ianzer *et al.*, 2011) which have been isolated from viper and crotalid venoms, L-type Ca^{2+} channels blockers (Garcia *et al.*, 2001) and natriuretic peptides (Vink *et al.*, 2012). The latter are also found in Australian inland taipan (*Oxyuranus microlepidotus*) venom and may contribute to cardiovascular collapse (Fry *et al.*, 2005).

The identification of the toxins that cause early cardiovascular collapse, following envenoming by brown snakes, is essential to delineate the mechanisms responsible for this effect and provides valuable insight into effective treatment strategies. Therefore, the aim of this study was to isolate and characterize the toxin(s) responsible for sudden collapse. We hypothesized that this toxin may act indirectly via depletable endogenous mediators.

2. Materials and Methods

2.1 Size-exclusion HPLC

Venom (3 mg) was applied to a Superdex G-75 column (13 µm; 10×300mm; GE Healthcare, Buckinghamshire, UK) equilibrated with ammonium acetate buffer (0.1 M; pH 6.8). The sample was eluted at a flow rate of 0.5 ml/min and the output was monitored at 280 nm. The fractions were collected and pooled, frozen at -80°C and then freeze-dried to remove the solvent.

2.2 Partial purification of toxin inducing early cardiovascular collapse (PTV3)

The toxin purification was performed by Margaret O'Leary at the Department of Clinical Pharmacology and Toxicology, Calvary Mater Hospital, Newcastle as follows.

Freeze-dried *P. textilis* venom (4 mg) was dissolved in water (180 µl) and applied to a column of hydroxyapatite (Bio-Rad HTP gel catalogue No. 130-0420, 4 x 1.7cm, packed in water) at a flow rate of 1 ml/min. Three fractions were collected and eluted with water (fraction 1), phosphate buffer (fraction 2; 0.15 M, pH 7), and phosphate buffer (fraction 3; 0.45 M, pH 7). Samples were collected and reapplied onto a Phenomenex Jupiter C18 column (5 µm; 300 Å; 250 mm × 4.6 mm) and eluted with 15% acetonitrile (MeCN)/ 0.1% trifluoroacetic acid (TFA) increasing to 54% MeCN, at t = 60 min at a flow rate of 0.5ml/min. The eluent was detected at 215 nm.

2.3 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS – PAGE)

Purified toxin which caused early collapse from 2.2 (11 µg) was resolved on a 4-12 % precast polyacrylamide gel, NuPAGE® Bis-Tris Precast Gel (Life Technology, Mulgrave, Australia) under reducing condition (5% β-mercaptoethanol in Laemmli's sample buffer (62.5 mM Tris-hydrochloride, 25% glycerol, 2% SDS, 0.01% bromophenol blue). Sample was heated for 5 min at 95˚C prior to resolving on SDS-PAGE. Protein bands were visualized by staining with BioSafe Coomassie G-250 solution (Bio-Rad Laboratories; Hercules, CA USA), followed by de-

staining in distilled water. Gel image was captured utilizing Typhoon Trio scanner (GE Healthcare; Uppsala, Sweden).

2.4 N-terminal sequencing

This determination was performed by Shane Reeve (Department of Biochemistry & Molecular Biology, Monash University, Australia) as follows.

2.4.1 Trypsin Digest

The gel pieces were washed and dehydrated with alternating washing cycles of 50 mM ammonium bicarbonate and acetonitrile and then digested with 0.5 µg trypsin (Promega corp., Madison, WI, USA) in 20 mM ammonium bicarbonate. Samples were incubated at 37° C overnight and sonicated for 2 min prior to analysis.

2.4.2 LCMS-MS

Tryptic digests were determined by LC-MS/MS using a HCT ULTRA ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany) coupled online with a nano HPLC (Ultimate 3000, Dionex Corporation, SunnyBrook, CA, USA). Samples were injected onto a Dionex pepmap100, 75 um id, 100Å pore size, reversed phase nano column with buffer A (5 % acetonitrile 0.1 % Formic acid) at a flow rate of 300 nl/minute. The peptides were eluted over a 30 min gradient to 55% B (90 % Acetonitrile 0.1 % formic acid). The eluent was nebulised and ionised using a Bruker electrospray source with a Low flow electrospray needle with a capillary voltage of 4000 V dry gas at 300 ºC, flow rate of 8 l/min and nebuliser gas pressure at 1500 mbar. Peptides were selected for MSMS analysis in autoMSn mode with smart parameter settings selected and active exclusion released after 1 min.

2.5 Anaesthetized rat preparation

As per manuscript in Chapter 7.

2.6 Procoagulant assay

As per manuscript in Chapter 6.

2.7 Rat mesenteric artery preparation

As per manuscript in Chapter 5.

2.8 Determination of PLA_2 Activity

As per manuscript in Chapter 6.

2.9 Chemicals and drugs

The following drugs and solutions were used: ACh, phenylephrine HCl, HOE140 and indomethacin, all from Sigma Chemical Co. (St Louis, MO, USA); mepyramine maleate (May & Baker, Dagenham, UK); and heparin sodium (Hospira, VIC, Australia).

2.10 Analysis of Results and Statistics

As per manuscript in Chapter 5.

3. Results

3.1 Size-exclusion HPLC

Size exclusion HPLC analysis of *P. textilis* venom indicated seven major peaks that were collected (Figure 1a) for further screening in the anaesthetized rat. A rapid decrease in MAP followed by collapse was observed following the administration of fraction A (i.e. all fractions from the venom recombined; 100 µg/kg, i.v.; Figure 1e). Then, each peak was individually tested in the anaesthetized rats. Peaks 3 and 4 (50 µg/kg, i.v.) caused transient hypotension without collapse. A higher dose of peak 4 (i.e. 100 µg/kg, i.v.; Figure 1d), but not peak 3 (Figure 1c), induced cardiovascular collapse in 4-5 min. The combination of peaks 3, 4 and 5 (100 µg/kg, i.v.) induced cardiovascular collapse within 10 min (Figure 1b). These results indicated a loss or

reduction of toxin activity. Thus, purification of the toxin using a column of hydroxyapatite was performed.

3.2 Identification of PTV3

On a column of hydroxyapatite, venom was separated into fractions 1-3. The fractions were reapplied onto a Phenomenex Jupiter C18 column (Figures 2b-d). Each fraction was tested for early cardiovascular collapse (Figures 4 and 5a). Only fraction 3 caused early collapse in the anaesthetized rat and was subsequently named as PTV3.

PTV3 was run on a 4-12% Bis-Tris gel and indicated the presence of 9-10 protein bands over a range of 2-100 kDa (Figure 3). The amino acid sequences of the protein bands within the gel were determined, based on their molecular mass showing on the gel.

Data from LC-MS/MS was exported in Mascot generic file format and searched against the NCBI and Swiss-Prot databases using the MASCOT search engine. Bands 1 (MW ~100 kDa) and 2 (MW 50-75 kDa) of PTV3 were identified as the non-catalytic subunit of prothrombin activator, pseutarin C of *P. textilis* venom while bands 5 (MW 25-37 kDa) and 6 (MW ~25 kDa) showed similarity in sequence to the catalytic subunit of pseutarin C. Band 10 (MW ~5 kDa) displayed similarity in sequence to the non-catalytic subunit of prothrombin activator, oscutarin C of *O. scutellatus* venom.

3.3 Effect of PTV3 on anaesthetized rats

PTV3 at 2-5 µg/kg (i.v., n = 4-5) caused a 59 ± 14% and 90 ± 7% decrease in MAP of the anaesthetized rats, respectively (Figure 5b). Cardiovascular collapse was observed following administration of higher doses of PTV3 (i.e. 10 or 20 µg/kg) (Figure 5a). At the time point of a 50% reduction of MAP, there was no significant change in heart rate (Figure 5c).

The prior administration of PTV3 (2 and 5 µg/kg or 2 discrete additions of 2 µg/kg, i.v.) prevented cardiovascular collapse induced by the subsequent administration of 10-20 µg/kg, i.v. PTV3 ($P < 0.05$, Student's unpaired t-test, n = 4; Figure 5b).

The prior administration of heparin (300 units/kg, i.v., n = 4) prevented cardiovascular collapse induced by PTV3 (10 µg/kg, i.v., $P < 0.05$, one-way ANOVA). However, mepyramine (2 mg/kg, i.v., n = 3) did not prevent cardiovascular collapse induced by PTV3 (10 µg/kg, i.v.; Figure 6).

In addition, prior administration of indomethacin (30 mg/kg, i.v., n = 4) or HOE140 (30 µg/kg, n = 3) also protected animals from PTV3-induced early cardiovascular collapse without a significant reduction in the decrease in MAP following the administration of PTV3 (Figure 6).

3.4 Procoagulant assay

A plot of clotting time versus venom/toxin concentration indicated a concentration-dependent procoagulant response over the range of 3.125-1000 ng/ml of PTV3 and *P. textilis* venom in human plasma (Figure 7).

3.5 Phospholipase A_2 activity

P. textilis venom had significantly higher PLA_2 activity (67.7 ± 4 µmol/min/mg) compared to PTV3 (2.4 ± 0.7 µmol/min/mg; n = 3; one-way ANOVA, $P < 0.05$). The positive control bee venom displayed PLA_2 activity of 317.5 ± 13.1 µmol/min/mg.

3.6 Isolated mesenteric artery

P. textilis venom (1 or 3 µg/ml) produced a relaxation response in rat mesenteric arteries (n = 6, $P < 0.05$, one-way ANOVA). In contrast, PTV3 (1 or 3 µg/ml) failed to produce significant vascular relaxation in rat mesenteric artery rings (n = 5, $P > 0.05$, one-way ANOVA; Figure 8).

4. Discussion

PTV3, a prothrombin activator like compound, was isolated from *P. textilis* venom. PTV3 induced sudden cardiovascular collapse in the anaesthetized rats, an effect mimicking that of the whole venom. PTV3 induced cardiovascular collapse without a significant change in heart rate suggesting a lack of direct effect on the heart. Prior administration of small priming doses of PTV3 protected against early cardiovascular collapse.

Screening of the size-exclusion HPLC fractions of *P. textilis* venom indicated that peak 4 caused cardiovascular collapse. However, this method for isolating the toxin was not optimal as either pooled fractions of whole venom or a high dose of peak 4 was required to induce collapse. This may indicate that collapse is generated by the synergistic effect of several compounds in the venom or that the efficacy of the responsible toxin(s) had been reduced during the purification procedure. Therefore, purification of the responsible toxin using a column of hydroxyapatite was performed. Fraction 3 of *P. textilis* venom from the hydroxyapatite column or PTV3 induced rapid cardiovascular collapse as indicated by unmeasurable MAP.

The LCMS-MS sequencing of trypsin digested protein bands from SDS-PAGE analysis indicated that the protein bands of PTV3 are similar to pseutarin C, the major prothrombin activator from *P. textilis* venom. Pseutarin C is a group C prothrombin activator complex (MW ~ 250 kDa), consisting of enzymatic and non-enzymatic subunits (Rao and Kini, 2002). The catalytic subunit of pseutarin C comprises a light chain and heavy chain linked together with a disulfide bond. The catalytic and non-catalytic subunits of pseutarin C display homology to mammalian factor Xa and factor Va, respectively, in the N-terminal sequence (Rao and Kini, 2002).

Previous studies reported that the administration of the prothrombin activator, from *P. textilis* venom (Masci *et al.*, 1988), or trocarin (Joseph *et al.*, 1999), a group D prothrombin activator from *Tropidechis carinatus* venom, caused death in animals within several minutes. Tibballs *et al.* (1991, 1992) suggested that brown snake venom caused depression of systemic blood pressure and cardiac output in anaesthetized dogs via the action of prothrombin activator-induced occlusion of major arteries. However, it is unlikely that coronary artery occlusion is

responsible for snake venom-induced cardiac collapse as chest pain is rarely reported, and sudden but transient hypotension is not a common feature of coronary artery occlusion.

The protective effect of prior administration of small doses of PTV3 can be attributed to the depletion of endogenous mediators involved in the hypotensive response. In the current study, administration of heparin prior to the injection of PTV3 prevented cardiovascular collapse suggesting the involvement of histamine or other relevant mediators as heparin has been shown to inhibit the release of histamine as well as the effects of bradykinin and/or prostaglandins (Carr, 1979; Inase *et al.*, 1993). Indeed, isolated PLA_2s or snake venom metalloproteinase also activate human mast cells resulting in histamine release, an effect inhibited by heparin (Wei *et al.*, 2006; Wei *et al.*, 2010). Mepyramine did not prevent cardiovascular collapse induced by PTV3 suggesting that H_1 receptor is unlikely to be responsible for brown snake venom/toxin-induced early collapse. Interestingly, prior administration of indomethacin or HOE140 also protected against early cardiovascular collapse, although a significant inhibition of the fall in MAP was not observed. This may suggest the involvement of prostacyclin and/or bradykinin in early collapse. Therefore, further investigation of the role of mast cell degranulation is required to better understand the role of endogenous compounds.

A number of snake venoms or phospholipase A_2s (PLA_2s) display vascular relaxant and hypotensive responses on isolated vasculature (Huang and Lee, 1985) and *in vivo* models (Huang, 1984), respectively. *P. textilis* venom, but not PTV3, induced relaxation in isolated rat mesenteric arteries suggesting the absence of a direct vascular effect of PTV3.

In conclusion, PTV3, a prothrombin activator-like toxin, appears to be a responsible toxin for, or contribute to, the early cardiovascular collapse observed *in vivo* in anaesthetized rats. The effect is due to the release of depletable endogenous mediators.

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References

Allen, GE, Brown, SG, Buckley, NA, O'Leary, MA, Page, CB, Currie, BJ, White, J, Isbister, GK (2012) Clinical effects and antivenom dosing in brown snake (*Pseudonaja* spp.) envenoming-Australian snakebite project (ASP-14). *PLoS One* 7: e53188.

Carr, J (1979) The anti-inflammatory action of heparin: heparin as an antagonist to histamine, bradykinin and prostaglandin E1. *Thromb Res* 16: 507-516.

Chaisakul, J, Isbister, GK, Konstantakopoulos, N, Tare, M, Parkington, HC, Hodgson, WC (2012) In vivo and in vitro cardiovascular effects of Papuan taipan (*Oxyuranus scutellatus*) venom: Exploring "sudden collapse". *Toxicol Lett* 213: 243-248.

Currie, BJ (2004) Snakebite in tropical Australia: a prospective study in the "Top End" of the Northern Territory. *Med J Aust* 181: 693-697.

Fry, BG, Wickramaratana, JC, Lemme, S, Beuve, A, Garbers, D, Hodgson, WC, Alewood, P (2005) Novel natriuretic peptides from the venom of the inland taipan (*Oxyuranus microlepidotus*): isolation, chemical and biological characterisation. *Biochem Biophys Res Commun* 327: 1011-1015.

Garcia, MC, Hernandez-Gallegos, Z, Escamilla, J, Sanchez, JA (2001) Calciseptine, a Ca^{2+} channel blocker, has agonist actions on L-type Ca^{2+} currents of frog and mammalian skeletal muscle. *J Membr Biol* 184: 121-129.

Huang, HC (1984) Effects of phospholipases A_2 from *Vipera russelli* snake venom on blood pressure, plasma prostacyclin level and renin activity in rats. *Toxicon* 22: 253-264.

Huang, HC and Lee, CY (1985) Relaxant effect of phospholipase A_2 from *Vipera russelli* snake venom on rat aorta. *Eur J Pharmacol* 118: 139-146.

Ianzer, D, Xavier, CH, Fraga, FC, Lautner, RQ, Guerreiro, JR, Machado, LT, Mendes, EP, de Camargo, AC, Santos, RA (2011) BPP-5a produces a potent and long-lasting NO-dependent antihypertensive effect. *Ther Adv Cardiovasc Dis* 5: 281-295.

Inase, N, Schreck, RE, Lazarus, SC (1993) Heparin inhibits histamine release from canine mast cells. *Am J Physiol* 264: L387-390.

Joseph, JS, Chung, MC, Jeyaseelan, K, Kini, RM (1999) Amino acid sequence of trocarin, a prothrombin activator from *Tropidechis carinatus* venom: its structural similarity to coagulation factor Xa. *Blood* 94: 621-631.

