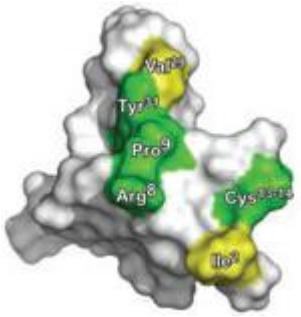
Interactions of insecticidal spider peptide neurotoxins with insect voltage- and neurotransmitter-gated ion channels



(Molecular representation of κ-HXTX-Hv1c including key binding residues, adapted from Gunning et al, 2008)

PhD Thesis

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UTS 2012

CERTIFICATE OF AUTHORSHIP/ORIGINALITY

I certify that the work in this thesis has not previously been submitted for a degree nor has it been submitted as part of requirements for a degree except as fully acknowledged within the text.

I also certify that the thesis has been written by me. Any help that I have received in my research work and the preparation of the thesis itself has been acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

Monique J. Windley
2012

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Abstract

Two families of peptide neurotoxins that target insect large-conductance calcium-activated potassium channels (BK_{Ca}) have been isolated from the venom of two unrelated spiders. The κ-TRTX-Ec2 toxins are a family of three homologous peptides isolated from the African tarantula, *Eucratoscelus longiceps* and κ-HXTX-Hv1c is the prototypic member of a family of insect-selective neurotoxins isolated from the venom of the Blue Mountains funnel-web spider, *Hadronyche versuta*. This thesis describes the characterisation of these insecticidal toxins using voltage-clamp and current-clamp analysis of cockroach dorsal unpaired neurons utilising the whole-cell patch-clamp technique. The ability of these toxins to modulate the gating and kinetics of both voltage- and neurotransmitter-gated ion channels were assessed. Insect bioassays were also utilised to validate the insecticidal activities of various toxins that target K_V channel subtypes in house crickets.

The κ-TRTX-Ec2 family of toxins were found to be high affinity blockers of the insect BK_{Ca} channel while failing to modify voltage-gated sodium (Na_V) and calcium (Ca_V) channels. κ-TRTX-Ec2a, -Ec2b and -Ec2c block cockroach BK_{Ca} channels with IC_{50} values of 3.7, 25.3 and 24.6 nM, respectively. Additionally, κ-TRTX-Ec2a was found to inhibit delayed-rectifier K_V channel currents ($I_{K(DR)}$), but only at significantly higher concentrations. κ-TRTX-Ec2 toxins induced voltage-independent channel block and are thus proposed to interact with the turret and/or loop region of the external vestibule of the insect BK_{Ca} channel.

κ-HXTX-Hv1c has also been characterised to block the insect BK_{Ca} channel, while failing to modulate insect Na_V and Ca_V channels. The unique insect-selective action of κ-HXTX-Hv1c involves a rare vicinal disulphide ring (Cys13-Cys14) that has been determined to act as part of the bioactive surface (pharmacophore) interacting with the molecular recognition site on the insect BK_{Ca} channel. However, despite the high affinity and selectivity for the BK_{Ca} channel it was discovered that the BK_{Ca} channel is unlikely to be the lethal target of κ-HXTX-Hv1c. Acute toxicity tests of classical non-phylum selective BK_{Ca} blockers such as paxilline, charybdotoxin and iberiotoxin did not induce acute toxicity in insects. Furthermore, while κ-HXTX-Hv1c was found to prolong action potential repolarisation, increase spontaneous firing frequency and reduce spike afterhyperpolarisation, these results were markedly reduced in the presence of the BK_{Ca} channel blocker iberiotoxin.

Subsequent testing of cockroach K_V channel currents revealed that κ-HXTX-Hv1c failed to modify sodium-activated or delayed-rectifier K_V channel currents, but 1 μM κ-HXTX-Hv1c did produce a 29% block of 'A-type' fast-transient K_V channel currents ($I_{K(A)}$). This suggests that κ-HXTX-Hv1c additionally targets insect K_V1- or K_V4-like channel subtypes. The lethal insecticidal action of 4-AP in crickets further supports an action of κ-HXTX-Hv1c to block $I_{K(A)}$. The results of co-application experiments revealed that κ-HXTX-Hv1c blocks the same channel as the non-phylum selective vertebrate K_V4 channel toxin, κ-sparatoxin-Hv1b. However, it was found that κ-sparatoxin-Hv1b, either alone or in combination with iberiotoxin, was not insecticidal and thus the K_V4 and BK_{Ca} channels are unlikely to be the lethal targets of κ-HXTX-Hv1c. To determine if the lethal target was a neurotransmitter-gated ion channel, the effects of κ-HXTX-Hv1c were investigated on chloride-gated $GABA_A$ (GABA-Cl) and glutamate (Glu-Cl) channel currents and nAChR channel currents. It was revealed that 1 μM κ-HXTX-Hv1c failed to modify $GABA_A$ channel currents while causing only a moderate 21% increase in Glu-Cl channel currents. Alternately, it was found that κ-HXTX-Hv1c caused a concentration-dependent (EC_{50} 183 nM) slowing of nicotinic acetylcholine receptor (nAChR) channel current decay and reversed channel desensitisation. In addition, κ-HXTX-Hv1c moderately increased nAChR sensitivity to nicotine. These findings are consistent with a positive allosteric modulation of insect nAChRs to slow receptor desensitisation. The nAChR is a validated insecticidal target for various agrochemical insecticides, including the allosteric modulator spinosyn A. Therefore it is believed that the lethal target of κ-HXTX-Hv1c is the insect nAChR, whose modulation would lead to an increase in neurotransmission consistent with the excitotoxic phenotype of the toxin. This action is possibly augmented by additional actions on BK_{Ca} and K_V4 like channels to increase neuronal excitability.

Abbreviations

4-AP	4-aminopyridine
α-BgTx	α-bungarotoxin
ACh	acetylcholine
AcNPV	*Autographa californica* nuclear polyhedrosis virus
AHP	afterhyperpolarisation
AP	action potential
ASICs	acid-sensing ion channels
ATP	adenosine tri-phosphate
BK_{Ca} channel	large-conductance Ca^{2+} and voltage-activated K^+ channel ($K_{Ca}1.1$, Maxi-K, BK, Slo1)
BSA	bovine albumin serum
Ca_V channel	voltage-activated Ca^{2+} channel
ChTx	charybdotoxin
DDH	disulfide-directed β-hairpin
DSE	dihedral strain energy
DUM	dorsal unpaired median
dSlo	*Drosophila* Slo-poke potassium channel
EC_{50}	median effective dose
EDTA	2,2',2",2"'-(Ethane-1,2-diyldinitrilo)tetraacetic acid
EGTA	ethylene glycol-bis(2-aminoethyl ether)-*N*,*N*,*N*'*N*'-tetraacetic acid

ESI-QTOF	electrospray ionization quadrupoletime-of-flight mass spectrometry
FBS	fetal bovine serum
GABA-Cl channel	γ-aminobutyric acid-activated chloride channel
Glu-Cl channel	glutamate-activated chloride channel
GNA	*Galanthus nivalis* agglutinin
GSH	glutathione
HEPES	N-hydroxyethylpiperazine-N-ethanesulfonic acid
hSlo	human Slo-poke potassium channel
HVA	high-voltage-activated
HXTX	hexatoxin (from the venom of spiders belonging to the family Hexathelidae)
IbTx	iberiotoxin
IC_{50}	median inhibitory concentration
ICK	inhibitory cystine knot
IK_{Ca}	intermediate-conductance Ca^{2+}-activated K^+ channel ($K_{Ca}3.1$, $IK_{Ca}1$)
$I_{BK(Ca)}$	Ca^{2+}-activated K^+ channel current
I_{Ca}	voltage-activated Ca^{2+} channel current
I_K	voltage-activated K^+ channel current
$I_{K(A)}$	transient ‘A-type’ K^+ current
$I_{K(DR)}$	delayed-rectifier K^+ current
$I_{K(Na)}$	Na^{2+}-activated K^+ channel current
I_{Glu-Cl}	glutamate-activated chloride current

$I_{GABA\text{-}Cl}$	γ-aminobutyric acid-activated chloride current
I_{Na}	voltage-activated Na^+ channel current
I_{nAChR}	nicotinic-acetylcholine receptor current
α-KTx	potassium channel scorpion toxin
KD_{50}	median knockdown dose
K_V channel	voltage-activated K^+ channel
LD_{50}	median lethal dose
LIT	latroinsectotoxin (from the venom of spiders belonging to the genus *Latrodectus*)
LJP	liquid junction potential
MALDI-TOF	matrix-assisted laser desorption/ionization time-of-flight
MAMPs	membrane-acting antimicrobial peptides
M-LVA	mid- to low-voltage-activated
MOPS	3-morpholinopropane-1-sulfonic acid
MSCs	mechanosensitive ion channels
mSlo	*Mus musculus* Slo-poke potassium channel
nAChD	desensitising nicotinic-acetylcholine channel current
nAChN	non-desensitising nicotinic-acetylcholine channel current
nAChR	nicotinic-acetylcholine receptor
Na_V channel	voltage-activated Na^+ channel
NIS	normal insect saline
NMR	nuclear magnetic resonance

PAMs	positive allosteric modulators
PDB	protein data base
pSlo	*Periplaneta* Slo-poke potassium channel
rp-HPLC	reversed phase high performance liquid chromatography
Sec	selenocysteine
SK_{Ca} channel	small-conductance Ca^{2+}-activated K^+ channel ($K_{Ca}2.x$)
SPRTX	sparatoxin (from the venom of spiders belonging to the family Sparassidae)
TAG	terminal abdominal ganglia
TEA	tetraethylammonium
TFA	2,2,2-trifluoroacetic acid
TRP	transient receptor potential
TRTX	theraphotoxin (from the venom of spiders belonging to the family Theraphosidae)
TTX	tetrodotoxin
VDR	vicinal disulfide ring
V_h	holding potential
$V_{1/2}$	voltage at half-maximal activation
V_{rev}	reversal potential

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Chapter One

Introduction: Spider venom peptides as bioinsecticides

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[2012]

1. 1 THE GLOBAL INSECT PEST PROBLEM

1.1.1 AGRICULTURAL PESTS

Arthropods are the most successful and diverse group of animals, with an estimated 2.8–10 million global species (Ødegaard, 2000). While only around 10,000 species are recognised as crop pests, approximately 14% of global crop loss and 20% of damage to stored food grains are due to insects (Oerke and Dehne, 2004; Pimentel, 2009). This results in an estimated US$100 billion in damage each year (Carlini and Grossi-de-Sá, 2002). Phytophagous (plant-eating) arthropods are the major cause of this crop loss. These include insect species from the Orders Coleoptera (beetles), Orthoptera (locusts and grasshoppers) and Lepidoptera (moths and butterflies) (Novotny, Basset et al., 2002). While the larval forms of lepidopterans are considered the most destructive (McCaffery, 1998), with 40% of chemical insecticides directed against heliothines (Brooks and Hines, 1999), insect species from the Order Diptera (true flies), Hemiptera (true bugs), Thysanoptera (thrips) and Acarina (mites) are also recognised as important crop pests (McCaffery, 1998; Nicholson, 2007a). The crop loss caused by insect pest damage diminishes our ability to meet the ever-increasing demand for food production to sustain the world's population, which is expected to grow from ~7 billion to around 9.31 billion people in the next 40 years (U.N. Department of Economic & Social Affairs; http://esa.un.org/unpd/wpp/unpp/panel_population.htm).