Masci, PP, Whitaker, AN, de Jersey, J (1988) Purification and characterization of a prothrombin activator from the venom of the Australian brown snake, *Pseudonaja textilis textilis*. *Biochem Int* 17: 825-835.

Myint, L, Warrell, DA, Phillips, RE, Tin Nu, S, Tun, P, Maung Maung, L (1985) Bites by Russell's viper (*Vipera russelli siamensis*) in Burma: haemostatic, vascular, and renal disturbances and response to treatment. *Lancet* 2: 1259-1264.

Rao, VS, Kini, RM (2002) Pseutarin C, a prothrombin activator from Pseudonaja textilis venom: its structural and functional similarity to mammalian coagulation factor Xa-Va complex. *Thromb Haemost* 88: 611-619.

Tibballs, J, Sutherland, SK, Kerr, S (1991) Studies on Australian snake venoms, Part II: The haematological effects of brown snake (*Pseudonaja*) species in the dog. *Anaesth Intensive Care* 19: 338-342.

Tibballs, J, Sutherland, SK, Rivera, RA, Masci, PP (1992) The cardiovascular and haematological effects of purified prothrombin activator from the common brown snake (*Pseudonaja textilis*) and their antagonism with heparin. *Anaesth Intensive Care* 20: 28-32.

Vink, S, Jin, AH, Poth, KJ, Head, GA, Alewood, PF (2012) Natriuretic peptide drug leads from snake venom. *Toxicon* 59: 434-445.

Wei, JF, Mo, YZ, Qiao, LY, Wei, XL, Chen, HQ, Xie, H, Fu, YL, Wang, WY, Xiong, YL, He, SH (2006) Potent histamine-releasing activity of atrahagin, a novel snake venom metalloproteinase. *Int J Biochem Cell Biol* 38: 510-520.

Wei, JF, Wei, XL, Chen, QY, He, SH (2010) Induction of inflammatory cell accumulation by TM-N49 and promutoxin, two novel phospholipase A_2. *Toxicon* 56: 580-588.

Figures

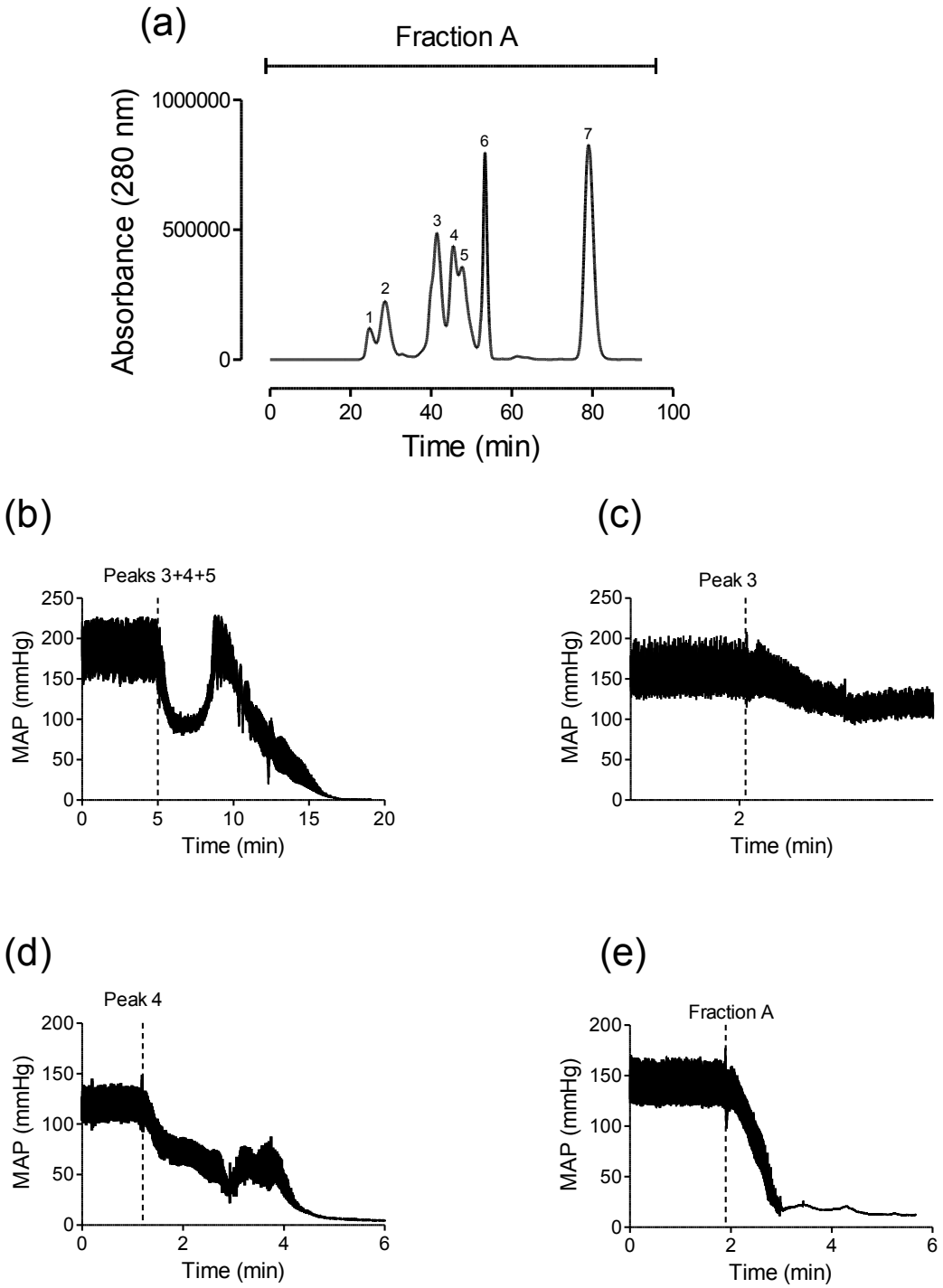

Figure 1: *Size exclusion chromatograph of P. textilis venom indicates peaks 1-7 and the area of fraction A (a). MAP of anaesthetized rats following the administration of mixture of (b) peaks 3-5; (c) peak 3; (d) peak 4 and (e) fraction A (100 µg/kg, i.v.; n = 2-3).*

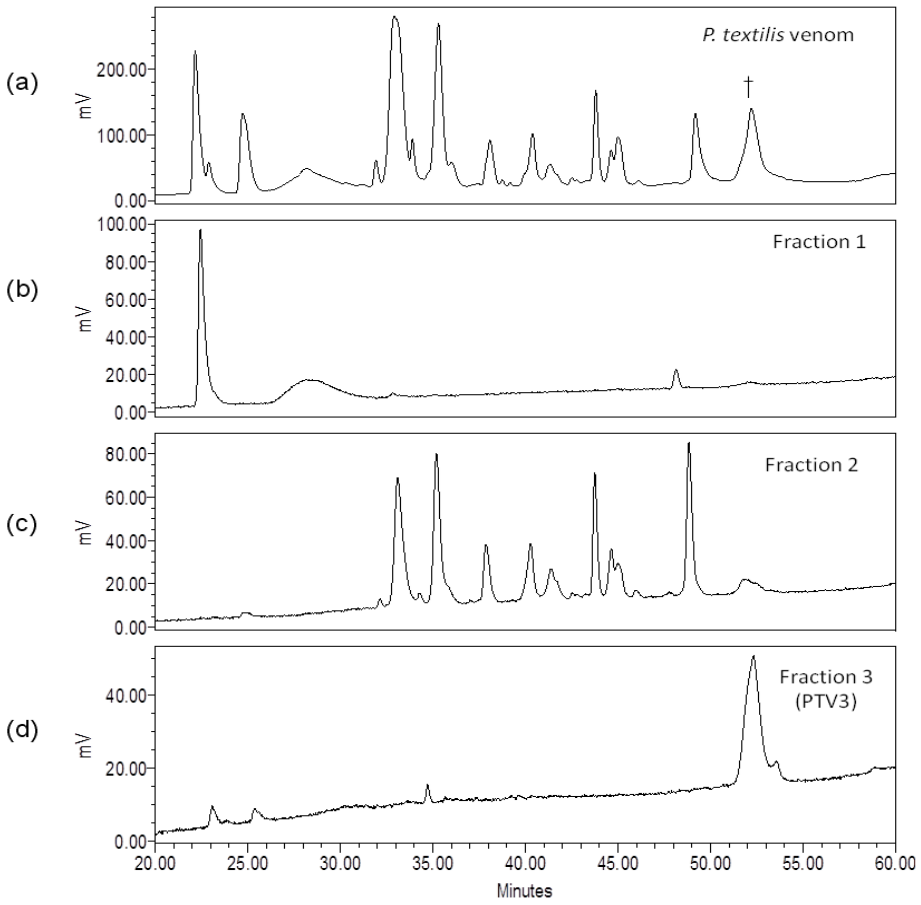

Figure 2: *(a) P. textilis venom († indicating PTV3), (b) fraction 1, (c) fraction 2 and (d) PTV3 on a Phenomenex Jupiter analytical C18 column, equilibrated with 15% MeCN/0.1% TFA increasing to 54% MeCN at t = 60, flow rate 0.5 ml/min. The samples were detected at 215 nm.*

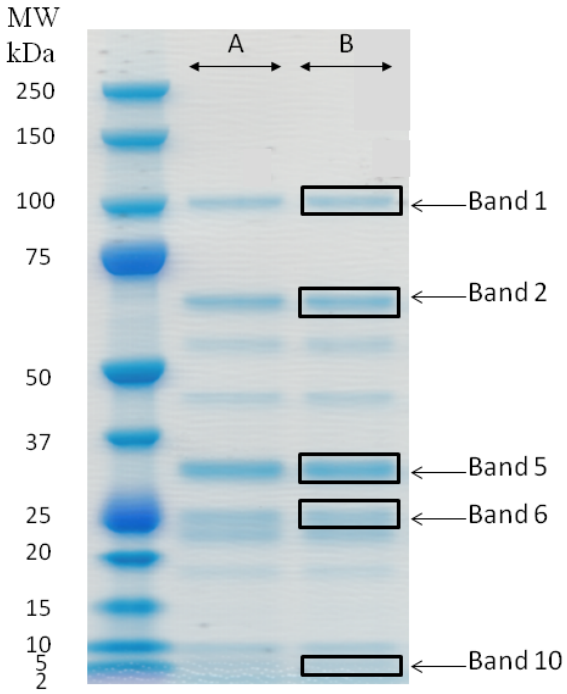

Figure 3: *A 4-12 % precast polyacrylamide gel showing profile of PTV3 under reducing condition (lane A) and bands cut out for N-terminal sequencing (lane B).*

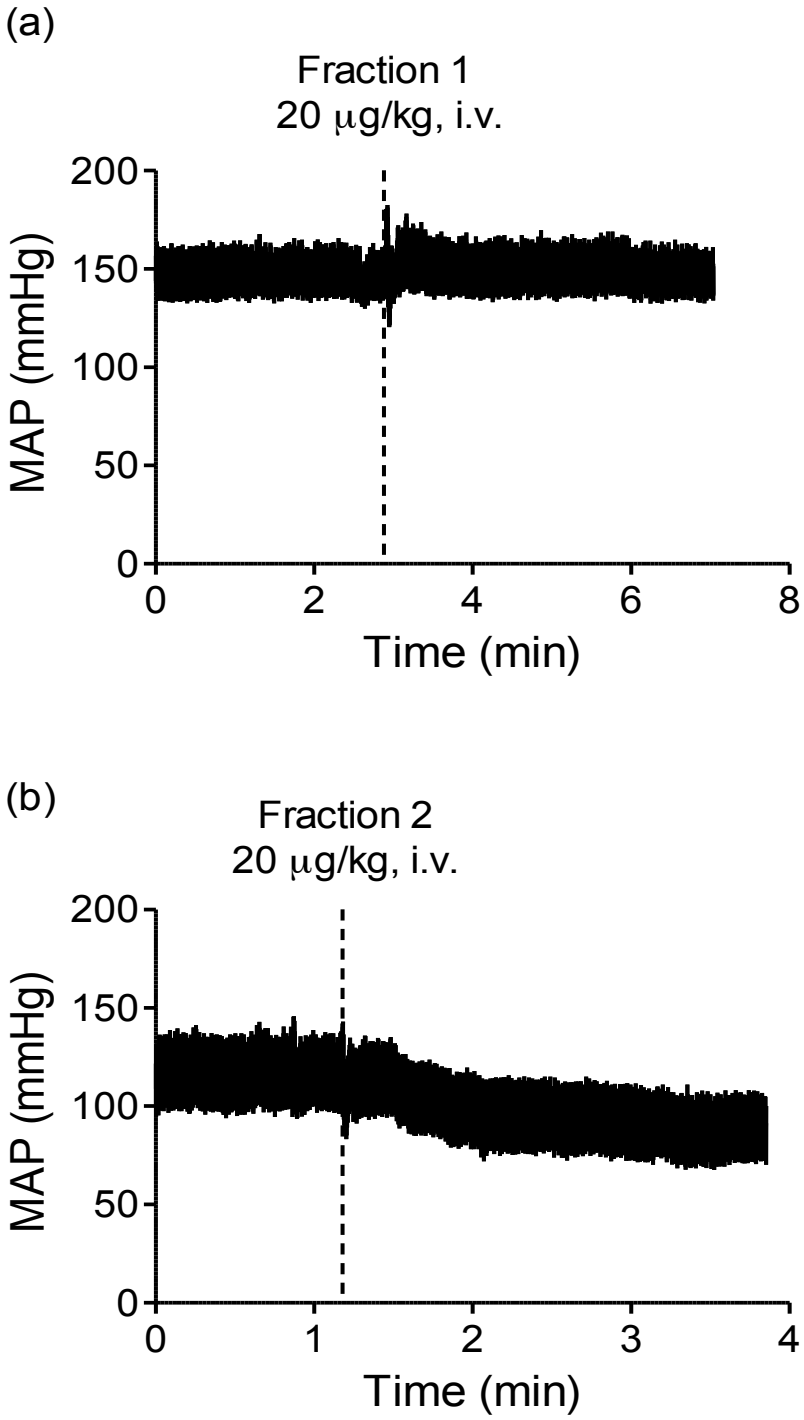

Figure 4: *Traces showing the effect (a) fraction 1 or (b) fraction 2 (20 μg/kg, i.v.) of P. textilis venom from the hydroxyapatite column on MAP of the anaesthetized rats.*

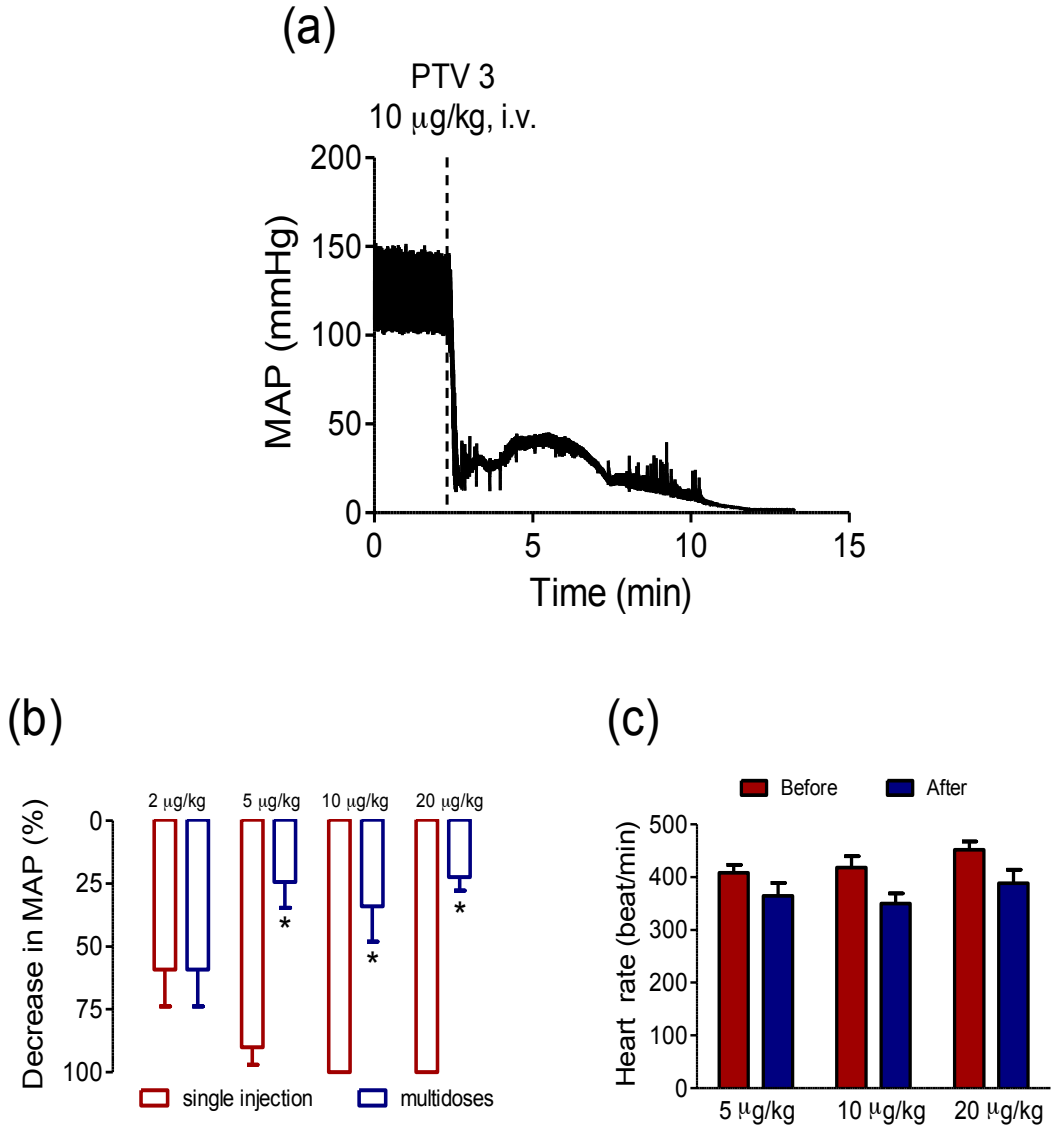

Figure 5: *(a) Effect of PTV3 (10 µg/kg, i.v.) on MAP in an anaesthetized rat. (b) Effect of PTV3 after single injection (2, 5, 10 and 20 µg/kg, i.v., n = 4-5) in different rats and sequential addition of PTV3 (2, 5, 10 and 20 µg/kg, i.v., n = 4) in the same rat on MAP. *P < 0.05, significantly different from single injection, Student's unpaired t-test.(c) A comparison of heart rate before and after single injection of PTV3 (measured at the point when MAP had decreased by 50%).*

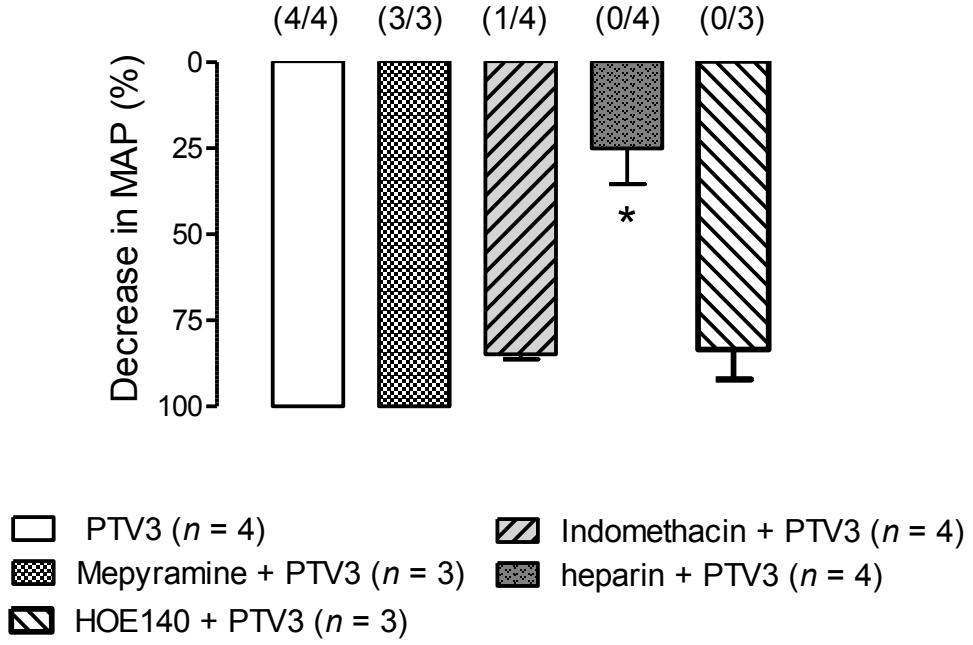

Figure 6: *The effect of PTV3 (10 µg/kg, i.v.) on MAP of the anaesthetized rats in the absence and presence of heparin (300 units/kg, i.v.), mepyramine (2 mg/kg, i.v.), indomethacin (30 mg/kg, i.v.) or HOE140 (30 µg/kg, i.v.). *P<0.05, significantly different from PTV3 alone, one-way ANOVA; (In brackets represents the number of animals with complete sudden collapse/ number of animals in each group).*

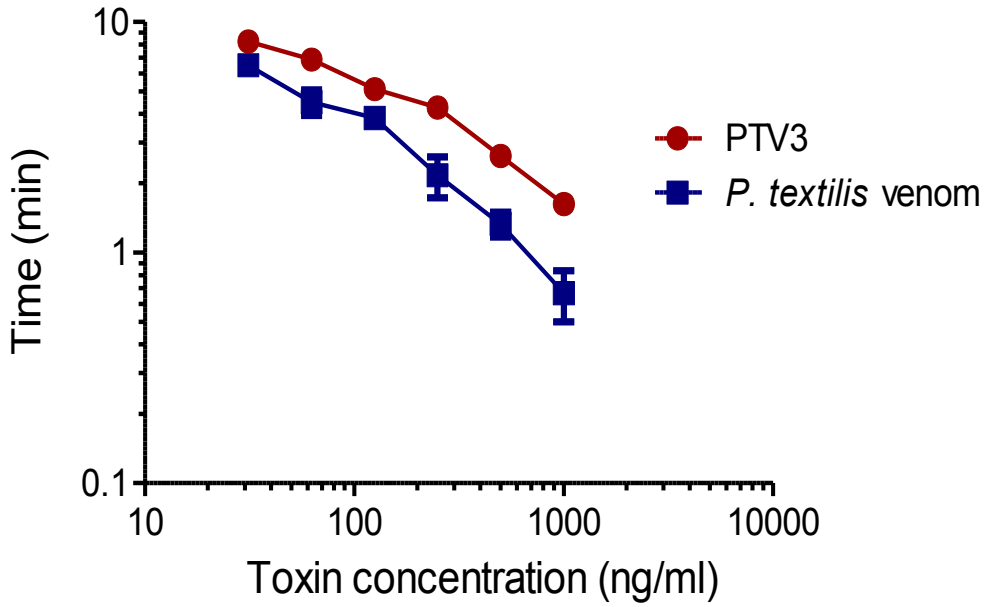

Figure 7: *Plots of clotting times versus concentrations of P. textilis venom and PTV3.*

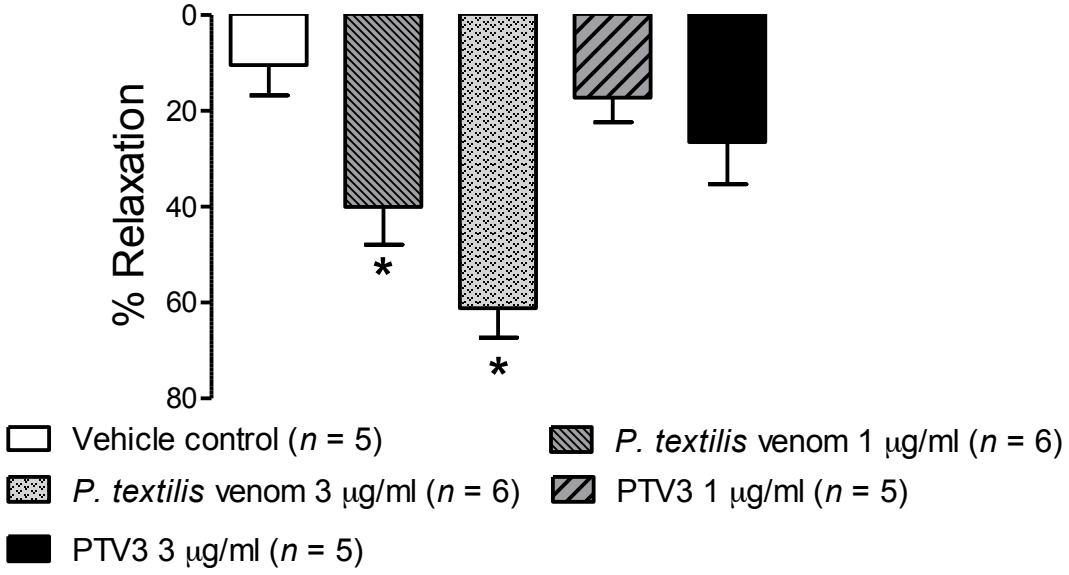

Figure 8: *Relaxation effects of P. textilis venom (1-3 μg/ml) and PTV3 (1-3 μg/ml) on endothelium-intact rat mesenteric arteries. *P < 0.05, significantly different from vehicle control, one-way ANOVA.*

Chapter 9

General Discussion

GENERAL DISCUSSION

Neurotoxicity and cardiovascular collapse are significant outcomes following envenoming by some Australasian elapids. Persistent neurotoxicity, following death adder envenoming, and early collapse resulting in death after brown snake envenoming have been reported in humans (Allen *et al.*, 2012; Johnston *et al.*, 2012). Identifying the mechanism(s) behind these life-threatening clinical complications is required to assist in the development of effective treatment strategies. In this thesis, the mechanisms responsible for neurotoxicity and cardiovascular collapse were explored.

Determination of Neurotoxicity

Based on the amount of venom required to kill 50% of a population of mice (i.e. murine LD_{50}) (Broad *et al.*, 1979), the Australo-Papuan region is home to many of the world's most venomous snakes. Whether this is an accurate representation of the risk of mortality to humans is open to debate. LD_{50} tests are becoming far less common due to ethical concerns and more strict regulatory requirements in many countries including Australia (Kuruppu *et al.*, 2008). In addition, it has been suggested that the value of LD_{50} studies in determining the toxicity of venoms is limited as they only measure the concentration of venom/toxin to cause 50% lethality of the animal population within a defined period but do not take into an account the duration or time frame of the toxicity (Hodgson and Wickramaratna, 2002). Therefore, *in vitro* studies using isolated tissue preparations e.g. mouse or rat phrenic nerve hemi-diaphragm preparations, toad rectus abdominis or CBCNM preparations have an important role in the pharmacological characterization of snake venoms/toxins (Hodgson and Wickramaratna, 2002). Given the presence of both focally- and multiply innervated skeletal muscle fibers, the CBCNM preparation is a very useful screening tool to distinguish between postsynaptic and presynaptic neurotoxicity, and to examine myotoxic activity by direct muscle stimulation (Ramasamy *et al.*, 2004). However, when studying crude venom, presynaptic neurotoxicity can be concealed by the rapid effect of α-neurotoxins present in the venom (Kuruppu *et al.*, 2008). The time taken to inhibit the

nerve-mediated twitches by 90% (i.e. t_{90} value) of skeletal muscle preparations is an indicator of the rank order of potency for venoms/toxins providing valuable insight into the kinetics of toxin-receptor binding. Therefore, the data obtained from t_{90} experiments is different, but complementary, to those obtained from LD_{50} studies (Kuruppu *et al.*, 2008).

Characterization of pre-synaptic neurotoxins from Australasian elapid venoms

Neurotoxicity is a common clinical characteristic of patients envenomed by snakes of genus *Oxyuranus* or genus *Acanthophis*, while this effect is likely to be absent following brown snake (genus *Pseudonaja*) envenoming despite the venom containing a pre-synaptic neurotoxin 'textilotoxin'. This anomaly is commonly referred to as the 'Brown snake paradox' (Currie, 2000). The lack of clinical neurotoxicity following brown snake envenoming may be due to the relatively small amount of the pre-synaptic neurotoxin in the venom and the apparent low potency of textilotoxin (Barber *et al.*, 2012). Species differences in the binding of post-synaptic neurotoxins to the skeletal muscle nAChRs have also been shown to contribute to differences observed in the onset of neurotoxicity (Hart *et al.*, 2008; 2011; 2013a). It has been demonstrated that venom of *Pseudechis* spp. (Black snakes) display poor binding activity at human and rat skeletal muscle nAChRs compared with their binding at nAChRs of chick skeletal muscle (Hart *et al.*, 2008; Hart *et al.*, 2011). *In vitro* experiments showed that an avian skeletal muscle preparation was more susceptible to the effects of these toxins compared with murine muscle preparations (Hart *et al.*, 2013a). This species difference in binding activity may help to explain the absence of neurotoxicity in *Pseudechis colletti* envenomed patients (Isbister *et al.*, 2006a).

Based on these studies, pre-synaptic neurotoxins, rather than post-synaptic neurotoxins, may be the major contributors toward the development of neurotoxicity in envenomed patients. In support of this, Johnston *et al.* (2012) reported persistent neurotoxicity in death adder envenomed patients which did not respond to large doses of antivenom or anticholinesterases. The onset of neurotoxicity was primarily observed more than 3 hours post-bite (Range; 0.5-15.5 hours). Early administration of antivenom was not associated with less severe neurotoxicity suggesting the

'irreversible' neurotoxicity induced by pre-synaptic neurotoxins (Johnston *et al.*, 2012). This indicates that post-synaptic neurotoxins, which normally respond well to antivenom (Barber *et al,* 2013; Tan *et al.*, 2006), are unlikely to be the major contributors to death adder-induced neurotoxicity. Indeed, pre-synaptic neurotoxins have been isolated and characterized from *A. antarcticus* (Blacklow *et al.*, 2010a), *A. praelongus* and *A. rugosus* venoms (Chapter 2).

Pre-synaptic neurotoxins from *A. praelongus* and *A. rugosus* were named P-EPTX-Ap1a and P-EPTX-Ar1a, respectively, based on the rational nomenclature system (King *et al.*, 2008). The prefix 'P' indicates a presynaptic PLA_2 activity, while 'EPTX' is the abbreviation of 'elapitoxin' which refers to the generic name for toxins from elapid venoms. 'Ap' and 'Ar' are the genus and species descriptors for *A. praelongus* and *A. rugosus*, respectively. '1' was chosen to represent multimeric snake pre-synaptic PLA_2 neurotoxin vs. '2' for monomeric snake pre-synaptic PLA_2 neurotoxin and 'a' denotes the first isoforms found (Blacklow *et al.*, 2010a).

Many snake venom pre-synaptic neurotoxins contain multiple subunits held together by noncovalent interactions. Dissociation of these subunits significantly attenuates the toxic activity of the neurotoxin (Fohlman *et al.*, 1976; Francis *et al.*, 1993). Using RP-HPLC, we have shown that P-EPTX-Ap1a and P-EPTX-Ar1a dissociate into 4 subunits i.e. γ, α, β1 and β2. The β1 and β2 subunits of P-EPTX-Ap1a and P-EPTX-Ar1a have similar molecular weights and are likely to be isoforms. Under the same conditions, cannitoxin, from the venom of the Papuan taipan, also dissociates into four peaks, two peaks being isoforms of the β subunit as well as γ and α subunits (Kuruppu *et al.*, 2005b). The γ subunit is acidic and glycosylated as evidenced by a broadening of the peak presented in the MALDI-TOF mass spectrometry (Chapters 2 and 3). The presence of a γ subunit in both death adder pre-synaptic neurotoxins was not observed initially when 40 µg protein of P-EPTX-Ap1a or P-EPTX-Ar1a was loaded on to the RP-HPLC. However, it should be noted that the venom profile is dependent on the pH of the mobile phase (Han *et al.*, 2012), the amount of loaded protein (Martins *et al.*, 2012; Skidan *et al.*, 2011), temperature, individual venom components and possible geographical variations in the locality of the snakes used for venom collection (Winter *et al.*, 2010).