1.1.2 VECTORS OF DISEASE

A number of arthropod pests act as disease vectors for the transmission of infectious diseases of human and veterinary health importance (Nauen, 2007). In particular, insects belonging to the Order Diptera, such as mosquitoes, midges and flies are major disease vectors (Gratz, 1999; Gubler, 2002; Hall and Gerhardt, 2009). Hematophagous (blood-sucking) dipterans are collectively responsible for a wide variety of infections known to cause human morbidity and mortality, including malaria, dengue fever, West Nile virus, yellow fever, filariasis, leishmaniasis, Japanese encephalitis and African trypanosomiasis (Gratz, 1999; Hall and Gerhardt, 2009). Other disease vectors include ticks, fleas, lice and triatomid bugs (Lounibos, 2002), which are responsible for the transmission of infectious diseases such as Lyme disease, ehrlichiosis, various rickettsioses, Rocky mountain spotted fever, tularemia, bubonic plague, Chagas disease

and *Bartonella* (Brogdon and McAllister, 1998; Gratz, 1999; Gayle and Ringdahl, 2001; Gubler, 2002; Billeter, Levy et al., 2008; Schofield and Kabayo, 2008). Newly emerging diseases, such as onchocerciasis, Barmah Forest virus, Japanese spotted fever and dengue-dengue hemorrhagic fever are also vectored by arthropods (Gratz, 1999). Of these infectious diseases, malaria best exemplifies the need for insect pest control due to the fact that 3.3 billion people—almost 35% of the world population—live in areas at risk of transmission (Centers for Disease Control and Prevention, 2010). There were 216 million cases of malaria in 2010, resulting in a death every minute, most of whom were children under the age of 5 (World Health Organization, 2010). Treatment is available, yet the infection still accounts for 20% of all childhood deaths in Africa (World Health Organization, 2008).

Arthropod-mediated viral, rickettsial, bacterial and protozoan diseases pose not only a threat to human health, but also have consequences for global food production. Poultry and livestock diseases such as African swine fever, Akabane disease, bovine ephemeral fever, equine encephalitis, blue tongue fever and epizootic hemorrhagic fever all have the potential to compromise animal health. Symptoms range from lameness, blindness, wasting, congenital defects, spontaneous abortion and sterility to death, with infected livestock often being destroyed (Committee on Foreign Animal Diseases of the United States Animal Health Association, 1998). Currently, arthropod pest control and eradication programs rely on synthetic chemical insecticides as a means of reducing, if not eliminating, the prevalence of these debilitating diseases in humans and animals.1.2 Agrochemical insecticides: current challenges to insect pest control

Chemical insecticides were first introduced in the 1940s and they remain the major method for controlling insect pests. Chemical insecticides were seen as promising tools for insect control with the remarkable success of DDT in malaria eradication programs (Casida and Quistad, 1998; Attaran, Roberts et al., 2000). Organophosphates were then introduced in the 1960s (Casida and Quistad, 1998). The widespread use of organophosphates and other chemical insecticides in agriculture and malaria eradication programs provided a quick and relatively cheap solution to the growing insect pest problem. However, major problems with the use of agrochemicals have arisen including (i) a lack of phyletic specificity resulting in human health and environmental impacts,

and (ii) a lack of diversity in the bioactivity of these compounds leading to insecticide resistance.

1.2 HEALTH CONSEQUENCES AND ENVIRONMENTAL IMPACTS

To provide effective and safe insect control it is important that insecticides act with high affinity only at specific sites within the target invertebrate. Unfortunately, the majority of current agrochemicals act on targets conserved across insects and non-target organisms, including humans. As a consequence, acute toxicity is well documented in both animal models and humans. For example, in the developing world, over 250,000 people die each year from suicide and deliberate self-harm using insecticides and other pesticides (Gunnell and Eddleston, 2003; Gunnell, Eddleston et al., 2007). These deaths are responsible for about a third of suicides globally (Gunnell, Eddleston et al., 2007) and the World Health Organization (WHO) now recognizes pesticide poisoning to be the single most important means of suicide worldwide (Bertolote, Fleischmann et al., 2006).

The effects of chronic exposure to residual chemical pesticides, however, remain controversial (Pimentel, 2005). Epidemiological studies have purported to show a link between exposure to chemical pesticides and the development of cancers including pancreatic cancer, multiple myeloma, leukaemia, ovarian cancer and prostate cancer (for a review see ref. (Eriksson, 1997)). However, the evidence is not substantial and currently only arsenic-containing insecticides are considered carcinogenic, while others are only suspected of carcinogenicity (Alavanja, Hoppin et al., 2004). There are also possible links between chronic pesticide exposure and congenital defects (Eriksson, 1997; Barone, Das et al., 2000), preterm birth (Longnecker, Klebanoff et al., 2001), Parkinson's disease (Semchuk, Love et al., 1992; Gorell, Johnson et al., 1998; Betarbet, Sherer et al., 2000; Priyadarshi A., Khuder S. A. et al., 2000; Sherer, Kim et al., 2003), and neuropsychological dysfunctions (for a review see (Kamel and Hoppin, 2004)).

Adverse environmental effects are also of concern. Due to the indiscriminate actions of some agrochemical insecticides, beneficial insects (e.g., pollinators such as bees and butterflies), birds, aquatic invertebrates and fish can also succumb to the toxic effects of these agents either through direct, or indirect, exposure in the form of spray drift, run-off or leaching (Schuler, Denholm et al., 2001; Pain, Gargi et al., 2004; Van

Wijngaarden, Brock et al., 2005). Some agrochemicals also persist in the environment, with insecticides such as DDT highlighting the deleterious effects of bioaccumulation. These environmental problems along with human health concerns have seen the de-registration or use-cancellation of 169 insecticides between January 2005 and December 2009, with only 9 new insecticides registered during the same period (Dale Kemery, U.S. EPA, pers. comm.).

1.2.1 INSECTICIDE RESISTANCE

The vast majority of agrochemicals act on a single target within the insect nervous system. Indeed, chemical insecticides interact with just one of five main targets—voltage gated sodium (Na_V) channels, glutamate receptors, γ-aminobutyric acid (GABA) receptors, nicotinic acetylcholine receptors and acetylcholinesterases (Casida, 2009)—although a new class of insecticides have recently been developed that target the ryanodine receptor (Sattelle, Cordova et al., 2008). As a result, the use of agrochemicals with so few targets has promoted the evolution of resistance to a number of insecticide families (Feyereisen, 1995; Brogdon and McAllister, 1998). There are multiple ways that this insecticide resistance can arise: (i) increased metabolic detoxification, (ii) decreased target sensitivity, and/or (iii) increased sequestration or lowered insecticide availability (Feyereisen, 1995; Brogdon and McAllister, 1998). The molecular mechanisms responsible for these increases in resistance include point mutations in the ion channel of the GABA receptor or Na_V channel, mutations in the active site of acetylcholinesterase, amplification of esterase genes, and mutations causing up-regulation of detoxification enzymes (Feyereisen, 1995; Brogdon and McAllister, 1998; Hemingway and Ranson, 2000; Hemingway, Hawkes et al., 2004). Unfortunately, resistance has now arisen in almost all insect vector species (Georghiou, 1990; World Health Organisation, 1992). In particular, increases in the number of surviving insect vectors following treatment with insecticides is predicted to directly influence the resurgence (Krogstad, 1996), or challenge the management, of vector-borne diseases (World Health Organisation, 1992).

These problems indicate the need to identify new and safe insecticidal lead compounds, validate novel insecticidal targets and develop alternate methods of effective insect control. Therefore, it is crucial that we identify novel insecticides that can exploit subtle

differences in targets that are conserved between insects and vertebrates, or agents that target structures only found in insects.

1.3 BIOINSECTICIDES AS NATURAL INSECT PEST CONTROL AGENTS

Bioinsecticides are being investigated as potentially more efficacious and safer alternatives to chemical insecticides. Bioinsecticides are natural organisms, or their metabolic products, that can be employed for the control of insect pests. Potential sources of biopesticides include microbes (viral, fungal, bacterial), entomophagous nematodes, plant-derived products, insect pheromones and insect resistance genes expressed in crops (for a review see ref. (Copping and Menn, 2000)). In particular, insecticidal toxins derived from insect predators and parasitoids are of growing interest in the development of bioinsecticides, and these include peptide neurotoxins derived from the venoms of scorpions (Froy, Zilberberg et al., 2000), parasitic wasps (Quistad and Skinner, 1994), the straw itch mite (Tomalski, Bruce et al., 1988), and spiders (King, 2007; Nicholson, 2007b). Currently, there is a great deal of interest in spider venoms as they comprise an extensive library of potent insecticidal, neurotoxic peptides

The development of bioinsecticides has been pursued with the view of assuaging concerns associated with the use chemical insecticides in regards to persistence within the environment, broad activities across non-target organisms and to subsequently provide new alternatives for insecticide-resistant pest insects (Nauen and Bretschneider, 2002). Furthermore, bioinsecticides have shown promise in improving the efficacy of current pest management programs, and in some cases have exhibited synergism with existing pest management techniques (Wratten, 2009).