The potency of snake pre-synaptic neurotoxins are different and depend on the tissue preparation used for testing and the snake species from which the toxin is isolated (Chang *et al.*, 1977). Comparing the pre-synaptic neurotoxins from death adder venoms, based on t_{90} values in an avian preparation, death adder pre-synaptic neurotoxins displayed very similar inhibitory activity (i.e. approximately 78 min; Table 9.1) although it should be noted that different concentrations were used in the different studies. Changing the temperature of the bathing solution or the frequency of nerve stimulation also affects the degree of neurotoxicity in *in vitro* studies (Su *et al.*, 1983). In the current studies, increasing the rate of indirect stimulation from 0.2 Hz to 1.0 Hz accelerated the inhibitory action of P-EPTX-Ar1a and P-EPTX-Ap1a. Increasing the rate of nerve stimulation may produce an increase in $[Ca^{2+}]_i$ resulting in facilitation of neurotransmitter depletion (Hodgson *et al.*, 2007; Su *et al.*, 1983) and producing a more rapid decrease in twitch height as evidenced by a lower t_{90} value.

Table 9.1: *Pre-synaptic neurotoxins isolated from some Australasian elapid venoms*

Species	Toxin	Molecular mass (Da)	Number of subunits	t_{90} (min)
O. microlepidotus (inland taipan)	Paradoxin[a]	45,000	3	129 ± 8 (65nM)[b]
O. s. scutellatus (Australian coastal taipan)	Taipoxin[c]	46,800	3	126 ± 12 (66 nM)[d]
O. scutellatus (Papuan taipan)	Cannitoxin[e]	44,770[h]	3	43 ± 2 (65 nM)
P. textilis (eastern brown snake)	Textilotoxin[f]	~80,000	5	350 (3 nM)
A. antarcticus (common death adder)	P-EPTX-Aa1a[g]	44,698	3	74 ± 3.5 (222 nM)
A. praelongus (northern death adder)	P-EPTX-Ap1a	43,285	3	79.5 ± 8 (100 nM)[i]
A. rugosus (Irian Jayan death adder)	P-EPTX-Ar1a	44,093[h]	3	79.6 ± 6 (100 nM)[i]

[a](Fohlman, 1979) [b](Hodgson *et al.*, 2007) [c](Fohlman *et al.*, 1977)
[d](Crachi *et al.*, 1999a) [e](Kuruppu *et al.*, 2005b) [f](Su *et al.*, 1983)
[g](Blacklow *et al.*, 2010a) [h](Chaisakul *et al.*, 2013) [i](Chaisakul *et al.*, 2010)

Studies examining the reversibility of the *in vitro* effects of pre-synaptic neurotoxin by antivenoms are limited. One study showed that the addition of antivenom at the t_{90} time point failed to reverse the inhibitory effect of Papuan taipan venom on the CBCNM preparation confirming the irreversible nature of pre-synaptic neurotoxicity (Kuruppu *et al.*, 2005b). In contrast, the present study shows that CSL death adder antivenom neutralized the pre-synaptic neurotoxins P-EPTX-Ar1a and P-EPTX-Ap1a when added to the skeletal muscle preparations prior to the toxins. This indicates that the commercial antivenom is able to interact with the pre-synaptic neurotoxins (Kuruppu *et al.*, 2005b), although timing is critical.

Myotoxicity and Cytotoxicity induced by snake pre-synaptic neurotoxins

Secreted PLA_2 enzymes have been studied in a number of physiological systems. The PLA_2s used in the current study are class I PLA_2s from elapid venoms while PLA_2s present in viperid venoms belong to class II. Class II PLA_2s are subdivided into Asp49 PLA_2 and Lys49 PLA_2. Asp49 PLA_2s induce apoptosis in cultured rat neurons by receptor-mediated internalization (Herkert *et al.*, 2001). Lys49 PLA_2s are devoid of enzymatic activity as the substitution of lysine for aspartate causes a loss of activity. This substitution affects calcium binding site and catalytic centre causing an inability of the protein to bind to Ca^{2+} (Lomonte *et al.*, 2003). The role of Lsy49 has not been investigated in elapid venoms (Lomonte and Rangel, 2012), although it has been suggested that the isolated myotoxins from the venom of *A. laevis* (previously known as death adder sp. Seram), acanmyotoxin 3 and acanmyotoxin 2 are Lys49 PLA_2 and Asp49 PLA_2, respectively (Hart *et al.*, 2005). However, the full amino acid sequencing of both toxins is required to discover the identity of the amino acid at position 49.

The death adder pre-synaptic neurotoxins isolated in the current study displayed marked PLA_2 activity. An investigation of the myotoxic and cytotoxic activities (Chapter 3) was subsequently undertaken. The venoms and pre-synaptic neurotoxins from the Irian Jayan death adder (*A. rugosus*) and Papuan taipan (*O. scutellatus*) were chosen due to the ability of these venoms to induce myotoxicity in envenomed patients and their geographical overlap.

Obvious differences in the myotoxic activity of *A. rugosus* and *O. scutellatus* venoms were observed. Interestingly, the pre-synaptic neurotoxin from each venom produced similar morphological changes in the chick skeletal muscle. This was characterized by vacuolation due to dilatation of the sarcoplasmic reticulum (Ownby *et al.*, 1976). Additional changes that included swelling of mitochondria, disorganized myofibrils, and degeneration of the myofilaments, which also indicate snake venom-induced pathological changes in skeletal tissues (Kuruppu *et al.*, 2005a; Wickramaratna *et al.*, 2003a), were not observed. However, assessment of blood creatine kinase levels following the administration of snake venoms/purified toxins to rodents may be an alternative method to evaluate the degree of myotoxic activity.

The cytotoxic effects of *A. rugosus* venom on the rat skeletal muscle cell line (L6 cell) was time dependent which is in agreement with a previous study performed by our laboratory (Kalam *et al.*, 2010). Kini (2003) has reported that the pharmacological effects of PLA_2 will incrementally increase in relation to incubation time (Kini, 2003). *O. scutellatus* venom and cannitoxin induced myotoxicity on the chick preparation, but not cytotoxicity in the L6 rat skeletal muscle cell line, suggesting species differences in the interaction of the PLA_2 toxin and target sites on the tissue (Kini, 2003).

Snake pre-synaptic PLA_2 neurotoxin-induced an increase in $[Ca^{2+}]_i$

As $[Ca^{2+}]_i$ plays an important role in the exocytosis of neurotransmitter release and cellular homeostasis, the effect of P-EPTX-Ar1a on the increase in $[Ca^{2+}]_i$ of mouse DRG neurons was determined using Ca^{2+} imaging techniques. Clinically, there is limited evidence to suggest that snake venoms/neurotoxins affect the CNS. Thus, mouse DRG neurons were chosen as they are more likely to represent the peripheral targets of snake neurotoxins. A rise in $[Ca^{2+}]_i$ was abolished as soon as the extracellular Ca^{2+} from the physiological solution was omitted or in the presence of L-type or P/Q type Ca^{2+} channel blockers including cationic channel blockers (i.e. SKF-96365, MK801). An NMDA receptor antagonist, MK801, also attenuated Ca^{2+} influx induced by P-EPTX-Ar1a. This suggests that the effect of P-EPTX-Ar1a on the increase in $[Ca^{2+}]_i$ may involve the

glutamatergic system which overstimulates the plasma membrane. This result supported the work of Tseng and Lin-Shiau (2003b) who demonstrated that neurotoxicity of β-bungarotoxin might be mediated through direct or indirect activation of NMDA receptors and Ca^{2+} channels. Although a number of studies have shown the involvement of pre-synaptic neurotoxins in the increase in $[Ca^{2+}]_i$ of neurons, there are few studies examining the effects of snake neurotoxins on ion channels. Electrophysiological examination indicated that P-EPTX-Ar1a may activate the opening of voltage-gated Na^+ channels causing membrane depolarization. In fact, P-EPTX-Ar1a displays a similar action to crotamine, a small basic polypeptide myotoxin from the venom of the South American rattlesnake (*Crotalus durissus terrificus*) (Radis-Baptista and Kerkis, 2011). Crotamine induces pathological changes in skeletal muscle e.g. vacuolation of SR, myonecrosis and spasticity in the hind limbs of rodents and dogs (Oguiura *et al.*, 2005). In addition, crotamine evokes membrane depolarization on skeletal muscle cells and influx of Na^+ which are inhibited by TTX and the attenuation of extracellular Na^+ (Chang and Tseng, 1978; Ownby *et al.*, 1988).

Cardiovascular effects

The mechanisms behind cardiovascular effects are described in Chapters 5, 6, 7 and 8. Snake venom cardiotoxins or 'direct lytic factors' have been reported to induce cardiac arrest, degeneration of cardiomyocytes (Tzeng and Chen, 1988) and cytolysis (Chen and Chu, 1988). However, these components appear to be absent in Australasian elapid venoms. In the current study (Chapter 5), *in vitro* experiments confirmed that *O. scutellatus* venom had no effect on the heart. Indeed, subsequent experiments outlined in this thesis indicate that the release of vasoactive substances is responsible for the sudden cardiovascular collapse which occasionally occurs following envenoming by some Australasian elapids.

The vascular effects of Australasian elapids venoms have been previously investigated in rat isolated aortic rings, with the relaxant effects observed being shown not to involve NO or cyclooxygenase metabolites (Bell *et al.*, 1999; Crachi *et al.*, 1999b). In the present study (i.e. Chapters 5 and 6), vascular relaxation of rat mesenteric arteries induced by *O. scutellatus* venom,

and an isolated fraction (i.e. OSC3), was shown to involve a cellular cascade of PGI_2 and PKA. The PLA_2 activity of OSC3 may mediate the hypotensive and relaxant effects through membrane hydrolysis leading to the generation of arachidonic acid (Trevisi *et al.*, 2002) which is converted to PGI_2 via the cyclooxygenase pathway (Mehta *et al.*, 1983). In Chapter 6, OSC3 induced a right-ward shift of the concentration-relaxation curve to ACh indicating that this snake venom PLA_2 may induce a direct effect on the vascular endothelial cells.

Sudden cardiovascular collapse induced by taipan venom is unlikely to be solely due to the effects of PLA_2 enzymes since the administration of purified PLA_2s did not produce cardiovascular collapse (Chapter 6). Interestingly, the pooled mixture of isolated fractions failed to replicate the sudden collapse induced by the whole venom. This may be explained by the fact that the toxins had been denatured during the purification procedure or there might be other components which have not been included in the 'mixture' which induce cardiovascular collapse.

How incrementally increasing the dose of venom prevents early cardiovascular collapse

The 'self-protective effect' of prior administration of priming doses of snake venoms from the same or different families (Chapters 7) suggested that early collapse is likely to involve a common mechanism/pathway activated by different snake venom e.g. venom-induced autopharmacological release of endogenous vasoactive compounds (e.g. histamine, serotonin and bradykinin) which result in severe, rapid hypotension and may appear similar to anaphylaxis (Myint *et al.*, 1985). However, the *in vivo* study presented in Chapter 5 indicated that sudden cardiovascular collapse following taipan envenoming was not reliant on histamine or serotonin involvement as the prior administration of the mixture of mepyramine (H_1 receptor antagonist) and cimetidine (H_2 receptor antagonist) or methysergide (5-HT_2 antagonist) failed to inhibit cardiovascular collapse.

It has been suggested that the resultant thrombi from VICC may occlude systemic vessels resulting in cardiac disturbance (Tibballs *et al.*, 1991; Tibballs *et al.*, 1992). In the current study, the venoms causing early cardiovascular collapse (i.e. from *Oxyuranus* spp. and *Pseudonaja* spp.)

contain activators of factors V and X (Speijer *et al.*, 1986) or, in the venom of *Daboia russelii*, procoagulant metalloproteinase (Mukherjee, 2008). It could be suggested that the self protective effect of snake venom is due to a deficiency of clotting factors following the administration of priming doses of the snake prothrombin activator or venom. However, our study (Chapter 5) indicates that protective effect of priming doses was 'lost' after about 1 hour while the re-synthesis of clotting factors takes 24 to 48 hours (Isbister *et al.*, 2010b; Tanos *et al.*, 2008). Therefore protective effect of priming doses of snake venom is much more likely to be due to a mechanism involving the release of endogenous mediators.

Efficacy of polyvalent antivenom on snake venom-induced early collapse

Administration of CSL snake antivenoms is the current mainstay treatment of systemic snake envenoming in Australia and Papua New Guinea. A previous study has indicated that CSL snake monovalent antivenoms are in fact polyvalent (O'Leary and Isbister, 2009). Therefore, CSL polyvalent snake antivenom was used in the current study. The efficacy of brown snake antivenom against brown snake-induced cardiovascular depression and coagulopathy has been demonstrated by incubation of venom and antivenom at 37°C for 30 min prior to administration to the animals (Tibballs and Sutherland, 1991). In the current study, prior administration of CSL polyvalent snake antivenom (i.e. 500 units/kg i.v.), protected animals from sudden collapse induced by *O. scutellatus* or *P. textilis* venoms. Moreover, Western blots analysis confirmed that CSL polyvalent antivenom was antigenic to key venom proteins. Administration of polyvalent antivenom, at the time point where MAP had decreased by 50%, prevented complete collapse (Chapter 7). This indicates the efficacy of polyvalent antivenom to reverse sudden collapse. Interestingly, this effect is in contrast to the early collapse induced by jellyfish venom which cannot be reversed by antivenom, perhaps indicating a difference in the time to onset of the toxin(s) involved (Winter *et al.*, 2009).

Future directions

Snake venom PLA_2 enzymes are associated with a vast array of pharmacological activities. Pharmacological effects can be induced by the binding activity between pharmacological sites of PLA_2 and target sites on tissue. Identification of the target protein for snake venom PLA_2s i.e. OSC3a or OSC3b on vascular smooth muscle or endothelial cells may help to elucidate the mechanism behind the vascular relaxant effect of PLA_2. In addition, patch clamp recording of specific cloned voltage-gated channels (e.g. VOCCs and K_V) may allow us to elucidate the activity of pre-synaptic neurotoxins on specific ion channels.

The protective effect of heparin may be related to the inhibition of vascular mediator release (Inase *et al.*, 1993). Therefore, determination of mast cell degranulation is required to investigate the effect of Australasian elapid venoms on mediator release as have been reported for cobra venom PLA_2 (Wei *et al.*, 2006; Wei *et al.*, 2010).

Conclusions

It is anticipated that the data from *in vivo* and *in vitro* studies of four Australasian elapid venoms, described in this thesis, will be of both clinical and experimental importance. The presence of pre-synaptic PLA_2 neurotoxins in some death adder venoms is likely to result in persistent or delayed neurotoxicity. P-PTX-Ar1a also induced cytotoxicity by mechanism involving an increase in $[Ca^{2+}]_i$. Interestingly, P-EPTX-Ar1a is the first pre-synaptic neurotoxin from Australasian elapid venom shown to affect cationic channels leading to membrane depolarization. In particular, it is expected that P-EPTX-Ar1a may be a useful research tool in the understanding the cellular processes that underlie neurotoxicity or other outcomes.

Finally, Australasian elapid venoms induce profound effects on the cardiovascular system including; (1) indirect vascular relaxation via vasoactive mediators, and (2) the presence of prothrombin activator-like compounds causing rapid cardiovascular collapse through a mechanism involving depletable endogenous mediators. Therefore, it is hoped that these studies will provide the treatment strategy for envenomed patients.

References

Ackermann, U, Khanna, J, Irizawa, TG (1988) Atrial natriuretic factor alters autonomic interactions in the control of heart rate in conscious rats. *Can J Physiol Pharmacol* 66: 930-936.

Adams, ZS, Gattullo, D, Losano, G, Marsh, NA, Vacca, G, Whaler, BC (1981) The effect of *Bitis gabonica* (gaboon viper) snake venom on blood pressure, stroke volume and coronary circulation in the dog. *Toxicon* 19: 263-270.

Akk, G and Auerbach, A (1996) Inorganic, monovalent cations compete with agonists for the transmitter binding site of nicotinic acetylcholine receptors. *Biophys J* 70: 2652-2658.