1.4 SPIDER VENOMS: SOURCES OF NOVEL BIOINSECTICIDES

Spiders are ancient creatures that evolved from an arachnid ancestor around 300 million years ago during the Carboniferous period. This highlights the long evolutionary timescale over which spiders have evolved their complex venom. Spiders are the most speciose venomous animals and along with predatory beetles are the most successful terrestrial predators, with over 42,000 extant species described to date (Platnick, 2011). This may be an under-representation of their true speciation, with about four times as many species predicted to exist, but not yet characterised (Coddington and Levi, 1991). One of the major features contributing to the overall success of spiders is the production

of highly toxic venom from their venom glands that they employ to subdue prey and deter predators. Since they rely completely on predation as a trophic strategy, spiders have evolved a complex pre-optimized combinatorial library of enzymes, neurotoxins and cytolytic compounds in their venom glands (Rash and Hodgson, 2002; Escoubas and Rash, 2004; Tedford, Sollod et al., 2004; Sollod, Wilson et al., 2005; Estrada, Garcia et al., 2007; Escoubas, Quinton et al., 2008; Vassilevski, Kozlov et al., 2009; Kuhn-Nentwig, Stöcklin et al., 2011). These venom components fall into three classes delineated by their molecular mass: (i) low molecular mass acylpolyamines and other nonpeptidic molecules (<1 kDa), (ii) disulfide-rich neurotoxins and linear cytolytic peptides (1–10 kDa), and (iii) high molecular mass proteins (>30 kDa) comprising mainly enzymes and neurotoxins. Most spider venoms are dominated by small disulfide-rich peptide neurotoxins (Figure1.1B), and these are the largest and most extensively studied group of spider toxins.

To date, around 800 peptide toxins from 78 spider species have been described in ArachnoServer (Herzig, Wood et al., 2011). These toxins were isolated from the venom of 20 of the 110 extant spider families, including representatives from the two major infraorders Araneomorphae ("modern" spiders) and Mygalomorphae ("primitive" spiders). Araneomorphs represent >90% of all known spider species, however, mygalomorphs are a more sustainable and convenient source of venom due to their large venom glands and their longevity (they can live for over 25 years). In recent years, it has become clear that spider venoms are considerably more complex than previously appreciated, with some venoms containing more than 1000 distinct peptides (Escoubas, Sollod et al., 2006). If one uses a conservative estimate of 100,000 species and 200 peptides per venom, then spider venoms may contain upwards of 10 million bioactive peptides (Escoubas and King, 2009). Less than 0.01% of this proteomic diversity has been tapped to date.

Spiders utilize their venoms to paralyse and/or kill prey or predators as rapidly as possible. Therefore their venoms are particularly rich in neurotoxins that rapidly modify ion conductance (ion channel toxins), and to a lesser extent affect neurotransmitter exocytosis (presynaptic toxins). However, like scorpion toxins, they appear to lack significant numbers of postsynaptic toxins that block the action of neurotransmitters, which are particularly common in the venom of snakes and, to a lesser extent, marine

cone snails. Many of these spider peptide toxins are selectively insecticidal. In particular, insect-selective toxins have been patented for their possible use as bioinsecticidal agents for the control of phytophagous pests or insect vectors (King, Sollod McFarland et al., 2005). The focus of this chapter is the discovery, processing, structure, and function of insecticidal spider venom peptides. In particular, it will detail the site and mechanism of their action, the molecular determinants for their pharmacology, and discuss the application of these peptides in the development of novel bioinsecticides.

1.5 SPIDER VENOM PEPTIDE NOMENCLATURE

Recently there has been an exponential increase in the number of spider toxins that have been reported in the literature (King, Gentz et al., 2008). This has resulted from the advent of modern high-throughput analytical techniques involving proteomic, transcriptomic and genomic approaches. As a result, a rational nomenclature system based on a Greek letter 'activity prefix' together with a toxin name based on the family, genus and species of the spider has been recently proposed (King, Gentz et al., 2008). This nomenclature has been adopted by UniProtKB and ArachnoServer (www.arachnoserver.org), a curated database containing available information on spider venom peptides and proteins (Wood, Miljenovic et al., 2009; Herzig, Wood et al., 2011). This chapter will employ this new nomenclature to facilitate identification of orthologs and paralogs, but will also provide original names of the toxin. In addition, readers are directed to relevant entries in the ArachnoServer 2.0 database for original literature references, biological activity, molecular targets, sequence and 3D structure (where known).

1.6 STRUCTURE OF THE PRECURSOR SPIDER VENOM PEPTIDE AND POST-TRANSLATIONAL PROCESSING

Similar to peptides from marine cone snails and sea anemones, spider venom peptides are translated as precursors that undergo post-translational modification to yield the mature toxin (Sollod, Wilson et al., 2005). These precursors are typically composed of an N-terminal signal peptide of 15–47 residues that generally precedes a propeptide region rich in acidic residues and of highly variable length, followed by a single

downstream copy of the mature toxin sequence. Notably, however, for some larger spider venom peptides, the toxin precursor does not contain a propeptide region.

It appears that during evolution toxin diversity is maintained through gene duplication followed by focal hypermutation in the mature peptide region while conserving the basic disulfide framework (Sollod, Wilson et al., 2005). Hypermutation of the mature toxin sequence often gives rise to new pharmacological activity. Peptide libraries of toxin paralogs are maintained, with spider species capable of expressing up to 26 variants (homologs/isoforms) of a single peptide toxin (e.g., U_2-AGTX-Ao1a to -Ao1z (Herzig, Wood et al., 2011)). Despite this high diversity, the signal sequence within the prepropeptide and the Cys residues responsible for correct protein folding are highly conserved (Sollod, Wilson et al., 2005). The signal peptide is presumably conserved since its role is to direct the precursor to a specific secretory pathway to ensure correct peptide folding. The specific role(s) of the propeptide region is still not understood but it may enhance folding of the mature toxin and provide signals for post-translational modifications (PTMs) such as N-terminal pyroglutamate formation, palmitoylation, and C-terminal trimming and amidation. However, insecticidal spider toxins have only been observed with palmitoylation and C-terminal trimming/amidation. In the case of the high molecular mass latroinsectoxins, from *Latrodectus* spp. (widow spiders), the N-terminal propeptide is absent. These mechanisms have allowed spiders to evolve vast libraries of peptides with variable pharmacological activity.

1.7 STRUCTURAL MOTIFS OF SPIDER VENOM PEPTIDES: VARIATIONS ON AN ANCESTRAL FOLD

Around 90% of spider venom toxins are compact globular proteins possessing several disulfide bridges. The numbers of disulfide bonds range from one to seven, but nearly 60% of all toxins have three disulfide bridges. These peptides, predominantly targeting voltage-activated ion channels, often contain a 'disulfide pseudo-knot' which places them in a class of toxins and inhibitory polypeptides with an 'inhibitor cystine-knot' (ICK) motif (Norton and Pallaghy, 1998). This structural motif is normally exemplified by a triple-stranded, antiparallel β-sheet stabilized by disulfide bridges. Since not all ICK peptides exhibit the N-terminal β-strand (β1 in Figure 1.1C), a modified definition composed of 'an antiparallel β-hairpin stabilized by a cystine-knot' without a mandatory third β-sheet has been proposed (Pallaghy, Norton et al., 1994; Norton and

Pallaghy, 1998; King, Tedford et al., 2002). The three disulfide bridges and intervening backbone form a pseudo-knot consisting of a ring (Cys_I-Cys_{IV}, Cys_{II}-Cys_V) penetrated by a third disulfide bridge (Cys_{III}-Cys_{VI}) (Norton and Pallaghy, 1998); see Figure 1.1. The ICK has a consensus sequence of -Cys-X_{3-7}-Cys-X_{3-8}-Cys-X_{0-7}-Cys-X_{1-4}-Cys-X_{4-13}-Cys- where X is any amino acid (Norton and Pallaghy, 1998). However within this fold-class, the biological activities of spider ICK toxins are quite diverse with activity at voltage-activated sodium (Na_V), calcium (Ca_V), and potassium (K_V) channels, acid-sensing ion channels (ASICs), transient receptor potential (TRP) channels, and mechanosensitive channels (MSCs) (see below). This highlights the observation that different biological functions are often grafted onto the same, or similar, structural scaffolds.

Another structural fold has been defined for spider toxins. The disulfide-directed β-hairpin (DDH) fold lacks the disulfide knot and is composed of a double-stranded antiparallel β-hairpin stabilized by two mandatory disulfide bridges with a current consensus sequence of -CysX_{4-19}-CysX_2[G or P]X_2-CysX_{4-19}-Cys-, where X is any amino acid (Figure 1.1A-B). The ICK motif appears to have evolved from this simpler canonical ancestral fold (Wang, Connor et al., 2000; Smith, Hill et al., 2011). This DDH fold has been observed in a range of peptides with unknown targets such as the MIT-like U_1-HXTX-Hv1a (Wen, Wilson et al., 2005) and U_1-TRTX-Lp1a and -Lp1b (Escoubas and Rash, 2004), and the insecticidal toxins U_1-TRTX-Hh1 toxins, U_1-TRTX-Asp1f and -Asp1g (Escoubas and Rash, 2004). The ICK fold in particular creates hyperstable mini-proteins that are typically resistant to extremes of pH, organic solvents, and high temperatures (Colgrave and Craik, 2004). However, from a bioinsecticide perspective, their most important property is their resistance to proteases. Specific differences in the DDH and ICK structural folds, determined by the spacing between cysteine residues and their connectivity, is critical for the presentation of key functional residues to the target. This together with their protease resistance and compact nature provides an effective scaffold for the design of bioinsecticides, including peptidomimetics, as well as molecular tools and therapeutics (Norton and Pallaghy, 1998). Finally, another structural motif that has been reported in spider venoms is the Kunitz-type toxin motif characterised by an N-terminal 3_{10} helix, C-terminal α-helix and a triple-stranded antiparallel β-sheet with a C_I–C_{VI}, C_{II}–C_{IV}, C_{III}–C_V disulphide bonding pattern. Peptides and proteins with this motif exhibit potassium

channel blocking activity and also act as serine protease inhibitors. This structural fold has been discovered in toxins from a variety of other venomous animals including cone snails, scorpions, sea anemones, snakes, ticks, and wasps, and but has so far only been reported in the venoms of two theraphosid spiders (*Haplopelma schmidti* and *H. hainanum*) and one araneid spider (*Araneus ventricosus*)(Fry, Roelants et al., 2009).