Akopian, AN, Sivilotti, L, Wood, JN (1996) A tetrodotoxin-resistant voltage-gated sodium channel expressed by sensory neurons. *Nature* 379: 257-262.

Allen, GE, Brown, SG, Buckley, NA, O'Leary, MA, Page, CB, Currie, BJ, White, J, Isbister, GK (2012) Clinical effects and antivenom dosing in brown snake (*Pseudonaja* spp.) envenoming - Australian snakebite project (ASP-14). *PLoS One* 7: e53188.

Alloatti, G, Gattullo, D, Marsh, NA, Pagliaro, P, Vono, P (1991) The mechanical and electrical effects of rhinoceros viper (*Bitis nasicornis*) venom on the isolated perfused guinea pig heart and atrial preparations. *Life Sciences* 49: 1539-1548.

Amininasab, M, Elmi, MM, Endlich, N, Endlich, K, Parekh, N, Naderi-Manesh, H, Schaller, J, Mostafavi, H, Sattler, M, Sarbolouki, MN, Muhle-Goll, C (2004) Functional and structural characterization of a novel member of the natriuretic family of peptides from the venom of *Pseudocerastes persicus*. *FEBS Lett* 557: 104-108.

Ariaratnam, CA, Sheriff, MH, Arambepola, C, Theakston, RD, Warrell, DA (2009) Syndromic approach to treatment of snake bite in Sri Lanka based on results of a prospective national hospital-based survey of patients envenomed by identified snakes. *Am J Trop Med Hyg* 81: 725-731.

Arni, RK and Ward, RJ (1996) Phospholipase A_2-a structural review. *Toxicon* 34: 827-841.

Barber, CM, Isbister, GK, Hodgson, WC (2013) Alpha neurotoxins. *Toxicon* 66: 47-58.

Barber, CM, Isbister, GK, Hodgson, WC (2012) Solving the 'Brown snake paradox': *in vitro* characterisation of Australasian snake presynaptic neurotoxin activity. *Toxicol Lett* 210: 318-323.

Bargmann, CI (1998) Neurobiology of the *Caenorhabditis elegans* genome. *Science* 282: 2028-2033.

Barone, F, Aguanno, S, D'Alessio, A, D'Agostino, A (2004) Sertoli cell modulates MAA-induced apoptosis of germ cells throughout voltage-operated calcium channels. *FASEB J* 18: 353-354.

Bear, MF, Connors, BW, Paradiso, MA (2007) Synaptic transmission. In: Williams, PC (ed). *Neuroscience Exploring the Brain*, 3rd edn. Lippincott Williams & Wilkins: Baltimore, MD, pp 102-166.

Becher, A, Drenckhahn, A, Pahner, I, Margittai, M, Jahn, R, Ahnert-Hilger, G (1999) The synaptophysin-synaptobrevin complex: a hallmark of synaptic vesicle maturation. *J Neurosci* 19: 1922-1931.

Bell, KL, Kemp, BK, McPherson, GA, Hodgson, WC (1999) The smooth muscle relaxant effects of venom from the inland taipan (*Oxyuranus microlepidotus*). *Toxicon* 37: 229-231.

Berridge, MJ (2005) Unlocking the secrets of cell signaling. *Annu Rev Physiol* 67: 1-21.

Berridge, MJ, Bootman, MD, Roderick, HL (2003) Calcium signalling: dynamics, homeostasis and remodelling. *Nat Rev Mol Cell Biol* 4: 517-529.

Berridge, MJ, Lipp, P, Bootman, MD (2000a) Signal transduction. The calcium entry pas de deux. *Science* 287: 1604-1605.

Berridge, MJ, Lipp, P, Bootman, MD (2000b) The versatility and universality of calcium signalling. *Nat Rev Mol Cell Biol* 1: 11-21.

Black, SA and Rylett, RJ (2012) Choline transporter CHT regulation and function in cholinergic neurons. *Cent Nerv Syst Agents Med Chem* 12: 114-121.

Blacklow, B, Escoubas, P, Nicholson, GM (2010a) Characterisation of the heterotrimeric presynaptic phospholipase A_2 neurotoxin complex from the venom of the common death adder (*Acanthophis antarcticus*). *Biochem Pharmacol* 80: 277-287.

Blacklow, B, Konstantakopoulos, N, Hodgson, WC, Nicholson, GM (2010b) Presence of presynaptic neurotoxin complexes in the venoms of Australo-Papuan death adders (*Acanthophis* spp.). *Toxicon* 55: 1171-1180.

Bonanomi, D, Pennuto, M, Rigoni, M, Rossetto, O, Montecucco, C, Valtorta, F (2005) Taipoxin induces synaptic vesicle exocytosis and disrupts the interaction of synaptophysin I with VAMP2. *Mol Pharmacol* 67: 1901-1908.

Brandt, BL, Hagiwara, S, Kidokoro, Y, Miyazaki, S (1976) Action potentials in the rat chromaffin cell and effects of acetylcholine. *J Physiol* 263: 417-439.

Broad, AJ, Sutherland, SK, Coulter, AR (1979) The lethality in mice of dangerous Australian and other snake venom. *Toxicon* 17: 661-664.

Bukoski, RD, Bergmann, C, Gairard, A, Stoclet, JC (1989) Intracellular Ca^{2+} and force determined simultaneously in isolated resistance arteries. *Am J Physiol* 257: H1728-1735.

Burdmann, EA, Woronik, V, Prado, EB, Abdulkader, RC, Saldanha, LB, Barreto, OC, Marcondes, M (1993) Snakebite-induced acute renal failure: an experimental model. *Am J Trop Med Hyg* 48: 82-88.

Busse, R, Edwards, G, Feletou, M, Fleming, I, Vanhoutte, PM, Weston, AH (2002) EDHF: bringing the concepts together. *Trends Pharmacol Sci* 23: 374-380.

Caccin, P, Rigoni, M, Bisceglie, A, Rossetto, O, Montecucco, C (2006) Reversible skeletal neuromuscular paralysis induced by different lysophospholipids. *FEBS Lett* 580: 6317-6321.

Camargo, AC, Ianzer, D, Guerreiro, JR, Serrano, SM (2011) Bradykinin-potentiating peptides: beyond captopril. *Toxicon* 59: 516-523.

Carafoli, E (2005) Calcium-a universal carrier of biological signals. *FEBS J* 272: 1073-1089.

Caratsch, CG, Miledi, R, Strong, PN (1985) Influence of divalent cations on the phospholipase-independent action of beta-bungarotoxin at frog neuromuscular junctions. *J Physiol* 363: 169-179.

Carlson, BB, Trevitt, JT, Salamone, JD (2000) Effects of H_1 antagonists on cholinomimetic-induced tremulous jaw movements: studies of diphenhydramine, doxepin, and mepyramine. *Pharmacology Biochemistry and Behavior* 65: 683-689.

Carr, J (1979) The anti-inflammatory action of heparin: Heparin as an antagonist to histamine, bradykinin and prostaglandin E1. *Thrombosis Research* 16: 507-516.

Catterall, WA, Goldin, AL, Waxman, SG (2005) International Union of Pharmacology. XLVII. Nomenclature and structure-function relationships of voltage-gated sodium channels. *Pharmacol Rev* 57: 397-409.

Chaisakul, J, Konstantakopoulos, N, Smith, AI, Hodgson, WC (2010) Isolation and characterisation of P-EPTX-Ap1a and P-EPTX-Ar1a: pre-synaptic neurotoxins from the venom of the northern (*Acanthophis praelongus*) and Irian Jayan (*Acanthophis rugosus*) death adders. *Biochem Pharmacol* 80: 895-902.

Chaisakul, J, Parkington, HC, Isbister, GK, Konstantakopoulos, N, Hodgson, WC (2013) Differential myotoxic and cytotoxic activities of pre-synaptic neurotoxins from Papuan taipan (*Oxyuranus scutellatus*) and Irian Jayan death adder (*Acanthophis rugosus*) venoms. *Basic Clin Pharmacol Toxicol* 112: 325-334.

Chang, CC, Lee, JD, Eaker, D, Fohlman, J (1977) The presynaptic neuromuscular blocking action of taipoxin. A comparison with β-bungarotoxin and crotoxin. *Toxicon* 15: 571-576.

Chang, CC and Su, MJ (1982) Presynaptic toxicity of the histidine-modified, phospholipase A_2-inactive, beta-bungarotoxin, crotoxin and notexin. *Toxicon* 20: 895-905.

Chang, CC and Tseng, KH (1978) Effect of crotamine, a toxin of South American rattlesnake venom, on the sodium channel of murine skeletal muscle. *Br J Pharmacol* 63: 551-559.

Chen, YH and Chu, ST (1988) Snake venom cardiotoxin induces G-actin polymerization. *Biochim Biophys Acta* 966: 266-268.

Chippaux, JP, Williams, V, White, J (1991) Snake venom variability: methods of study, results and interpretation. *Toxicon* 29: 1279-1303.

Cicala, C and Cirino, G (1993) Phospholipase A_2-induced hypotension in the rat and its pharmacological modulation. *Gen Pharmacol* 24: 1197-1202.

Clarke, C, Kuruppu, S, Reeve, S, Ian Smith, A, Hodgson, WC (2006) Oxylepitoxin-1, a reversible neurotoxin from the venom of the inland taipan (*Oxyuranus microlepidotus*). *Peptides* 27: 2655-2660.

Cole, SW and Lundquist, LM (2013) Icatibant for the treatment of hereditary angioedema. *Ann Pharmacother* 47: 49-55.

Coleman, RA, Smith, WL, Narumiya, S (1994) International Union of Pharmacology classification of prostanoid receptors: properties, distribution, and structure of the receptors and their subtypes. *Pharmacol Rev* 46: 205-229.

Coruzzi, G, Adami, M, Guaita, E, de Esch, IJP, Leurs, R (2007) Antiinflammatory and antinociceptive effects of the selective histamine H_4-receptor antagonists JNJ7777120 and VUF6002 in a rat model of carrageenan-induced acute inflammation. *European Journal of Pharmacology* 563: 240-244.

Crachi, MT, Hammer, LW, Hodgson, WC (1999a) The effects of antivenom on the in vitro neurotoxicity of venoms from the taipans *Oxyuranus scutellatus*, *Oxyuranus microlepidotus* and *Oxyuranus scutellatus canni*. *Toxicon* 37: 1771-1778.

Crachi, MT, Hammer, LW, Hodgson, WC (1999b) A pharmacological examination of venom from the Papuan taipan (*Oxyuranus scutellatus canni*). *Toxicon* 37: 1721-1734.

Cummins, TR, Dib-Hajj, SD, Black, JA, Akopian, AN, Wood, JN, Waxman, SG (1999) A novel persistent tetrodotoxin-resistant sodium current in SNS-null and wild-type small primary sensory neurons. *J Neurosci* 19: RC43.

Currie, BJ (2000) Snakebite in tropical Australia, Papua New Guinea and Irian Jaya. *Emergency Medicine* 12: 285-294.

Damerau, B, Lege, L, Oldigs, HD, Vogt, W (1975) Histamine release, formation of prostaglandin-like activity (SRS-C) and mast cell degranulation by the direct lytic factor (DLF) and phospholipase A of cobra venom. *Naunyn Schmiedebergs Arch Pharmacol* 287: 141-156.

Dattolo, L, Keller, EL, Carta, G (2010) pH transients in hydroxyapatite chromatography columns-Effects of operating conditions and media properties. *J Chromatogr A* 1217: 7573-7578.

Davie, EW (2003) A brief historical review of the waterfall/cascade of blood coagulation. *J Biol Chem* 278: 50819-50832.

de Oliveira, M, Cavalcante, WL, Arruda, EZ, Melo, PA, Dal-Pai Silva, M, Gallacci, M (2003) Antagonism of myotoxic and paralyzing activities of bothropstoxin-I by suramin. *Toxicon* 42: 373-379.

de Roodt, AR, Lago, NR, Stock, RP (2012) Myotoxicity and nephrotoxicity by *Micrurus* venoms in experimental envenomation. *Toxicon* 59: 356-364.

de Weille, JR, Schweitz, H, Maes, P, Tartar, A, Lazdunski, M (1991) Calciseptine, a peptide isolated from black mamba venom, is a specific blocker of the L-type calcium channel. *Proc Natl Acad Sci U S A* 88: 2437-2440.

Delaey, C, Boussery, K, Breyne, J, Vanheel, B, Van de Voorde, J (2007) The endothelium-derived hyperpolarising factor (EDHF) in isolated bovine choroidal arteries. *Exp Eye Res* 84: 1067-1073.

Dib-Hajj, SD, Black, JA, Cummins, TR, Kenney, AM, Kocsis, JD, Waxman, SG (1998a) Rescue of alpha-SNS sodium channel expression in small dorsal root ganglion neurons after axotomy by nerve growth factor in vivo. *J Neurophysiol* 79: 2668-2676.

Dib-Hajj, SD, Tyrrell, L, Black, JA, Waxman, SG (1998b) NaN, a novel voltage-gated Na channel, is expressed preferentially in peripheral sensory neurons and down-regulated after axotomy. *Proc Natl Acad Sci U S A* 95: 8963-8968.

Dixon, RW and Harris, JB (1996) Myotoxic activity of the toxic phospholipase, notexin, from the venom of the Australian tiger snake. *J Neuropathol Exp Neurol* 55: 1230-1237.

Doughty, P, Maryan, B, Donnellan, SC, Hutchinson, MN (2007) A new species of taipan (Elapidae: *Oxyuranus*) from central Australia. *Zootaxa* 1422: 45-58.

Duan, Y, Haugabook, SJ, Sahley, CL, Muller, KJ (2003) Methylene blue blocks cGMP production and disrupts directed migration of microglia to nerve lesions in the leech CNS. *J Neurobiol* 57: 183-192.

Dufton, MJ and Harvey, AL (1989) The long and the short of snake toxins. *Trends Pharmacol Sci* 10: 258-259.

Dutta, NK and Narayanan, KG (1952) Release of histamine from rat diaphragm by cobra venom. *Nature* 169: 1064-1065.

Dvir, H, Silman, I, Harel, M, Rosenberry, TL, Sussman, JL (2010) Acetylcholinesterase: from 3D structure to function. *Chem Biol Interact* 187: 10-22.

Edwards, JC and Kahl, CR (2010) Chloride channels of intracellular membranes. *FEBS Lett* 584: 2102-2111.

Endo, T and Tamiya, N (1991) *Structure-function relationships of postsynaptic neurotoxins from snake venom*. Pergamon Press: New York.

Ertel, EA, Campbell, KP, Harpold, MM, Hofmann, F, Mori, Y, Perez-Reyes, E, Schwartz, A, Snutch, TP, Tanabe, T, Birnbaumer, L, Tsien, RW, Catterall, WA (2000) Nomenclature of voltage-gated calcium channels. *Neuron* 25: 533-535.

Evangelista, IL, Martins, AM, Nascimento, NR, Havt, A, Evangelista, JS, de Noroes, TB, Toyama, MH, Diz-Filho, EB, Toyama Dde, O, Fonteles, MC, Monteiro, HS (2010) Renal and cardiovascular effects of *Bothrops marajoensis* venom and phospholipase A_2. *Toxicon* 55: 1061-1070.

Fathi, B, Harvey, AL, Rowan, EG (2011) Suramin inhibits the early effects of PLA_2 neurotoxins at mouse neuromuscular junctions: A twitch tension study. *J Venom Res* 2: 6-10.

Fathi, HB, Rowan, EG, Harvey, AL (2001) The facilitatory actions of snake venom phospholipase A_2 neurotoxins at the neuromuscular junction are not mediated through voltage-gated K^+ channels. *Toxicon* 39: 1871-1882.

Faure, G, Copic, A, Le Porrier, S, Gubensek, F, Bon, C, Krizaj, I (2003) Crotoxin acceptor protein isolated from Torpedo electric organ: binding properties to crotoxin by surface plasmon resonance. *Toxicon* 41: 509-517.

Ferreira, SH, Bartelt, DC, Greene, LJ (1970) Isolation of bradykinin-potentiating peptides from *Bothrops jararaca* venom. *Biochemistry* 9: 2583-2593.

Fletcher, JE and Lizzo, FH (1987) Contracture induction by snake venom cardiotoxin in skeletal muscle from humans and rats. *Toxicon* 25: 1003-1010.