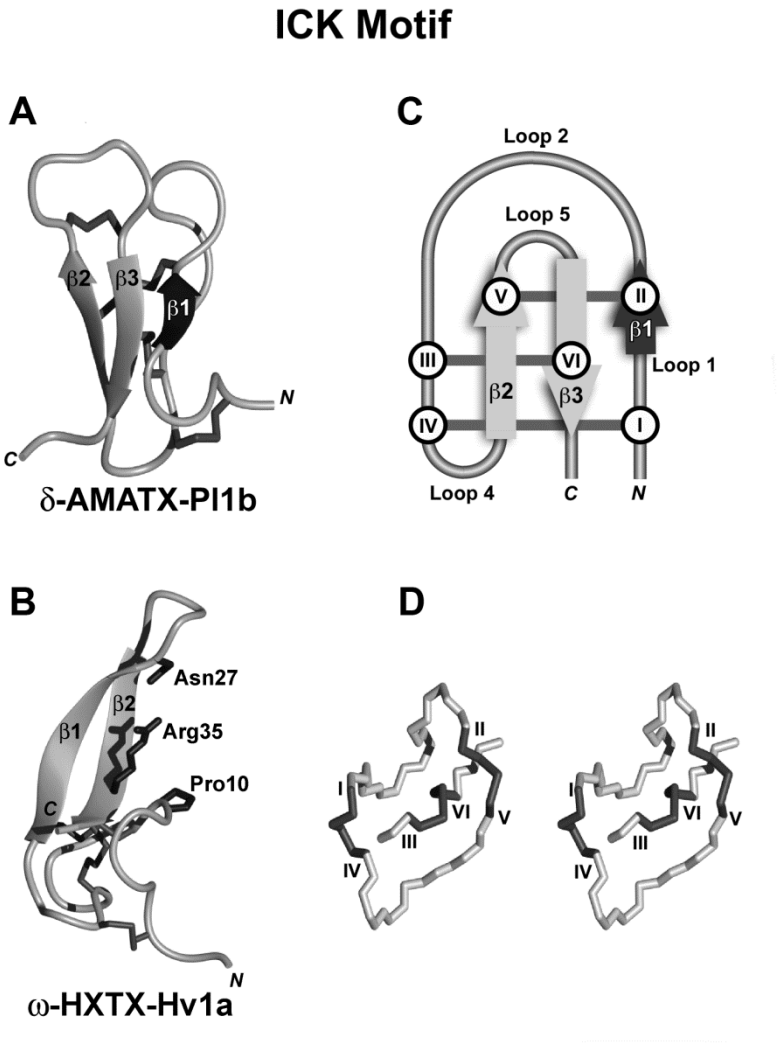


Figure 1.1: The ICK structural motif. Left-hand panels (A-B) show a schematic view of the 3D structures of typical representatives of the ICK structural motif. **(A)** The insecticidal peptide δ-AMATX-Pl1b (PDB 1V91) and **(B)** the insecticidal peptide ω-HXTX-Hv1a (formerly ω-ACTX-Hv1a; PDB 1AXH) showing the major pharmacophore residues. Panel **(C)** shows a schematic representation of the ICK motif depicting the formation of the cystine-knot and possible addition of the third β-strand. The dark arrow (β1) represents the additional β-strand not always present in ICK spider venom peptides (i.e. present in A but not B). **(D)** Stereoview of the cystine-knot motif of κ-TRTX-Scg1a (formerly SGTx1). In all panels, β-strands are shown as gray arrows and disulfide bridges connecting cysteine residues are shown as dark gray lines with roman numerals.

1.8 INSECTICIDAL TARGETS OF SPIDER NEUROTOXINS

There are predicted to be at least 10 million bioactive spider venom peptides (Escoubas and King, 2009) of which only 800 have been characterized. Of the 800 peptides in the ArachnoServer 2.0 Database, 136 are insecticidal with 38 being insect-selective, 34 non-selective and 64 of unknown phyletic selectivity (these data do not include homologs whose activity and phyletic selectivity is yet to be determined). Of the insecticidal spider toxins the molecular target has only been identified for 85 (63%). To date, the most common identified targets of insecticidal spider venom toxins are Na_V channels (n = 33), Ca_V channels (n = 33), the lipid bilayer (n = 11), calcium-activated potassium (K_{Ca}) channels (n = 7), presynaptic nerve terminals (n = 2) and *N*-methyl-D-aspartate (NMDA) receptors (n = 1). However, these statistics may be skewed by the rather limited range of targets that have been assayed to date. With advances in venom screening technologies (Vetter, Davis et al., 2011), it is likely that spider toxins with novel molecular targets will be discovered in the near future. In the subsequent sections, the structure and pharmacology of some of the insect-selective toxins that have been identified in spider venoms has been reviewed.

1.8.1 SPIDER VENOM PEPTIDES TARGETING INSECT NA_V CHANNELS

Mammalian and insect Na_V channels mediate inward sodium conductance during the depolarisation phase of the action potential and regulate a wide range of physiological processes (Hodgkin and Huxley, 1952; Hille, 2001). The crystal structure of a bacterial Na_V channel has recently been determined and that could potentially shed some light on the structural basis for voltage-dependent gating, ion selectivity and drug block of the channel (Payandeh, Scheuer et al., 2011). Unfortunately, the gating mechanism of mammalian Na_V channels appears to be quite different from their bacterial counterparts, subsequently, little is still known in regards to the structural basis for voltage-gating in mammalian Na_V channels. Nevertheless, it is known that Na_V channel contains a pore forming α-subunit, associated with one or two auxiliary β-subunits (Catterall, 2000). The α-subunit has four homologous domains (I–IV) that are further divided into six transmembrane sections (S1–S6). The voltage-sensing domain is composed of the S1–S4 segments that flank the pore module comprising the S5 and S6 segments. The re-entrant P-loop between S5 and S6 forms the narrow ion-selectivity filter at the extracellular end of the pore (Catterall, 2000). The S1-S4 segment acts as the voltage

sensor by locating charged amino acids within the membrane electric field that undergo outward displacement in response to depolarization and initiate opening of the ion pore (Catterall, 2010). Sodium channel inactivation is mediated by a short intracellular loop connecting domains III and IV (West, Patton et al., 1992; Davies, Field et al., 2007).

To date, nine mammalian Na_V channels ($Na_V1.1$–1.9) have been cloned and, in all cases except $Na_V1.9$, functionally expressed (Goldin, Barchi et al., 2000). Consequently the structural, functional and pharmacological diversity of mammalian Na_V channels is achieved primarily through expression of multiple genes. In contrast, insects appear to rely upon extensive alternative splicing and RNA editing of a single *para* Na_V channel gene to provide channels with different functional properties. For example, gene splicing of the Na_V channels has been observed at 9 different sites in *Drosophila* (Tan, Liu et al., 2002; Song, Liu et al., 2004). This has the potential of leading to 100 distinct variants of the insect Na_V channel.

The wide range of *para* Na_V channels are highly conserved across various insect orders, with sequence identities of 87–98% (King, Escoubas et al., 2008). Hence, insecticides targeting insect Na_V channels have broad toxicity across diverse insect orders. In contrast, *para* Na_V channels have only low levels of sequence identity (50–60%) with mammalian $Na_V1.1$–1.9 channels (King, Escoubas et al., 2008). As a result, insect and mammalian Na_V channels are distinguishable pharmacologically by the selective action of several chemical insecticides. These include insect Na_V channel selective; pyrethroids, DDT *N*-alkylamides, oxadiazines and dihydropyazoles (Bloomquist, 1996; Raymond-Delpech, Matsuda et al., 2005; Casida, 2009), as well as a growing range of insect-selective Na_V channel toxins including those derived from spider venoms. Much of the structure and function of Na_V channels have also been determined using toxins derived from a range of animal venoms and plants. These molecular probes have enabled identification of at least seven allosterically coupled neurotoxin binding sites, referred to as neurotoxin receptor sites 1–7, of which three sites bind spider venom peptides (Cestèle and Catterall, 2000; Catterall, Cestèle et al., 2007; Nicholson, 2007b). Toxins targeting Na_V channels are expressed in most families of araneomorph and mygalomorph spiders, suggesting an early development during venom evolution. These spider toxins modulate neuronal excitability, resulting in paralysis (both flaccid and excitatory) and death in insects. Importantly, some spider toxins are highly selective for

these three neurotoxin receptor sites on insect Na_V channels and thus insect-selective spider neurotoxins have potential to be developed as bioinsecticides. Importantly, the three neurotoxin receptor sites do not correspond to the site targeted by pyrethroids, DDT or DDT analogues (site 7) (Cestèle and Catterall, 2000) so the possibility of cross resistance between spider toxins and pyrethroids/DDT is negligible.