Fletcher, JE, Tripolitis, L, Beech, J (1993) Species difference in modulation of calcium release by *Naja naja kaouthia* snake venom cardiotoxin in terminal cisternae from human and equine skeletal muscle. *Toxicon* 31: 43-51.

Fohlman, J (1979) Comparison of two highly toxic Australian snake venoms: The taipan (*Oxyuranus S. scutellatus*) and the fierce snake (*Parademansia microlepidotus*). *Toxicon* 17: 170-172.

Fohlman, J, Eaker, D, Dowdall, MJ, Lullmann-Rauch, R, Sjodin, T, Leander, S (1979) Chemical modification of taipoxin and the consequences for phospholipase activity, pathophysiology, and inhibition of high-affinity choline uptake. *Eur J Biochem* 94: 531-540.

Fohlman, J, Eaker, D, Karlsoon, E, Thesleff, S (1976) Taipoxin, an extremely potent presynaptic neurotoxin from the venom of the australian snake taipan (*Oxyuranus s. scutellatus*). Isolation, characterization, quaternary structure and pharmacological properties. *Eur J Biochem* 68: 457-469.

Fohlman, J, Lind, P, Eaker, D (1977) Taipoxin, an extremely potent presynaptic snake venom neurotoxin. Elucidation of the primary structure of the acidic carbohydrate-containing taipoxin-subunit, a prophospholipase homolog. *FEBS Lett* 84: 367-371.

Francis, B, Williams, ES, Seebart, C, Kaiser, II (1993) Proteins isolated from the venom of the common tiger snake (*Notechis scutatus scutatus*) promote hypotension and hemorrhage. *Toxicon* 31: 447-458.

Fry, BG (1999) Structure-function properties of venom components from Australian elapids. *Toxicon* 37: 11-32.

Fry, BG, Wickramaratana, JC, Lemme, S, Beuve, A, Garbers, D, Hodgson, WC, Alewood, P (2005) Novel natriuretic peptides from the venom of the inland taipan (*Oxyuranus microlepidotus*): isolation, chemical and biological characterisation. *Biochem Biophys Res Commun* 327: 1011-1015.

Furchgott, RF and Zawadzki, JV (1980) The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature* 288: 373-376.

Garcia, ML, Garcia-Calvo, M, Hidalgo, P, Lee, A, MacKinnon, R, 1994. Purification and characterization of three inhibitors of voltage-dependent K^+ channels from *Leiurus quinquestriatus var. hebraeus* venom. *Biochemistry* 33, 6834-6839.

Glusa, E and Pertz, HH (2000) Further evidence that 5-HT-induced relaxation of pig pulmonary artery is mediated by endothelial 5-HT_{2B} receptors. *Br J Pharmacol* 130: 692-698.

Glusa, E and Roos, A (1996) Endothelial 5-HT receptors mediate relaxation of porcine pulmonary arteries in response to ergotamine and dihydroergotamine. *Br J Pharmacol* 119: 330-334.

Goldin, AL (2001) Resurgence of sodium channel research. *Annu Rev Physiol* 63: 871-894.

Goldin, AL, Barchi, RL, Caldwell, JH, Hofmann, F, Howe, JR, Hunter, JC, Kallen, RG, Mandel, G, Meisler, MH, Netter, YB, Noda, M, Tamkun, MM, Waxman, SG, Wood, JN, Catterall, WA (2000) Nomenclature of voltage-gated sodium channels. *Neuron* 28: 365-368.

Gosling, M, Poll, C, Li, S (2005) TRP channels in airway smooth muscle as therapeutic targets. *Naunyn Schmiedebergs Arch Pharmacol* 371: 277-284.

Gunja, N, Ling, M, Dowsett, R, Murray, L, Isbister, GK (2007) Antivenom and neostigmine failure in death adder envenoming. *Clin Toxicol* 45: 389.

Gutierrez, JM, Williams, D, Fan, HW, Warrell, DA (2010) Snakebite envenoming from a global perspective: Towards an integrated approach. *Toxicon* 56: 1223-1235.

Han, S-y, Liang, C, Zou, K, Qiao, J-q, Lian, H-z, Ge, X (2012) Influence of variation in mobile phase pH and solute pKa with the change of organic modifier fraction on QSRRs of hydrophobicity and RP-HPLC retention of weakly acidic compounds. *Talanta* 101: 64-70.

Hansen, KB and Brauner-Osborne, H (2009) FLIPR assays of intracellular calcium in GPCR drug discovery. *Methods Mol Biol* 552: 269-278.

Harris, JB (1991) Phospholipases in snake venoms and their effects on nerve and muscle. In: Harvey, AL (ed). *Snake toxins*. Pergamon Press Inc: New York, pp 91-129.

Harris, JB and Maltin, CA (1982) Myotoxic activity of the crude venom and the principal neurotoxin, taipoxin, of the Australian taipan, *Oxyuranus scutellatus*. *Br J Pharmacol* 76: 61-75.

Hart, A, Scott-Davey, T, Harris, J (2008) Venom of Collett's snake (*Pseudechis colletti*) blocks the binding of alpha-bungarotoxin to acetylcholine receptors at chick but not human neuromuscular junctions: a histochemical study. *Toxicon* 52: 647-650.

Hart, AJ, Hodgson, WC, Scott-Davey, T, Harris, JB (2011) Neuromuscular toxicology of the venom of Collett's snake (*Pseudechis colletti*): a histopathological study. *Muscle Nerve* 43: 552-559.

Hart, AJ, Isbister, GK, Hodgson, WC (2013a) In vitro neurotoxic effects of *Pseudechis* spp. venoms: A comparison of avian and murine skeletal muscle preparations. *Toxicon* 63: 112-115.

Hart, AJ, Isbister, GK, O'Donnell, P, Williamson, NA, Hodgson, WC (2013b) Species differences in the neuromuscular activity of post-synaptic neurotoxins from two Australian black snakes (*Pseudechis porphyriacus* and *Pseudechis colletti*). *Toxicol Lett* 219: 262-268.

Hart, AJ, Smith, AI, Reeve, S, Hodgson, WC (2005) Isolation and characterisation of acanmyotoxin-2 and acanmyotoxin-3, myotoxins from the venom of the death adder *Acanthophis* sp. Seram. *Biochem Pharmacol* 70: 1807-1813.

He, WQ, Qiao, YN, Zhang, CH, Peng, YJ, Chen, C, Wang, P, Gao, YQ, Chen, X, Tao, T, Su, XH, Li, CJ, Kamm, KE, Stull, JT, Zhu, MS (2011) Role of myosin light chain kinase in regulation of basal blood pressure and maintenance of salt-induced hypertension. *Am J Physiol Heart Circ Physiol* 301: H584-591.

Hecker, M, Porsti, I, Bara, AT, Busse, R (1994) Potentiation by ACE inhibitors of the dilator response to bradykinin in the coronary microcirculation: interaction at the receptor level. *Br J Pharmacol* 111: 238-244.

Henning, RH, Nelemans, A, Scaf, AH, Van Eekeren, J, Agoston, S, Den Hertog, A (1992) Suramin reverses non-depolarizing neuromuscular blockade in rat diaphragm. *Eur J Pharmacol* 216: 73-79.

Herkert, M, Shakhman, O, Schweins, E, Becker, CM (2001) Beta-bungarotoxin is a potent inducer of apoptosis in cultured rat neurons by receptor-mediated internalization. *Eur J Neurosci* 14: 821-828.

Hiriart, M and Matteson, DR (1988) Na channels and two types of Ca channels in rat pancreatic B cells identified with the reverse hemolytic plaque assay. *J Gen Physiol* 91: 617-639.

Ho, C-L and Lee, CY (1981) Cardiovascular effects of phospholipases A_2 purified from various snake venoms. *Proc Natl Sci Counc, Repub. China* 5: 181-189.

Hodgson, WC, Dal Belo, CA, Rowan, EG (2007) The neuromuscular activity of paradoxin: a presynaptic neurotoxin from the venom of the inland taipan (*Oxyuranus microlepidotus*). *Neuropharmacology* 52: 1229-1236.

Hodgson, WC and Wickramaratna, JC (2002) In vitro neuromuscular activity of snake venoms. *Clin Exp Pharmacol Physiol* 29: 807-814.

Hodgson, WC and Wickramaratna, JC (2006) Snake venoms and their toxins: an Australian perspective. *Toxicon* 48: 931-940.

Huang, CJ, Schild, L, Moczydlowski, EG (2012) Use-dependent block of the voltage-gated Na^+ channel by tetrodotoxin and saxitoxin: effect of pore mutations that change ionic selectivity. *J Gen Physiol* 140: 435-454.

Huang, HC (1984) Effects of phospholipases A_2 from *Vipera russelli* snake venom on blood pressure, plasma prostacyclin level and renin activity in rats. *Toxicon* 22: 253-264.

Huang, HC and Lee, CY (1985) Relaxant effect of phospholipase A_2 from *Vipera russelli* snake venom on rat aorta. *Eur J Pharmacol* 118: 139-146.

Ichinose, A (2001) Physiopathology and regulation of factor XIII. *Thromb Haemost* 86: 57-65.

Inase, N, Schreck, RE, Lazarus, SC (1993) Heparin inhibits histamine release from canine mast cells. *Am J Physiol* 264: L387-390.

Isbister, GK (2010) Antivenom efficacy or effectiveness: the Australian experience. *Toxicology* 268: 148-154.

Isbister, GK, Halkidis, L, O'Leary, MA, Whitaker, R, Cullen, P, Mulcahy, R, Bonnin, R, Brown, SG (2010a) Human anti-snake venom IgG antibodies in a previously bitten snake-handler, but no protection against local envenoming. *Toxicon* 55: 646-649.

Isbister, GK, Hooper, MR, Dowsett, R, Maw, G, Murray, L, White, J (2006a) Collett's snake (*Pseudechis colletti*) envenoming in snake handlers. *QJM* 99: 109-115.

Isbister, GK, Scorgie, FE, O'Leary, MA, Seldon, M, Brown, SG, Lincz, LF (2010b) Factor deficiencies in venom-induced consumption coagulopathy resulting from Australian elapid envenomation: Australian Snakebite Project (ASP-10). *J Thromb Haemost* 8: 2504-2513.

Isbister, GK, Williams, V, Brown, SG, White, J, Currie, BJ (2006b) Clinically applicable laboratory end-points for treating snakebite coagulopathy. *Pathology* 38: 568-572.

Itoh, T, Seki, N, Suzuki, S, Ito, S, Kajikuri, J, Kuriyama, H (1992) Membrane hyperpolarization inhibits agonist-induced synthesis of inositol 1,4,5-trisphosphate in rabbit mesenteric artery. *J Physiol* 451: 307-328.

Jentsch, TJ, Stein, V, Weinreich, F, Zdebik, AA (2002) Molecular structure and physiological function of chloride channels. *Physiol Rev* 82: 503-568.

Jiang, M-S, Fletcher, JE, Smith, LA (1989) Effects of divalent cations on snake venom cardiotoxin-induced hemolysis and 3H-deoxyglucose-6-phosphate release from human red blood cells. *Toxicon* 27: 1297-1305.

Johns, DG, Ao, Z, Heidrich, BJ, Hunsberger, GE, Graham, T, Payne, L, Elshourbagy, N, Lu, Q, Aiyar, N, Douglas, SA (2007) *Dendroaspis* natriuretic peptide binds to the natriuretic peptide clearance receptor. *Biochem Biophys Res Commun* 358: 145-149.

Johnston, CI, O'Leary, MA, Brown, SG, Currie, BJ, Halkidis, L, Whitaker, R, Close, B, Isbister, GK (2012) Death Adder Envenoming Causes Neurotoxicity Not Reversed by Antivenom - Australian Snakebite Project (ASP-16). *PLoS Negl Trop Dis* 6: e1841.

Joseph, JS, Chung, MC, Jeyaseelan, K, Kini, RM (1999) Amino acid sequence of trocarin, a prothrombin activator from *Tropidechis carinatus* venom: its structural similarity to coagulation factor Xa. *Blood* 94: 621-631.

Joseph, R, Pahari, S, Hodgson, WC, Kini, RM (2004) Hypotensive agents from snake venoms. *Curr Drug Targets Cardiovasc Haematol Disord* 4: 437-459.

Kaczorowski, GJ and Garcia, ML (1999) Pharmacology of voltage-gated and calcium-activated potassium channels. *Curr Opin Chem Biol* 3: 448-458.

Kalam, Y, Isbister, GK, Mirtschin, P, Hodgson, WC, Konstantakopoulos, N (2010) Validation of a cell-based assay to differentiate between the cytotoxic effects of elapid snake venoms. *J Pharmacol Toxicol Methods* 63: 137-142.

Kallen, RG, Sheng, ZH, Yang, J, Chen, LQ, Rogart, RB, Barchi, RL (1990) Primary structure and expression of a sodium channel characteristic of denervated and immature rat skeletal muscle. *Neuron* 4: 233-242.

Kasturiratne, A, Wickremasinghe, AR, de Silva, N, Gunawardena, NK, Pathmeswaran, A, Premaratna, R, Savioli, L, Lalloo, DG, de Silva, HJ (2008) The global burden of snakebite: a literature analysis and modelling based on regional estimates of envenoming and deaths. *PLoS medicine* 5: e218.

Keating, DJ, Chen, C, Pritchard, MA (2006) Alzheimer's disease and endocytic dysfunction: clues from the Down syndrome-related proteins, DSCR1 and ITSN1. *Ageing Res Rev* 5: 388-401.

King, GF, Gentz, MC, Escoubas, P, Nicholson, GM (2008) A rational nomenclature for naming peptide toxins from spiders and other venomous animals. *Toxicon* 52: 264-276.

Kini, RM (2003) Excitement ahead: structure, function and mechanism of snake venom phospholipase A_2 enzymes. *Toxicon* 42: 827-840.

Kini, RM and Doley, R (2010) Structure, function and evolution of three-finger toxins: mini proteins with multiple targets. *Toxicon* 56: 855-867.

Kini, RM and Evans, HJ (1989) A model to explain the pharmacological effects of snake venom phospholipases A_2. *Toxicon* 27: 613-635.

Kirkpatrick, LL, Matzuk, MM, Dodds, DC, Perin, MS (2000) Biochemical interactions of the neuronal pentraxins. Neuronal pentraxin (NP) receptor binds to taipoxin and taipoxin-associated calcium-binding protein 49 via NP1 and NP2. *J Biol Chem* 275: 17786-17792.

Kochegarov, AA (2003) Pharmacological modulators of voltage-gated calcium channels and their therapeutical application. *Cell Calcium* 33: 145-162.

Koh, CY and Kini, RM (2012) From snake venom toxins to therapeutics-cardiovascular examples. *Toxicon* 59: 497-506.

Kornhauser, R, Hart, AJ, Reeve, S, Smith, AI, Fry, BG, Hodgson, WC (2010) Variations in the pharmacological profile of post-synaptic neurotoxins isolated from the venoms of the Papuan (*Oxyuranus scutellatus canni*) and coastal (*Oxyuranus scutellatus scutellatus*) taipans. *Neurotoxicology* 31: 239-243.

Kuruppu, S, Isbister, GK, Hodgson, WC (2005a) Phospholipase A_2-dependent effects of the venom from the New Guinean small-eyed snake *Micropechis ikaheka*. *Muscle Nerve* 32: 81-87.

Kuruppu, S, Reeve, S, Banerjee, Y, Kini, RM, Smith, AI, Hodgson, WC (2005b) Isolation and pharmacological characterization of cannitoxin, a presynaptic neurotoxin from the venom of the Papuan Taipan (*Oxyuranus scutellatus canni*). *J Pharmacol Exp Ther* 315: 1196-1202.

Kuruppu, S, Reeve, S, Smith, AI, Hodgson, WC (2005c) Isolation and pharmacological characterisation of papuantoxin-1, a postsynaptic neurotoxin from the venom of the Papuan black snake (*Pseudechis papuanus*). *Biochem Pharmacol* 70: 794-800.