1.8.1.1 Spider venom peptides targeting insect Na_V channel site-1: pore blockers

Site-1 neurotoxins, like the guanidinium-containing alkaloid neurotoxin tetrodotoxin (TTX), physically occlude the pore region of the channel and are referred to as pore blockers. μ-Theraphotoxin-Hhn2b (μ-TRTX-Hhn2b; formerly hainantoxin-I) is the most abundant component within the crude venom of the Chinese black earth tiger tarantula *Haplopelma hainanum* (Liang, Peng et al., 1999) and blocks insect channels with high affinity (Li, Xiao et al., 2003). It displays 15-fold selectivity for the *Drosophila para* ($DmNa_V1$) channel compared with rat $Na_V1.2$, with no effect on rat $Na_V1.1$ and $Na_V1.4$–1.8 channels (Li, Xiao et al., 2003). It does not appear to alter ion selectivity, nor alter the voltage-dependence of activation or inactivation kinetics. However, μ-TRTX-Hhn2b is associated with a hyperpolarizing shift in the voltage dependence of steady-state Na_V channel inactivation that stabilizes the channel in the inactivated (closed) state and inhibits Na^+ conductance (Li, Xiao et al., 2003). It has been claimed that μ-TRTX-Hhn2b is the first spider toxin to selectively block Na^+ conductance via an interaction with site-1. However, the significant shift in steady-state inactivation suggests a remote allosteric site of action to inhibit ion conductance rather than a pore block. Using a panel of alanine mutants, it was found that the key residues responsible for the interaction of μ-TRTX-Hhn1b (formerly HNTX-IV), a structurally related toxin with similar actions on mammalian Na_V channels, are Lys27, Arg29, His28, Lys32, Phe5 and Trp30 (Li, Xiao et al., 2004). Interestingly, His28 is substituted by the negatively charged Asp26 in μ-TRTX-Hhn2b, thus providing a possible molecular basis for the selectivity of μ-TRTX-Hhn2b for the insect Na channel.

1.8.1.2 Spider venom peptides targeting insect Na_V channel site-3: gating modifiers of inactivation

Site-3 toxins induce a block or slow Na_V channel inactivation and are referred to as gating modifiers of inactivation. The block or slowing of Na_V inactivation generally

produces an excitatory effect due to the increased activity of Na_V channels (Cestèle and Catterall, 2000; Catterall, Cestèle et al., 2007). δ-Ctenitoxin-Pn1a [δ-CNTX-Pn1a; formerly Tx4(6-1)] was isolated from *Phoneutria nigriventer* venom and has significant selectivity towards dipterans (ED_{50} of 36 pmol/g) and blattarians (ED_{50} of 95–477 pmol/g), with no neurotoxic effects in lepidopterans or coleopterans (Figueiredo, Garcia et al., 1995). δ-CNTX-Pn1a specificity was further highlighted by the absence of effects on mammalian $Na_V1.2$ and $Na_V1.4$ channels (Figueiredo, Garcia et al., 1995; de Lima, Stankiewicz et al., 2002). δ-CNTX-Pn1a is an excitatory toxin resulting in immediate knockdown, with trembling and uncoordinated movements (Figueiredo, Garcia et al., 1995). It has been definitively established that δ-CNTX-Pn1a interacts with site 3 of the insect Na_V channel using competition binding assays where it displaces the site-3 ligand BomIV, an α-like scorpion toxin (de Lima, Stankiewicz et al., 2002).

Insecticidal toxins have also been isolated from the Japanese funnel web spider *Macrothele gigas* (Corzo, Gilles et al., 2003). In particular, μ-hexatoxin-Mg1a (μ-HXTX-Mg1a; formerly Magi-2) demonstrates high affinity and selectivity for the insect Na_V channel that results in flaccid paralysis of insect larvae. In comparison, the toxin fails to induce any neurotoxic symptoms in mice (Corzo, Gilles et al., 2003). The displacement of ^{125}I-LqhαIT binding, another classical ligand of insect site-3, from cockroach neurons further implies that an interaction with site 3 is likely. μ-HXTX-Mg1a shares 68% sequence identity with an inactive homolog μ-HXTX-Mg1a (formerly Magi-1). A comparative study of these two toxins theorised that the string of cationic residues Lys16–Lys19 in μ-HXTX-Mg1a may be critical for toxin affinity (Corzo, Gilles et al., 2003).

1.8.1.3 Spider venom peptides targeting insect Na_V channel site-4: gating modifiers of activation

Toxins interacting with site 4 typically alter the threshold for action potential generation. Hyperpolarising shifts in the voltage dependence of activation result in the activation of Na_V channels at, or near, resting membrane potentials, and result in an excitatory phenotype. In contrast, a depolarising shift in activation threshold results in a depressant phenotype due to the greater depolarisation required to open the channel. Consequently, toxins interacting with neurotoxin receptor site 4 are classed as either depressant or excitatory toxins.

Four insecticidal peptides, δ-amaurobitoxins (δ-AMATX-Pl1a to -Pl1d; formerly PaluIT toxins), from the venom of *Pireneitega luctuosa* demonstrate high selectivity for insect Na_V channels (Corzo, Escoubas et al., 2000). None of the δ-AMATX-Pl1 toxins demonstrate activity following intracerebroventricular injection into mice (Corzo, Escoubas et al., 2000). Using native and cloned para/tipE insect Na_V channels, δ-AMATX-Pl1 toxins have been shown to slow insect Na_V channel inactivation without any significant shifts in the voltage dependence of channel activation. However they fail to modulate the activity of mammalian $Na_V1.2$ channels at concentrations up to 10 µM (Ferrat, Bosmans et al., 2005). This action is similar to site-3 neurotoxins. Despite this they have been shown to displace the site-4 excitatory scorpion α-toxin, Bj-xtrIT, but not the site-3 ligand LqhαIT, on cockroach membranes (Corzo, Escoubas et al., 2005). In reciprocal experiments, Bj-xtrIT and the depressant scorpion α-toxin LqhIT2 also displaced ^{125}I-AMATX-Pl1b binding (Corzo, Escoubas et al., 2005). Thus δ-AMATX-Pl1 toxins represent the first spider toxins that definitively bind to site-4 on insect Na_V channels but modulate Na_V channel inactivation, an action typically associated with site-3 toxins.

The active site of the δ-AMATX-Pl1 toxins consists of a discontinuous string of residues. A main hot spot of positively charged Arg residues (8, 26, 32 and 34) surrounded by aromatic Tyr residues (22, 30) stands distinct from another aromatic region (Trp12) that is considered critical for activity (Corzo, Escoubas et al., 2005). Asp19 also appears to play an important role in maintaining toxin activity; however it seems unimportant for target affinity. A similar feature has been observed on the scorpion toxin Bj-xtrIT with Glu15 playing a part in trapping the Na_V channel voltage-sensor during channel activation (Cohen, Karbat et al., 2004). An action such as this may, in part, account for disparity between toxin activity and target affinity. These results contribute to the theory that the channel target site is more complex than originally perceived.

The µ-agatoxin-Aa1 toxins (µ-AGTX-Aa1a to -Aa1f; formerly µ-Aga I–VI) are a family of terminally amidated 36–38 residue peptides isolated from the venom of the Western grass spider *Agelenopsis aperta* (Adams, Herold et al., 1989; Skinner, Adams et al., 1989). The six members of this family share a high degree of homology with the δ-AMATX-Pl1 toxins (Corzo, Escoubas et al., 2000) and belong to a larger group of µ-

agatoxin-1 toxins from *Agelena orientalis*, *Agelena opulenta* and *Hololena curta* that are the most potent toxins to modulate the activity of Na_V channels (Adams, Herold et al., 1989; Skinner, Adams et al., 1989; Stapleton, Blankenship et al., 1990; Quistad, Reuter et al., 1991; Kozlov, Malyavka et al., 2005). μ-Agatoxin-1 family toxins are insect-selective neurotoxins that cause a convulsive paralysis in insects. They are also specific to certain insect orders, being very potent in dipterans (LD_{50} of 30–1380 pmol/g), moderately potent in orthopterans (LD_{50} of 944–4875 pmol/g), but only weakly active in lepidopterans (LD_{50} of 6565–18258 pmol/g). This action is the result of repetitive firing in insect axons resulting in a marked increase in spontaneous neurotransmitter release (Adams, Herold et al., 1989). This results from a hyperpolarizing shift in the voltage-dependence of Na_V channel activation (Cohen, Bale et al., 1993; Norris, Lee et al., 1995). This action is analogous to that reported for site-4 excitatory scorpion β-toxins (Wang and Strichartz, 1983) and therefore it is likely that this family targets site-4, although this awaits further radioligand binding studies. However, μ-AGTX-1 toxins also slow Na_V channel inactivation in insect motoneurons (Cohen, Bale et al., 1993; Norris, Lee et al., 1995) an action shared by δ-AMATX-Pl1 toxins. The similarities in primary structure and pharmacology of these toxins provide further support for the hypothesis that the insect site-4 is a macrosite, which may be allosterically linked to both channel activation and inactivation.

1.8.1.4 SPIDER VENOM TOXINS WITH AN UNKNOWN SITE OF ACTION ON INSECT NA_V CHANNELS

A family of 56–59 residue μ-diguetoxin-1 toxins have been isolated from the weaving spider, *Diguetia canities* (Krapcho, Kral et al., 1995). These toxins share moderate homology with each other however they do not appear to show any significant homology with any other venom peptides. This family consists of three toxins, isolated as a result of their potent insect paralytic activities, designated μ-DGTX-Dc1a to -Dc1c (formerly DTX9.2, DTX11 and DTX12). μ-DGTX-Dc1a demonstrates strong to moderately potent activity with a PD_{50} value of 380 pmol/g in lepidopterans (Krapcho, Kral et al., 1995; Bloomquist, Kinne et al., 1996). In mice, μ-DGTX-Dc1a did not show any activity at 657 pmol/g after intraperitoneal injection. While the toxin produced an excitatory effect with increasing muscle spasms until paralysis, the toxin was not lethal. Interestingly, it was apparent that even if larvae recovered from the symptoms of toxicity, feeding was inhibited (Krapcho, Kral et al., 1995). This is an important

distinction in terms of developing insecticides for crop protection. Studies performed on neuromuscular preparations from *Musca domestica* (house flies) were used to further analyse the rapid and potent activities of these toxins. The application of µ-DGTX-1 toxins induced excitatory postsynaptic potentials (Bloomquist, Kinne et al., 1996). Due to a TTX-dependent effect on cockroach action potentials it is likely that µ-DGTX-1 toxins target insect Na_V channels (Bloomquist, Kinne et al., 1996), however further studies are necessary to definitively ascertain the site of toxin action.

1.8.2 SPIDER VENOM PEPTIDES TARGETING INSECT Ca_V CHANNELS

Ca_V channels are key signal transducers that convert depolarization of the cell membrane into an influx of extracellular calcium ions. This ion influx then triggers muscle contraction, hormone and neurotransmitter release, enzymatic activities and patterns of gene expression (Catterall, Cestèle et al., 2007). Many channel subtypes have been identified in both vertebrates and invertebrates. Insect Ca_V channels are divided into two broad families based on their voltage-dependence of activation. Low-voltage-activated (LVA) Ca_V channels are activated by small membrane depolarisations and show rapid voltage-dependent inactivation, whereas high-voltage-activated (HVA) Ca_V channels are only activated by larger depolarisations and inactivate more slowly.

HVA Ca_V channels are composed of a pore forming unit (α_1), an extracellular subunit (α_2) linked to a transmembrane δ domain through a disulfide bridge, an intracellular β subunit and a transmembrane γ subunit (Bourinet and Zamponi, 2005; Catterall, Perez-Reyes et al., 2005). The transmembrane topology of the α_1 subunit of Ca_V channels is similar to Na_V channels and voltage dependence of activation is modulated by a similar mechanism (Tanabe, Takeshima et al., 1987). In contrast, LVA Ca_V channels are simpler in structure as they appear to comprise only the α_1 subunit (Bourinet and Zamponi, 2005; Catterall, Perez-Reyes et al., 2005).

Insects have a much smaller repertoire of Ca_V channels than vertebrates. For example, whereas the human genome encodes 10 pore-forming α_1 subunits, four β subunits, four α_2-δ complexes and seven γ subunits, the genome of the fruit fly *Drosophila melanogaster* appears to encode only three α_1 subunits, a single β subunit, three α_2-δ subunits and possibly a single γ subunit (Littleton and Ganetzky, 2000). However, insects are able to expand their array of functional Ca_V channels through alternative

splicing and RNA editing (King, 2007). Amino acid sequence comparisons indicate that the three α_1 subunits produced by *Drosophila*, designated Dmca1D, Dmca1A and Ca-α1T, can likely be classified as HVA Ca_V1-, and Ca_V2- channels and LVA Ca_V3-type channels, respectively (King, 2007). The fact that insects express only a single ortholog of each of three subtypes of Ca_V channels (King, Escoubas et al., 2008) might explain why loss-of-function mutations in the genes encoding Dmca1D and Dmca1A are embryonic lethal (Eberl, Ren et al.; Kawasaki, Collins et al., 2002). In contrast, the larger repertoire of Ca_V channels in vertebrates permits at least some functional plasticity since mice that harbour a knockout of the gene encoding the α1 subunit of many Ca_V channel subtypes are viable (King, Escoubas et al., 2008). This critical role in insects, coupled with <68% homology with their vertebrate counterparts and substantial differences in pharmacological sensitivities (Wicher, Walther et al., 2001), makes insect Ca_V channels an ideal target for the development of bioinsecticides. However the weaker conservation of insect Ca_V channels across insect orders (King, Escoubas et al., 2008) suggests that it might be more challenging to develop blockers of these channels that have a broad spectrum of activity. Of course, the potentially beneficial corollary of this is that it may be easier to develop Ca_V channel blockers that target specific groups of insect pests without harming beneficial insects such as pollinators.

1.8.2.1 SPIDER VENOM PEPTIDES THAT BLOCK INSECT CA_V1 CHANNELS

ω-Hexatoxin-Hv1a (ω-HXTX-Hv1a, formerly ω-atracotoxin-Hv1a) is the prototypic member of a large family of toxins from the venom of Australian funnel-web spiders with high affinity and specificity for insect Ca_V channels (Atkinson, Vonarx et al., 1996; Fletcher, Smith et al., 1997; Wang, Smith et al., 1999; Chong, Hayes et al., 2007). These toxins have low ED_{50} values in Orthoptera, Hemiptera, Dictyoptera, Diptera, Coleoptera, Acarina and Lepidoptera (Atkinson, Vonarx et al., 1996; Fletcher, Smith et al., 1997; Bloomquist, 2003) with no effect in vertebrates at up to 10,000-fold higher concentrations (Atkinson, Vonarx et al., 1996; Wang, Smith et al., 1999; Khan, Zafar et al., 2006; Chong, Hayes et al., 2007). It has been proposed that insect Ca_V1 channels are the primary target of ω-HXTX-Hv1a (Tedford, Maggio et al., 2007). However, recent studies revealed that ω-HXTX-Hv1a is a moderately potent blocker of both MVA and HVA (putative Ca_V2) currents in cockroach DUM neurons (Chong, Hayes et al., 2007). Thus it appears that ω-HXTX-Hv1a has high affinity for insect Ca_V1 channels (which

may not be present or present only at very low levels, in DUM neurons) and only moderate affinity for Ca_V2 channels. Thus, ω-HXTX-Hv1a might be a useful pharmacological agent for simultaneous block of all insect HVA Ca_V channel subtypes. In contrast to its effect on insect HVA channels, the toxin has no effect on calcium currents in rat trigeminal neurons (Fletcher, Smith et al., 1997), nor does it block rat $Ca_V1.2$, $Ca_V2.1$ and $Ca_V2.2$ HVA channels at concentrations as high as 10 μM (Tedford, Gilles et al., 2004).

Importantly, the topical and oral activity of ω-HXTX-Hv1a challenges the belief that it would be difficult to develop peptides as commercially viable insecticides. Studies have shown that transgenic expression of ω-HXTX-Hv1a in tobacco plants results in protection from *Helicoverpa armigera* and *Spodoptera littoralis* larvae (Khan, Zafar et al., 2006). Moreover, topical application of recombinant thioredoxin-ω-HXTX-Hv1a has been shown to be lethal to these caterpillar species (Khan, Zafar et al., 2006). Most importantly however, are the orally active properties demonstrated by ω-HXTX-Hv1a in ticks (Mukherjee, Sollod et al., 2006). Although this is the first spider toxin characterised to demonstrate such properties, ω-HXTX-Hv1a illustrates that it is possible for peptides to exhibit topical and oral activities against insects, and is amenable to the construction of a topically active peptidomimetic.

1.8.2.2 SPIDER VENOM PEPTIDES THAT BLOCK INSECT CA_V2 CHANNELS

ω-Plectotoxin-Pt1a (ω-PLTX-Pt1a, formerly Plectreurys toxin-II) is a toxin from the venom of *Plectreurys tristis* containing an unusual C-terminal *O*-palmitoyl threonine amide residue that is critical for toxin activity (Branton, Kolton et al., 1987; Branton, Rudnick et al., 1993; Bodi, Nishio et al., 1995). The toxin is assumed to be insecticidal as it blocks presynaptic Ca_V channel currents in *Drosophila* nerve terminals (Branton, Kolton et al.; Leung, Branton et al.), most likely through specific block of the Ca_V2 (Dmca1A) channel (Kuromi, Honda et al.). This results in a block of neurotransmitter release (Branton, Kolton et al., 1987). In contrast, ω-PLTX-Pt1a has no effect on K_V and Na_V channels (Kuromi, Honda et al., 2004) and it fails to block synaptic transmission at frog neuromuscular junctions (Leung, Branton et al.). At higher concentrations ω-PLTX-Pt1a also begins to disrupt endocytosis (Kuromi, Honda et al., 2004), suggesting that it might block additional insect Ca_V channel subtypes.

Nevertheless, low concentrations of ω-PLTX-Pt1a appear to be a defining pharmacology for the *Drosophila*, and possibly other, insect Ca_V2 channels.

A second family of insect-selective neurotoxins have also been isolated from *Hadronyche versuta* with a 10,000-fold preference for insect over vertebrate Ca_V channels. ω-Hexatoxin-Hv2a (ω-HXTX-Hv2a, formerly ω-atracotoxin-Hv2a) is the prototypic member of a family of 42–45-residue insect-selective neurotoxins (Wang, Connor et al., 2001). ω-HXTX-Hv2a induces immediate and sustained paralysis when injected into crickets with an ED_{50} of 160 pmol/g (Wang, Connor et al., 2001). This contrasts with the slow onset of paralysis following injection of ω-HXTX-Hv1a (Fletcher, Smith et al., 1997). The toxin is lethal to ticks (Mukherjee, Sollod et al., 2006) but it causes no adverse effects when injected into newborn mice (Wang, Connor et al., 2001). Injection of ω-HXTX-Hv2a into insects induces instantaneous paralysis and application of picomolar doses of toxin results in significant inhibition of Ca_V currents in bee brain neurons (IC_{50} 130 pM) (Wang, Connor et al., 2001). ω-HXTX-Hv2a is considered the most potent blocker of insect Ca_V channels reported thus far. The insect Ca_V channel subtype targeted by ω-HXTX-Hv2a has not been determined, but several lines of evidence suggest it is likely to be Ca_V2 (reviewed in ref. (King, 2007)). Unfortunately a recombinant expression system has never been developed for ω-HXTX-Hv2a so this is yet to be confirmed.

ω-Theraphotoxin-Hh2a (ω-TRTX-Hh2a, formerly huwentoxin-V) is a 35-residue peptide toxin isolated from the venom of the Chinese tarantula *Haplopelma schmidti* (Zhang, Chen et al., 2003). Small amounts of toxin induce a reversible paralysis when injected into locusts and cockroaches (PD_{50} = 4 nmol/g) whereas much larger doses (>24 nmol/g) are lethal. The neurotoxic effects of the peptide appear to be insect-specific since mice injected with high doses of toxin (7–49 nmol/g) via the intra-abdominal or intracerebroventricular route are unaffected (Zhang, Chen et al., 2003). ω-TRTX-Hh2a has no effect on Na_V, K_V and MVA Ca_V channel currents in cockroach DUM neurons, but it blocks HVA Ca_V currents with an IC_{50} of 219 nM (Deng, Luo et al.). This toxin therefore appears to be a moderately potent, but selective, blocker of insect Ca_V2 channels, although its effect on insect Ca_V1 and Ca_V3 channels remains to be examined. Nevertheless, ω-TRTX-Hh2a might prove to be a valuable

pharmacological tool for the study of insect Ca_V2 channels, especially for dissecting out currents mediated by different Ca_V2 isoforms.