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

Lalloo, DG, Trevett, AJ, Black, J, Mapao, J, Saweri, A, Naraqi, S, Owens, D, Kamiguti, AS, Hutton, RA, Theakston, RDG, Warrell, DA (1996) Neurotoxicity, anticoagulant activity and evidence of rhabdomyolysis in patients bitten by death adders (*Acanthophis* sp.) in Southern Papua New Guinea. *Toxicon* 34: 735-735.

Lalloo, DG, Trevett, AJ, Korinhona, A, Nwokolo, N, Laurenson, IF, Paul, M, Black, J, Naraqi, S, Mavo, B, Saweri, A, et al. (1995a) Snake bites by the Papuan taipan (*Oxyuranus scutellatus canni*): paralysis, hemostatic and electrocardiographic abnormalities, and effects of antivenom. *Am J Trop Med Hyg* 52: 525-531.

Lalloo, DG, Trevett, AJ, Nwokolo, N, Laurenson, IF, Naraqi, S, Kevau, I, Kemp, MW, James, R, Hooper, L, David, R, Theakston, G, Warrell, D (1997) Electrocardiographic abnormalities in patients bitten by taipans (*Oxyuranus scutellatus canni*) and other elapid snakes in Papua New Guinea. *Trans R Soc Trop Med Hyg* 91: 53-56.

Lalloo, DG, Trevett, AJ, Owens, D, Minei, J, Naraqi, S, Saweri, A, Hutton, RA, Theakston, RD, Warrell, DA (1995b) Coagulopathy following bites by the Papuan taipan (*Oxyuranus scutellatus canni*). *Blood Coagul Fibrinolysis* 6: 65-72.

Lambeau, G, Schmid-Alliana, A, Lazdunski, M, Barhanin, J (1990) Identification and purification of a very high affinity binding protein for toxic phospholipases A_2 in skeletal muscle. *J Biol Chem* 265: 9526-9532.

Landon, DN, Westgaard, RH, MacDermot, J, Thompson, EJ (1980) The morphology of rat soleus neuromuscular junctions treated *in vitro* with purified beta-bungarotoxin. *Brain Res* 202: 1-20.

Leist, M and Nicotera, P (1998) Calcium and neuronal death. *Rev Physiol Biochem Pharmacol* 132: 79-125.

Leonard, RJ, Garcia, ML, Slaughter, RS, Reuben, JP, 1992. Selective blockers of voltage-gated K^+ channels depolarize human T lymphocytes: mechanism of the antiproliferative effect of charybdotoxin. *Proc Natl Acad Sci* 89, 10094-10098.

Leung, AT, Imagawa, T, Campbell, KP (1987) Structural characterization of the 1,4-dihydropyridine receptor of the voltage-dependent Ca^{2+} channel from rabbit skeletal muscle. Evidence for two distinct high molecular weight subunits. *J Biol Chem* 262: 7943-7946.

Lewis, RJ, Dutertre, S, Vetter, I, Christie, MJ (2012) Conus venom peptide pharmacology. *Pharmacol Rev* 64: 259-298.

Lin, MJ, Tan, CT, Lee, SY, Lin-Shiau, SY (2000) Suramin protects the murine motor nerves from the toxic effects of presynaptic Ca^{2+} channel inhibitors. *Neurosci Lett* 287: 97-100.

Liu, M and Dilger, JP (2009) Site selectivity of competitive antagonists for the mouse adult muscle nicotinic acetylcholine receptor. *Mol Pharmacol* 75: 166-173.

Lomonte, B, Angulo, Y, Calderon, L (2003) An overview of lysine-49 phospholipase A_2 myotoxins from crotalid snake venoms and their structural determinants of myotoxic action. *Toxicon* 42: 885-901.

Lomonte, B and Rangel, J (2012) Snake venom Lys49 myotoxins: From phospholipases A_2 to non-enzymatic membrane disruptors. *Toxicon* 60: 520-530.

Luisi, R, Panza, E, Barrese, V, Iannotti, FA, Viggiano, D, Secondo, A, Canzoniero, LM, Martire, M, Annunziato, L, Taglialatela, M (2009) Activation of pre-synaptic M-type K^+ channels inhibits [3H]D-aspartate release by reducing Ca^{2+} entry through P/Q-type voltage-gated Ca^{2+} channels. *J Neurochem* 109: 168-181.

Marionneau, C, Lichti, CF, Lindenbaum, P, Charpentier, F, Nerbonne, JM, Townsend, RR, Merot, J (2012) Mass spectrometry-based identification of native cardiac Nav1.5 channel alpha subunit phosphorylation sites. *J Proteome Res* 11: 5994-6007.

Martins, SM, Wendling, T, Gonçalves, VMF, Sarmento, B, Ferreira, DC (2012) Development and validation of a simple reversed-phase HPLC method for the determination of camptothecin in animal organs following administration in solid lipid nanoparticles. *Journal of Chromatography B* 880: 100-107.

Mayrleitner, M, Timerman, AP, Wiederrecht, G, Fleischer, S (1994) The calcium release channel of sarcoplasmic reticulum is modulated by FK-506 binding protein: Effect of FKBP-12 on single channel activity of the skeletal muscle ryanodine receptor. *Cell Calcium* 15: 99-108.

McCue, JT, Cecchini, D, Hawkins, K, Dolinski, E (2007) Use of an alternative scale-down approach to predict and extend hydroxyapatite column lifetimes. *J Chromatogr A* 1165: 78-85.

McGain, F, Limbo, A, Williams, DJ, Didei, G, Winkel, KD (2004) Snakebite mortality at Port Moresby General Hospital, Papua New Guinea, 1992-2001. *Med J Aust* 181: 687-691.

McGuire, JJ, Ding, H, Triggle, CR (2001) Endothelium-derived relaxing factors: a focus on endothelium-derived hyperpolarizing factor(s). *Can J Physiol Pharmacol* 79: 443-470.

McIntosh, JM, Foderaro, TA, Li, W, Ireland, CM, Olivera, BM (1993) Presence of serotonin in the venom of *Conus imperialis*. *Toxicon* 31: 1561-1566.

McMichael, M (2012) New models of hemostasis. *Top Companion Anim Med* 27: 40-45.

Mehta, J, Mehta, P, Ostrowski, N (1983) Effect of different amounts of arachidonic acid on vessel wall-generated PGI_2 and TXA_2. *Prostaglandins Leukot Med* 12: 253-259.

Melo, PA, Burns, CF, Blankemeyer, JT, Ownby, CL (2004) Membrane depolarization is the initial action of crotoxin on isolated murine skeletal muscle. *Toxicon* 43: 111-119.

Mendelowitz, D, Reynolds, PJ, Andresen, MC (1995) Heterogeneous functional expression of calcium channels at sensory and synaptic regions in nodose neurons. *J Neurophysiol* 73: 872-875.

Menezes, MC, Furtado, MF, Travaglia-Cardoso, SR, Camargo, AC, Serrano, SM (2006) Sex-based individual variation of snake venom proteome among eighteen *Bothrops jararaca* siblings. *Toxicon* 47: 304-312.

Miller, C., 1995. The charybdotoxin family of K^+ channel-blocking peptides. Neuron 15, 5-10.

Mirtschin, P and Davis, R (1982) The snakes described. In: Pedler, H (ed). *Dangerous snakes of Australia*., Rigby Publishers Limited, Adelaide, pp 81-145.

Miyoshi, S, Kawata, K, Hosokawa, M, Tomochika, K, Shinoda, S (2003) Histamine-releasing reaction induced by the N-terminal domain of *Vibrio vulnificus* metalloprotease. *Life Sci* 72: 2235-2242.

Moncada, S, Radomski, MW, Palmer, RM (1988) Endothelium-derived relaxing factor. Identification as nitric oxide and role in the control of vascular tone and platelet function. *Biochem Pharmacol* 37: 2495-2501.

Montecucco, C and Rossetto, O (2000) How do presynaptic PLA_2 neurotoxins block nerve terminals? *Trends Biochem Sci* 25: 266-270.

Mora, R, Valverde, B, Diaz, C, Lomonte, B, Gutierrez, JM (2005) A Lys49 phospholipase A_2 homologue from *Bothrops asper* snake venom induces proliferation, apoptosis and necrosis in a lymphoblastoid cell line. *Toxicon* 45: 651-660.

Morecroft, I, Heeley, RP, Prentice, HM, Kirk, A, MacLean, MR (1999) 5-hydroxytryptamine receptors mediating contraction in human small muscular pulmonary arteries: importance of the 5-HT1B receptor. *Br J Pharmacol* 128: 730-734.

Morita, T, Iwanaga, S, Suzuki, T (1976) The mechanism of activation of bovine prothrombin by an activator isolated from *Echis carinatus* venon and characterization of the new active intermediates. *J Biochem* 79: 1089-1108.

Mukherjee, AK (2008) Characterization of a novel pro-coagulant metalloprotease (RVBCMP) possessing alpha-fibrinogenase and tissue haemorrhagic activity from venom of *Daboia russelli russelli* (Russell's viper): evidence of distinct coagulant and haemorrhagic sites in RVBCMP. *Toxicon* 51: 923-933.

Muller, F and Renne, T (2008) Novel roles for factor XII-driven plasma contact activation system. *Curr Opin Hematol* 15: 516-521.

Murakami, MT, Arruda, EZ, Melo, PA, Martinez, AB, Calil-Elias, S, Tomaz, MA, Lomonte, B, Gutierrez, JM, Arni, RK (2005) Inhibition of myotoxic activity of *Bothrops asper* myotoxin II by the anti-trypanosomal drug suramin. *J Mol Biol* 350: 416-426.

Myint, L, Warrell, DA, Phillips, RE, Tin Nu, S, Tun, P, Maung Maung, L (1985) Bites by Russell's viper (*Vipera russelli siamensis*) in Burma: haemostatic, vascular, and renal disturbances and response to treatment. *Lancet* 2: 1259-1264.

Nelson, MT, Patlak, JB, Worley, JF, Standen, NB (1990) Calcium channels, potassium channels, and voltage dependence of arterial smooth muscle tone. *Am J Physiol* 259: C3-18.

Nishimura, J and van Breemen, C (1989) Possible involvement of actomyosin ADP complex in regulation of Ca^{2+} sensitivity in alpha-toxin permeabilized smooth muscle. *Biochem Biophys Res Commun* 165: 408-415.

Nowycky, MC, Fox, AP, Tsien, RW (1985) Three types of neuronal calcium channel with different calcium agonist sensitivity. *Nature* 316: 440-443.

O'Leary, MA and Isbister, GK (2009) Commercial monovalent antivenoms in Australia are polyvalent. *Toxicon* 54: 192-195.

O'Shea, M (1996) *A Guide to the Snakes of Papua New Guinea*. Independent Group Pty Ltd.: Singapore, Singapore.

Oguiura, N, Boni-Mitake, M, Radis-Baptista, G (2005) New view on crotamine, a small basic polypeptide myotoxin from South American rattlesnake venom. *Toxicon* 46: 363-370.

Ownby, CL, Aird, SD, Kaiser, II (1988) Physiological and immunological properties of small myotoxins from the venom of the midget faded rattlesnake (*Crotalus viridis concolor*). *Toxicon* 26: 319-323.

Ownby, CL, Cameron, D, Tu, AT (1976) Isolation of myotoxic component from rattlesnake (*Crotalus viridis viridis*) venom. Electron microscopic analysis of muscle damage. *Am J Pathol* 85: 149-166.

Palouzier-Paulignan, B, Chamoin, MC, Ternaux, JP (1991) Choline uptake in cholinergic nodose cell bodies. *Neuroscience* 43: 687-696.

Parker, HW and Grandison, AGC (1977) *Snakes-A Natural History*. University of Queensland Press, Queensland.

Pasino, E, Buffelli, M, Arancio, O, Busetto, G, Salviati, A, Cangiano, A (1996) Effects of long-term conduction block on membrane properties of reinnervated and normally innervated rat skeletal muscle. *J Physiol* 497: 457-472.

Pedersen, SF, Owsianik, G, Nilius, B (2005) TRP channels: an overview. *Cell Calcium* 38: 233-252.

Penner, R and Dreyer, F (1986) Two different presynaptic calcium currents in mouse motor nerve terminals. *Pflugers Arch* 406: 190-197.

Penner, R, Petersen, M, Pierau, FK, Dreyer, F (1986) Dendrotoxin: a selective blocker of a non-inactivating potassium current in guinea-pig dorsal root ganglion neurones. *Pflugers Arch* 407: 365-369.

Petersen, M, Penner, R, Pierau, FK, Dreyer, F (1986) Beta-bungarotoxin inhibits a non-inactivating potassium current in guinea pig dorsal root ganglion neurones. *Neurosci Lett* 68: 141-145.

Ponraj, D and Gopalakrishnakone, P (1995) Morphological changes induced by a generalized myotoxin (myoglobinuria-inducing toxin) from the venom of *Pseudechis australis* (king brown snake) in skeletal muscle and kidney of mice. *Toxicon* 33: 1453-1467.

Prasarnpun, S, Walsh, J, Awad, SS, Harris, JB (2005) Envenoming bites by kraits: the biological basis of treatment-resistant neuromuscular paralysis. *Brain* 128: 2987-2996.

Queiroz, GP, Pessoa, LA, Portaro, FC, Furtado Mde, F, Tambourgi, DV (2008) Interspecific variation in venom composition and toxicity of Brazilian snakes from *Bothrops* genus. *Toxicon* 52: 842-851.

Radis-Baptista, G and Kerkis, I (2011) Crotamine, a small basic polypeptide myotoxin from rattlesnake venom with cell-penetrating properties. *Curr Pharm Des* 17: 4351-4361.

Ramasamy, S, Isbister, GK, Hodgson, WC (2004) The efficacy of two antivenoms against the in vitro myotoxic effects of black snake (*Pseudechis*) venoms in the chick biventer cervicis nerve-muscle preparation. *Toxicon* 44: 837-845.

Randall, A and Tsien, RW (1995) Pharmacological dissection of multiple types of Ca^{2+} channel currents in rat cerebellar granule neurons. *J Neurosci* 15: 2995-3012.

Rang, HP, Dale, MM, Ritter, JM (1999) The vascular system. In: Simmons, B (ed), 4th edn Pharmacology. Churchill Livingstone: Edinburgh, pp 278-290.

Rao, VS, Joseph, JS, Kini, RM (2003) Group D prothrombin activators from snake venom are structural homologues of mammalian blood coagulation factor Xa. *Biochem J* 369: 635-642.

Rao, VS and Kini, RM (2002) Pseutarin C, a prothrombin activator from *Pseudonaja textilis* venom: its structural and functional similarity to mammalian coagulation factor Xa-Va complex. *Thromb Haemost* 88: 611-619.

Renganathan, M, Dib-Hajj, S, Waxman, SG (2002) $Na_V1.5$ underlies the 'third TTX-R sodium current' in rat small DRG neurons. *Brain Res Mol Brain Res* 106: 70-82.

Rigoni, M, Pizzo, P, Schiavo, G, Weston, AE, Zatti, G, Caccin, P, Rossetto, O, Pozzan, T, Montecucco, C (2007) Calcium influx and mitochondrial alterations at synapses exposed to snake neurotoxins or their phospholipid hydrolysis products. *J Biol Chem* 282: 11238-11245.

Rizo, J and Sudhof, TC (2012) The membrane fusion enigma: SNAREs, Sec1/Munc18 proteins, and their accomplices-guilty as charged? *Annu Rev Cell Dev Biol* 28: 279-308.

Rizzo, MT, Nguyen, E, Aldo-Benson, M, Lambeau, G (2000) Secreted phospholipase A_2 induces vascular endothelial cell migration. *Blood* 96: 3809-3815.

Robertson, B (1997) The real life of voltage-gated K^+ channels: more than model behaviour. *Trends Pharmacol Sci* 18: 474-483.

Rosing, J and Tans, G (1992) Structural and functional properties of snake venom prothrombin activators. *Toxicon* 30: 1515-1527.

Rossetto, O, Morbiato, L, Caccin, P, Rigoni, M, Montecucco, C (2006) Presynaptic enzymatic neurotoxins. *J Neurochem* 97: 1534-1545.

Rowan, EG (2001) What does beta-bungarotoxin do at the neuromuscular junction? *Toxicon* 39: 107-118.