1.8.2.3 SPIDER VENOM PEPTIDES THAT BLOCK INSECT CA_V3 CHANNELS

To date, no toxins have been described that block insect Ca_V3 channels. Furthermore, biophysical and pharmacological characterization of these channels is sadly lacking, with not a single study of insect Ca_V3 channels reported in the scientific literature. It remains to be determined whether some of the LVA Ca_V currents recorded from insect neurons are mediated by Ca_V3 channels.

1.8.3 SPIDER VENOM PEPTIDES TARGETING INSECT K_V CHANNELS

Voltage-activated potassium (K_V) channels are involved in cellular signalling processes, regulation of neurotransmitter release and heart rate, insulin secretion, neuronal excitability, epithelial electrolyte transport, smooth muscle contraction and cell volume regulation (Wei, Gutman et al., 2005). Given this highly diverse range of functions it is not surprising that more than 75 human genes encoding various K_V channel subunits have been cloned (Coetzee, Amarillo et al., 1999). There are six known families of voltage-activated potassium (K_V) channels in *Drosophila*. These have been classified as Shaker (mammalian K_V1-related), Shaw (K_V2-related), Shal (K_V3-related), Shab (K_V4-related), and EAG, ERG, and ELK (KCNH-related) (Chandy and Gutman, 1993). Additionally, the *slo* gene family encodes large-conductance, Ca^{2+}-activated K_V (BK_{Ca}) channels and Na^+-activated K_V (K_{Na}) channels. These channels activate in response to voltage changes, as well as to changes in the intracellular concentration of calcium (BK_{Ca}) and sodium (K_{Na}) (Adelman, Shen et al., 1992; Meera, Wallner et al., 1997).

Unlike Na_V and Ca_V channels, K_V channels are tetramers with a four-fold symmetry around a central pore (Jiang et al., 2002). Each subunit consists of an α-helical transmembrane domain that is made of six transmembrane segments (S1–S6) arranged into two types of domains: a single pore domain formed by the S5–S6 regions from the four subunits, and four surrounding voltage-sensing domains (S1–S4) from a single subunit (Jiang et al., 2002) The pore domain contains the K^+-selective ion conduction pathway and the receptor for pore-blocking toxins that bind to the extracellular vestibule near the selectivity filter. BK_{Ca} channels are an exception as they have seven transmembrane segments (Meera, Wallner et al., 1997). K_V channels may contain

homotetrameric or heterotetrameric α-subunit assemblies, explaining the diversity of these ion channels (Pongs, Leicher et al., 1999), although Shaker, Shal, Shab and Shaw are all homotetramers (Pongs, Leicher et al., 1999). While there are many K_V channel families, only one is of importance to this discussion—the insect BK_{Ca} channel.

The κ-hexatoxin-1 family of toxins, isolated from Australian funnel-web spiders (Wang, Connor et al., 2000), were the first spider toxins discovered to selectively target insect potassium channels (Gunning, Maggio et al., 2008). These 33–37 residue peptides were originally named 'Janus-faced' atracotoxins after the two-faced god Janus from Roman mythology due to the striking asymmetric distribution of hydrophobic and charged residues on opposite surfaces of the molecule (Maggio and King, 2002a; Maggio and King, 2002b). The most insecticidal member of this family, κ-HXTX-Hv1c, was recently identified as a high affinity blocker (IC_{50} of 2 nM) of insect BK_{Ca} channels with a lack of effect on insect Na_V, Ca_V as well as other subtypes of K_V channels (Gunning, Maggio et al., 2008). Channel block displayed a lack of voltage-dependence, in contrast with many other spider toxins targeting vertebrate K_V channels (for a review see ref. (Diochot, 2005)). Like the μ-agatoxin-1 family, toxins from the κ-hexatoxin-1 family are specific to certain insect orders, being very potent in dipterans (LD_{50} of 91–319 pmol/g), moderately potent in orthopterans (LD_{50} of 167–1022 pmol/g), but only weakly active in lepidopterans (LD_{50} of 3070–3195 pmol/g). However, the κ-hexatoxin-1 family is not toxic against newborn mice, adult rabbits or isolated preparations of chick biventer cervicis or rat vas deferens (Maggio and King, 2002a; Gunning, Maggio et al., 2008). Therefore κ-HXTX-1 toxins are highly insect-selective.

Although several insect-selective hexatoxins have been isolated from the venom of spiders belonging to the family Hexathelidae, there have been only a limited number of investigations of insecticidal toxins from spiders of the family Theraphosidae (Li, Xiao et al., 2003; Corzo, Diego-García et al., 2008). This is despite the characterization of a number of toxins from theraphosid ("tarantula") spiders that target mammalian ASICs, MSCs, K_V, Na_V, and Ca_V channels. Recently a family of three κ-theraphotoxin-Ec2 toxins (κ-TRTX-Ec2a to -Ec2c) from the venom of the East African tarantula *Eucratoscelus constrictus* were found to block insect BK_{Ca} channels (Windley, Escoubas et al., 2011) but not other insect K_V, Na_V, and Ca_V channels. κ-TRTX-Ec2a

induces complete paralysis of orthopterans within 5 min, and death within 15 min at 1100 pmol/g. κ-TRTX-Ec2a and κ-TRTX-Ec2b cause no activity in mice after intracranial injection. Interestingly, κ-TRTX-Ec2c, which shows >80% sequence identity with other members of this toxin family, is not insect specific. It induces strong neurotoxic symptoms including convulsions, tonic paralysis, general ataxia, and respiratory paralysis in mice (Windley, Escoubas et al., 2011). κ-TRTX-Ec2c could therefore yield insights into which residues are important for insect specificity. It is noteworthy that although these toxins share their target with κ-HXTX-Hv1c, they possess no obvious sequence homology, implying that they may interact with different sites on the insect BK_{Ca} channel. The κ-hexatoxin-1 and κ-theraphotoxin-1 families will be useful for probing the biological role of BK_{Ca} channels in insects and are potential lead compounds for the development of insect-selective biopesticides.

1.8.4 LINEAR SPIDER PEPTIDE TOXINS TARGETING THE CELL MEMBRANE

To date, several groups of cytolytic peptides with antimicrobial activity have been discovered in araneomorph spider venoms. These have been classified as membrane-acting antimicrobial peptides (MAMPs) with the activity prefix 'M' (for a review see ref. (Vassilevski, Kozlov et al., 2009)). These cytolytic peptides are short (<50 residues), highly cationic, amphipathic peptides lacking cysteine residues. While these peptides are highly active against Gram-negative and Gram-positive bacteria, the reported insecticidal effect of MAMPs on insects is negligible with very high LD_{50} values (5–10 nmol/g). In contrast to these short MAMPs, *Lachesana tarabaevi* (Zodariidae) venom also contains a family of long, linear M-ZDTX-Lt toxins (cyto-insectotoxins) with much more potent insecticidal activity, in addition to cytolytic and antimicrobial activity. These are more than twice the length of typical MAMPs and possess very high charge at neutral pH. They still retain an α-helical motif but appear to be composed of two short MAMPs joined together in a 'head-to-tail' configuration by a short four-residue linker (Vassilevski, Kozlov et al., 2008).

1.8.5 SPIDER VENOM TOXINS TARGETING PRESYNAPTIC NERVE TERMINALS

Venoms from widow spiders of the genus *Latrodectus* (Theridiidae) contain five insect-specific proteins, known as latroinsectotoxins (LIT) α, β, γ, δ and ε (Grishin, 1998; Graudins, Little et al., 2011), with phylum-selective insecticidal actions. There is also a

vertebrate-specific neurotoxin, α-latrotoxin (α-LTX; for a review see ref. (Ushkaryov, Rohou et al., 2008)), and a toxin affecting crustaceans, α-latrocrustatoxin (α-LCT)(Krasnoperov, Shamotienko et al., 1990a). Two of the latroinsectoxins have been cloned and fully sequenced: α-LIT-Lt1a (Kiyatkin, Dulubova et al., 1993) and δ-LIT-Lt1a (Dulubova, Krasnoperov et al., 1996). They are high-molecular mass proteins with masses of 111 and 130 kDa, respectively. All latrotoxins whose structures have been determined are highly homologous and have a similar domain architecture, which consists of a unique N-terminal sequence and a large domain composed of 13–22 ankyrin repeats. It is believed that these toxins induce paralysis in insect prey by stimulating massive neurotransmitter exocytosis from nerve terminals. They act by (i) binding to specific receptors, some of which cause exocytosis, and (ii) inserting themselves into the terminal membrane to form non-selective cation channels (for a review see ref. (Rohou, Nield et al., 2007)). Specific receptors for LITs have yet to be identified, but all three classes of vertebrate receptors known to bind α-LTX are also present in insects. LITs are the most potent spider venom toxins known, with LD_{50} values of <1 pmol/g in lepidopterans and dipterans (Krasnoperov, Shamotienko et al., 1990a; Dulubova, Krasnoperov et al., 1990b). Furthermore, as LITs form ion channels upon membrane insertion it is unlikely that only a short fragment of the protein can be found that mimics the broad range of activity of the entire toxin, given that this fragment would need to encode information regarding targeting, membrane insertion and oligomerisation.

1.8.6 SPIDER VENOM TOXINS TARGETING GLUTAMATE RECEPTORS

In vertebrates the neuronal actions of L-glutamate are mediated by two distinct glutamate neurotransmitter receptor classes in the CNS: ionotropic (Stawski, Janovjak et al., 2010) and metabotropic (Nicoletti, Bockaert et al., 2011). All known vertebrate ionotropic L-glutamate receptors comprise cation channels and are classified into three subtypes: α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA), kainate (KA) and *N*-methyl-D-aspartate (NMDA) receptors. In insects, however, at least two ionotropic L-glutamate-gated cation channels mediate neurotransmission at the neuromuscular junction, rather than in the CNS (Usherwood, 1994; DiAntonio, 2006). In addition, two insect ionotropic L-glutamate receptors that gate chloride channels (Glu-Cls) have been discovered in insect CNS neurons (Raymond, Sattelle et al., 2000).

These anion-gated Glu-Cls are important sites of action of insecticides such as ivermectin (Kane, Hirschberg et al., 2000), and in some cases fipronil (Raymond, Sattelle et al., 2000).