Roy, ML, Narahashi, T (1992) Differential properties of tetrodotoxin-sensitive and tetrodotoxin-resistant sodium channels in rat dorsal root ganglion neurons. *J Neurosci* 12: 2104-2111.

Sandow, SL (2004) Factors, fiction and endothelium-derived hyperpolarizing factor. *Clin Exp Pharmacol Physiol* 31: 563-570.

Satin, J, Kyle, JW, Chen, M, Rogart, RB, Fozzard, HA (1992) The cloned cardiac Na^+ channel alpha-subunit expressed in Xenopus oocytes show gating and blocking properties of native channels. *J Membr Biol* 130: 11-22.

Satin, J, Schroder, EA, Crump, SM (2011) L-type calcium channel auto-regulation of transcription. *Cell Calcium* 49: 306-313.

Schiffman, S, Theodor, I, Rapaport, SI (1969) Separation from Russell's viper venom of one fraction reacting with factor X and another reacting with factor V. *Biochemistry* 8: 1397-1405.

Schneggenburger, R, Han, Y, Kochubey, O (2012) Ca^{2+} channels and transmitter release at the active zone. *Cell Calcium* 52: 199-207.

Schweitz, H, Vigne, P, Moinier, D, Frelin, C, Lazdunski, M (1992) A new member of the natriuretic peptide family is present in the venom of the green mamba (*Dendroaspis angusticeps*). *J Biol Chem* 267: 13928-13932.

Seabrook, GR, Bowery, BJ, Hill, RG (1995) Bradykinin receptors in mouse and rat isolated superior cervical ganglia. *Br J Pharmacol* 115: 368-372.

Sebia-Amrane, F and Laraba-Djebari, F (2013) Pharmaco-modulations of induced edema and vascular permeability changes by *Vipera lebetina* venom: Inflammatory Mechanisms. *Inflammation* 36: 434-43.

Senthilkumaran, S, Meenakshisundaram, R, Thirumalaikolundusubramanian, P (2012) Cardiac tamponade in Russell viper (*Daboia russelii*) bite: case report with brief review. *J Emerg Med* 42: 288-290.

Shankarraman, V, Davis-Gorman, G, McDonagh, PF, Caplan, MR (2012) Intracellular signaling controls endothelial cell prostacyclin secretion and regulation of blood clotting time. *J Biomed Mater Res A* 100: 3374-3383.

Shimomura, T, Irie, K, Nagura, H, Imai, T, Fujiyoshi, Y (2011) Arrangement and mobility of the voltage sensor domain in prokaryotic voltage-gated sodium channels. *J Biol Chem* 286: 7409-7417.

Skidan, I, Grunwald, J, Thekkedath, R, Degterev, A, Torchilin, V (2011) A HPLC method for the quantitative determination of N-(2-hydroxy-5-nitrophenylcarbamothioyl)-3,5-dimethylbenzamide in biological samples. *Journal of Chromatography B* 879: 1610-1616.

Somlyo, AP and Somlyo, AV (1994) Signal transduction and regulation in smooth muscle. *Nature* 372: 231-236.

Sosnina, NA, Golubenko, Z, Akhunov, AA, Kugaevskaia, EV, Eliseeva Iu, E, Orekhovich, VN (1990) [Bradykinin-potentiating peptides from the spider *Latrodectus tredecimguttatus*-inhibitors of carboxycathepsin and of a preparation of karakurt venom kininase]. *Dokl Akad Nauk SSSR* 315: 236-239.

Speijer, H, Govers-Riemslag, JW, Zwaal, RF, Rosing, J (1986) Prothrombin activation by an activator from the venom of *Oxyuranus scutellatus* (Taipan snake). *J Biol Chem* 261: 13258-13267.

Stocker, K, Hauer, H, Muller, C, Triplett, DA (1994) Isolation and characterization of Textarin R, a prothrombin activator from eastern brown snake (*Pseudonaja textilis*) venom. *Toxicon* 32: 1227-1236.

Strydom, DJ (1977) Snake venom toxins. The amino-acid sequence of a short-neurotoxin homologue from *Dendroaspis polylepis polylepis* (black mamba) venom. *Eur J Biochem* 76: 99-106.

Su, MJ, Coulter, AR, Sutherland, SK, Chang, CC (1983) The presynaptic neuromuscular blocking effect and phospholipase A_2 activity of textilotoxin, a potent toxin isolated from the venom of the Australian brown snake, *Pseudonaja textilis*. *Toxicon* 21: 143-151.

Sutton, RB and Sprang, SR (1998) Structure of the protein kinase Cbeta phospholipid-binding C2 domain complexed with Ca^{2+}. *Structure* 6: 1395-1405.

Tan, LC, Kuruppu, S, Smith, AI, Reeve, S, Hodgson, WC (2006) Isolation and pharmacological characterisation of hostoxin-1, a postsynaptic neurotoxin from the venom of the Stephen's banded snake (*Hoplocephalus stephensi*). *Neuropharmacology* 51: 782-788.

Tanos, PP, Isbister, GK, Lalloo, DG, Kirkpatrick, CM, Duffull, SB (2008) A model for venom-induced consumptive coagulopathy in snake bite. *Toxicon* 52: 769-780.

Tans, G, Govers-Riemslag, JW, van Rijn, JL, Rosing, J (1985) Purification and properties of a prothrombin activator from the venom of *Notechis scutatus scutatus*. *J Biol Chem* 260: 9366-9372.

Tare, M, Parkington, HC, Coleman, HA, Neild, TO, Dusting, GJ (1990) Hyperpolarization and relaxation of arterial smooth muscle caused by nitric oxide derived from the endothelium. *Nature* 346: 69-71.

Tatalovic, M, Glazebrook, PA, Kunze, DL (2012) Expression of the P/Q ($Ca_V2.1$) calcium channel in nodose sensory neurons and arterial baroreceptors. *Neuroscience Letters* 520: 38-42.

Tedesco, E, Rigoni, M, Caccin, P, Grishin, E, Rossetto, O, Montecucco, C (2009) Calcium overload in nerve terminals of cultured neurons intoxicated by alpha-latrotoxin and snake PLA_2 neurotoxins. *Toxicon* 54: 138-144.

Thomas, CJ, Allen, AM, McAllen, RM, Woods, RL (2002) ANP potentiates nonarterial baroreflex bradycardia: evidence from sinoaortic denervation in rats. *Auton Neurosci* 97: 89-98.

Tibballs, J (1998) The cardiovascular, coagulation and haematological effects of tiger snake (*Notechis scutatus*) venom. *Anaesth Intensive Care* 26: 529-535.

Tibballs, J and Sutherland, S (1991) The efficacy of antivenom in prevention of cardiovascular depression and coagulopathy induced by brown snake (*Pseudonaja*) species venom. *Anaesth Intensive Care* 19: 530-534.

Tibballs, J, Sutherland, S, Kerr, S (1989) Studies on Australian snake venoms. Part 1: The haemodynamic effects of brown snake (*Pseudonaja*) species in the dog. *Anaesth Intensive Care* 17: 466-469.

Tibballs, J, Sutherland, SK, Kerr, S (1991) Studies on Australian snake venoms, Part II: The haematological effects of brown snake (*Pseudonaja*) species in the dog. *Anaesth Intensive Care* 19: 338-342.

Tibballs, J, Sutherland, SK, Rivera, RA, Masci, PP (1992) The cardiovascular and haematological effects of purified prothrombin activator from the common brown snake (*Pseudonaja textilis*) and their antagonism with heparin. *Anaesth Intensive Care* 20: 28-32.

Trakhtenberg, EF and Goldberg, JL (2012) The role of serotonin in axon and dendrite growth. *Int Rev Neurobiol* 106: 105-126.

Treppmann, P, Brunk, I, Afube, T, Richter, K, Ahnert-Hilger, G (2011) Neurotoxic phospholipases directly affect synaptic vesicle function. *J Neurochem* 117: 757-764.

Trevett, AJ, Lalloo, DG, Nwokolo, NC, Naraqi, S, Kevau, IH, Theakston, RD, Warrell, DA (1995) Electrophysiological findings in patients envenomed following the bite of a Papuan taipan (*Oxyuranus scutellatus canni*). *Trans R Soc Trop Med Hyg* 89: 415-417.

Trevisi, L, Bova, S, Cargnelli, G, Ceolotto, G, Luciani, S (2002) Endothelin-1-induced arachidonic acid release by cytosolic phospholipase A_2 activation in rat vascular smooth muscle via extracellular signal-regulated kinases pathway. *Biochem Pharmacol* 64: 425-431.

Tse, A and Hille, B (1993) Role of voltage-gated Na^+ and Ca^{2+} channels in gonadotropin-releasing hormone-induced membrane potential changes in identified rat gonadotropes. *Endocrinology* 132: 1475-1481.

Tseng, WP and Lin-Shiau, SY (2003a) Calcium-activated NO production plays a role in neuronal death induced by beta-bungarotoxin in primary cultures of cerebellar granular neurons. *Naunyn Schmiedebergs Arch Pharmacol* 367: 451-461.

Tseng, WP and Lin-Shiau, SY (2003b) Neuronal death signaling by beta-bungarotoxin through the activation of the N-methyl-D-aspartate (NMDA) receptor and L-type calcium channel. *Biochem Pharmacol* 65: 131-142.

Tzeng, WF and Chen, YH (1988) Suppression of snake-venom cardiotoxin-induced cardiomyocyte degeneration by blockage of Ca^{2+} influx or inhibition of non-lysosomal proteinases. *Biochem J* 256: 89-95.

Vargas, M, Segura, A, Herrera, M, Villalta, M, Estrada, R, Cerdas, M, Paiva, O, Matainaho, T, Jensen, SD, Winkel, KD, Leon, G, Gutierrez, JM, Williams, DJ (2011) Preclinical evaluation of caprylic acid-fractionated IgG antivenom for the treatment of Taipan (*Oxyuranus scutellatus*) envenoming in Papua New Guinea. *PLOS Negl Trop Dis* 5: e1144.

Verano-Braga, T, Rocha-Resende, C, Silva, DM, Ianzer, D, Martin-Eauclaire, MF, Bougis, PE, de Lima, ME, Santos, RA, Pimenta, AM (2008) *Tityus serrulatus* Hypotensins: a new family of peptides from scorpion venom. *Biochem Biophys Res Commun* 371: 515-520.

Verkhratsky, A (2007) Calcium and cell death. *Subcell Biochem* 45: 465-480.

Villalobos, JC, Mora, R, Lomonte, B, Gutierrez, JM, Angulo, Y (2007) Cytotoxicity induced in myotubes by a Lys49 phospholipase A_2 homologue from the venom of the snake *Bothrops asper*: evidence of rapid plasma membrane damage and a dual role for extracellular calcium. *Toxicol In Vitro* 21: 1382-1389.

Vink, S, Jin, AH, Poth, KJ, Head, GA, Alewood, PF (2012) Natriuretic peptide drug leads from snake venom. *Toxicon* 59: 434-445.

Volwerk, JJ, Pieterson, WA, de Haas, GH (1974) Histidine at the active site of phospholipase A_2. *Biochemistry* 13: 1446-1454.

Wang, JP and Teng, CM (1989) Roles of mast cells and PMN leukocytes in cardiotoxin-induced rat paw edema. *Eur J Pharmacol* 161: 9-18.

Wang, M-C, Dolphin, A, Kitmitto, A (2004) L-type voltage-gated calcium channels: understanding function through structure. *FEBS Letters* 564: 245-250.

Watanabe, TX, Itahara, Y, Kuroda, H, Chen, YN, Kimura, T, Sakakibara, S (1995) Smooth muscle relaxing and hypotensive activities of synthetic calciseptine and the homologous snake venom peptide FS2. *Jpn J Pharmacol* 68: 305-313.

Wei, JF, Mo, YZ, Qiao, LY, Wei, XL, Chen, HQ, Xie, H, Fu, YL, Wang, WY, Xiong, YL, He, SH (2006) Potent histamine-releasing activity of atrahagin, a novel snake venom metalloproteinase. *Int J Biochem Cell Biol* 38: 510-520.

Wei, JF, Wei, XL, Chen, QY, He, SH (2010) Induction of inflammatory cell accumulation by TM-N49 and promutoxin, two novel phospholipase A_2. *Toxicon* 56: 580-588.

White, J (1998) Envenoming and antivenom use in Australia. *Toxicon* 36: 1483-1492.

White, J, Edmonds, C, Zborowski, P (1998) Venomous snake identification. In: Torr, G (ed). *Australia's most dangerous spiders, snakes and marine creatures*. Australian geographic Pty Ltd: NSW, pp 23-53.

White, J (2005) Snake venoms and coagulopathy. *Toxicon* 45: 951-967.

White, J (1991) Snakebite: an Australian perspective. *Journal of Wilderness Medicine* 2: 219-244.

Wickramaratna, JC, Fry, BG, Aguilar, MI, Kini, RM, Hodgson, WC (2003a) 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.

Wickramaratna, JC, Fry, BG, Hodgson, WC (2003b) Species-dependent variations in the in vitro myotoxicity of death adder (*Acanthophis*) venoms. *Toxicol Sci* 74: 352-360.

Wickramaratna, JC, Fry, BG, Loiacono, RE, Aguilar, MI, Alewood, PF, Hodgson, WC (2004) Isolation and characterization at cholinergic nicotinic receptors of a neurotoxin from the venom of the *Acanthophis* sp. Seram death adder. *Biochem Pharmacol* 68: 383-394.

Winter, KL, Isbister, GK, Jacoby, T, Seymour, JE, Hodgson, WC (2009) An in vivo comparison of the efficacy of CSL box jellyfish antivenom with antibodies raised against nematocyst-derived *Chironex fleckeri* venom. *Toxicology letters* 187: 94-98.

Winter, KL, Isbister, GK, McGowan, S, Konstantakopoulos, N, Seymour, JE, Hodgson, WC (2010) A pharmacological and biochemical examination of the geographical variation of *Chironex fleckeri* venom. *Toxicol Lett* 192: 419-424.

Wuster, W, Dumbrell, AJ, Hay, C, Pook, CE, Williams, DJ, Fry, BG (2005) Snakes across the Strait: trans-Torresian phylogeographic relationships in three genera of Australasian snakes (Serpentes: Elapidae: *Acanthophis*, *Oxyuranus*, and *Pseudechis*). *Mol Phylogenet Evol* 34: 1-14.

Wyrembek, P, Lebida, K, Mercik, K, Szczuraszek, K, Szczot, M, Pollastro, F, Appendino, G, Mozrzymas, JW (2010) Block and allosteric modulation of GABAergic currents by oenanthotoxin in rat cultured hippocampal neurons. *Br J Pharmacol* 160: 1302-1315.

Yamada, D, Sekiya, F, Morita, T (1996) Isolation and characterization of carinactivase, a novel prothrombin activator in *Echis carinatus* venom with a unique catalytic mechanism. *J Biol Chem* 271: 5200-5207.

Yamashita, T (2012) Ca^{2+}-dependent regulation of synaptic vesicle endocytosis. *Neurosci Res* 73: 1-7.

Yang, CC and King, K (1980) Chemical modification of the histidine residue in basic phospholipase A_2 from the venom of *Naja nigricollis*. *Biochim Biophys Acta* 614: 373-388.

Yang, JS, Sladky, JT, Kallen, RG, Barchi, RL (1991) TTX-sensitive and TTX-insensitive sodium channel mRNA transcripts are independently regulated in adult skeletal muscle after denervation. *Neuron* 7: 421-427.

Yoon, J, Ben-Ami, HC, Hong, YS, Park, S, Strong, LL, Bowman, J, Geng, C, Baek, K, Minke, B, Pak, WL (2000) Novel mechanism of massive photoreceptor degeneration caused by mutations in the trp gene of *Drosophila*. *J Neurosci* 20: 649-659.

Zamorano, B, Bruzzone, ME, Martinez, JL (1995) Vascular smooth muscle reactivity to norepinephrine in ovariectomized rats: Relationship to vascular PGE_2/PGF_2 ratio. *General Pharmacology: The Vascular System* 26: 1613-1618.

Zhou, W, Lu, T, Spector, AA, Lee, H-C (2006) Inhibition of PGI_2 signaling by miconazole in vascular smooth muscle cells. *Prostaglandins & Other Lipid Mediators* 80: 28-34.