While there have been a wide range of glutamate receptor antagonists found in spider venoms these are mostly acylpolyamines (in particular α-agatoxins (Adams, 2004)). Thus far, only one spider toxin, Γ-CNTX-Pn1a (formerly PnTx4(5–5)) from the venom of the Brazilian armed spider *Phoneutria nigriventer*, has been found to inhibit vertebrate glutamate receptors, in this case specifically inhibiting NMDA- but not the KA- or AMPA-subtypes (de Figueiredo, de Lima et al., 2001). Γ-CNTX-Pn1a is highly insecticidal, with <50 pmol/g causing neurotoxic effects immediately after intrathoracic injection in blattarians, orthopterans, and dipterans. This suggests that the effects of Γ-CNTX-Pn1a may be mediated via insect glutamate receptors. Importantly, Γ-CNTX-Pn1a had no effect when injected i.c.v. into mice at a dose of 290 pmol/g (de Figueiredo, de Lima et al., 2001).

1.9 INSECTICIDES TARGETING NEUROTRANSMITTER RECEPTORS

While no spider toxins have presently been characterised to target other neurotransmitter receptors, two in particular are considered important targets of commercial insecticides. Nicotinic acetylcholine (nACh) and γ-aminobutyric acid (GABA) receptors are members of the cys-loop superfamily of ionotrophic neurotransmitter receptors and are targeted by a number of insecticides. Notably, like glutamate, nicotine and GABA mediate fast chemical neurotransmission in the insect nervous system.

1.9.1 INSECTICIDES TARGETING NACH RECEPTORS

Nicotinic acetylcholine receptors (nAChRs) are the most abundant excitatory neurotransmitter receptors found in insects. Evidence suggests that the insect gene family encoding for nAChR is diverse in nature. For instance, 10 nAChR subunits encoding genes have been described in *Drosophila melanogaster* (Sattelle, Jones et al., 2005), 10 in *Anopheles gambiae* (Jones, Grauso et al., 2005), 11 in *Apis mellifera* (Jones, Raymond-Delpech et al., 2006) and 12 in *Tribolium castaneum* (Jones and Sattelle, 2007). However, these numbers are believed to be conservative estimates of

diversity, as many of these genes additionally undergo alternate splicing and post-translational modifications of the subunit mRNA (Sattelle, Jones et al., 2005). Although the identification and characterisation of insect nAChR has been limited, at least four subtypes have been identified in cockroach neurons. Firstly, two groups of insect nAChR have been identified based on sensitivity to the snake neurotoxin α-bungarotoxin (α-BgTx) (Tomizawa and Yamamoto, 1992; Tornøe, Bai et al., 1995). Moreover, varying sensitivities of α-BgTx sensitive nAChR to various nicotinic agonists reveal two additional subtypes correlated with a desensitizing (nAChD) or non desensitizing (nAChN) time course (Bai and Sattelle, 1993; Buckingham, Lummis et al., 1993; Su and O'Dowd, 2003; Salgado and Saar, 2004). α-BgTx insensitive are similarly divided into two group based on agonist sensitivity, namely nAChR1 and nAChR2 (Courjaret and Lapied, 2001; Courjaret, Grolleau et al., 2003; Thany, Courjaret et al., 2008).

The nAChR consists of five homologous peptide subunits forming around a central pore (Karlin, 2002). Two classes of subunits have been classified based on the presence of two adjacent cysteine residues in one of the extracellular loops. The adjacent Cys residues actively contribute to the ACh binding site and are only found in α-subunits and subsequently non α-subunits do not contain this feature (see (Karlin, 2002) for review). nAChRs are the targets of several commercially available insecticides including; cartap, imidacloprid and spinosad. Cartap was the first commercial insecticide derived from nereistoxin (Hagiwara, Numata et al., 1965). Nereistoxin was isolated from the marine annelid *Lumbriconereis heteropoda* and similarly to cartap, is a potent non-competitive agonist of insect nAChR, however these insecticides also induce effective block of vertebrate nAChR (Sattelle, Harrow et al., 1985; Shebl, Amira et al., 1986; Lee, Tomizawa et al., 2003; Raymond Delpech, Ihara et al., 2003). Imidacloprid on the other hand, is a partial agonist of nAChR, possesses low mammalian toxicity and is responsible for fast growing sales worldwide (Matsuda, Buckingham et al., 2001; Tomizawa and Casida, 2003). Furthermore, other related members of the neonicotiniod family of insecticides have been shown to depolarise the postsynaptic membrane and block excitatory post synaptic potentials at insect cholinergic synapses (Sattelle, Buckingham et al., 1989; Bai, Lummis et al., 1991; Benson, 1992; Buckingham, Lapied et al., 1997). Finally, spinosad is a newer insecticide derived from the bacterium *Saccharopolyspora spinosa.* Spinosad induces

muscle contractions and tremors in insects and is believed to allosterically enhance nAChR function (Salgado, 1998; Salgado, Sheets et al., 1998; Salgado and Saar, 2004; Lewer, Hahn et al., 2009; Orr, Shaffner et al., 2009; Watson, Chouinard et al., 2010).

1.9.2 INSECTICIDES TARGETING GABA RECEPTORS

Unlike nAChR, ionotropic $GABA_A$ receptors mediate inhibitory neurotransmission in the insect nervous system. Similarly to Glu-Cl receptors, GABA receptors are permeable to chloride ions (Sattelle, Lummis et al., 1991). GABA receptors consist of four transmembrane domains and possess an extracellular ligand binding domain (Hosie, Sattelle et al., 1997). Vertebrate GABA receptors are divided into three subtypes based on structure and pharmacology. $GABA_A$ and $GABA_C$ receptors are coupled to chloride channels while $GABA_B$ receptors modulate potassium or calcium channels through a G-protein linked second messenger system (Barnard, Skolnick et al., 1998). However, vertebrate GABA receptor classification cannot be applied to insects as their receptors possess different pharmacologies. Only two GABA-activated chloride (GABA-Cl) channel subtypes have been identified in DUM neurons, namely picrotoxin-sensitive and picrotoxin-insensitive GABA-Cl channels (Hue, 1998; Le Corronc, Alix et al., 2002).

Although little is still known in regards to the GABA receptor subtypes expressed in insects these receptors are important targets of a number of insecticidal compounds. Picrotoxin, fipronil, dieldrin, ivermectin are all insecticidal compounds that are characterised to target insect $GABA_A$ receptors (Buckingham, Biggin et al., 2005). Organochlorine insecticides such as dieldren were once used as insecticides due to their ability to block GABA receptors however their usage has since been banned due to persistence within the environment leading to bioaccumulation up the food chain (Narahashi, Frey et al., 1992; Le Corronc, Alix et al., 2002; Zhao, Salgado et al., 2003). Fipronil is a phenyl pyrazole that acts on insect GABA receptors and also has additional action on the Glu-Cl (Le Corronc, Alix et al., 2002; Zhao, Salgado et al., 2003; Narahashi, Zhao et al., 2010). Furthermore, the activity of ivermectin (an avermectin) is also implicated in the enhancement of both GABA and Glu-Cl receptor function (Rohrer, Birzin et al., 1995; Kane, Hirschberg et al., 2000).

1.10 BIOINSECTICIDE LEAD SELECTION

There are a number of requirements to be met for a spider venom peptide to be considered as a bioinsecticide lead compound. The most conspicuous reason to pursue a peptide as a lead is the potency against insect target(s). Particularly, because the potency of a toxin is inversely proportional to the volume needed to be deployed in the field. Selectivity is also crucial, for instance, a toxin that is potent in insects, but also lethal to vertebrates would not be considered as appropriate, although it might still be used to elucidate the key residues for determining phyletic selectivity. Good examples of such non-phylum-selective toxins are the δ-hexatoxins-1 toxins from Australian funnel-web spiders. δ-Hexatoxin-Ar1a and -Hv1a are potent insecticides, but they are also responsible for the lethal effect of these hexathelid venoms in primates (Nicholson, Graudins et al., 2006).

Size is also an important consideration. The most potent insecticidal toxins reported so far are the α- and δ-latroinsectotoxins from *L. tredecimguttatus* (LD_{50} 0.11 pmol/g and 0.45–0.54 pmol/g, respectively). However, these peptides consist of 1170 and 991 amino-acid residues (respectively) and are therefore not suitable for production on a large scale due to the large amounts of protein that would be required. Among the low mass peptide toxins, U_1-CUTX-As1c from *Apomastus schlingeri* is the most potent in lepidopterans (LD_{50} of 2.4 pmol/g), followed by its paralog U_1-CUTX-As1d (LD_{50} of 7.2 pmol/g) and U_1-PLTX-Pt1a from *Plectreurys tristis* with an LD_{50} of 13.8 pmol/g, however the target sites of these toxins still remain unknown.

In addition, to be developed as a bioinsecticide lead, a toxin should only target a narrow range of pest species while not harming other potentially beneficial insects (e.g., pollinators and natural predators of the target pest species). Unfortunately, most studies fail to determine if insecticidal spider toxins are toxic to beneficial or protected species of certain beetles, dipterans and lepidopterans. Interestingly, although high sequence homology of targets between insect orders would indicate that this might be difficult to achieve (King, Escoubas et al., 2008), potency differences have been observed in acute toxicity assays across different insect orders. For example, μ-AGTX-Aa1d was reported to be 317 times more potent in dipterans than in lepidopterans. Sometimes activity may even vary considerably within one insect order. For example, U_1-PLTX-Pt1a is 309

times more potent against *Manduca sexta* than its lepidopteran relative Heliothis *virescens*.

Furthermore, unless the toxin is to be delivered via a vehicle such as an entomopathogen, it is also necessary that the toxin is orally- or contact-active. Unfortunately, the insecticidal activity of most spider venom peptides has been determined by injection and in most cases the presence of oral activity unknown (an exception being the ω-HXTX-1 toxins). Moreover, the toxin must be sufficiently stable under field conditions and involve complete degradation to harmless metabolites over time to avoid adverse impacts on the environment via biomagnification and bioaccumulation.

Despite the discovery of 136 insecticidal spider toxins, so far less than 25 are adequately potent, specific for insects and sufficiently characterized (i.e., determination of the full sequence and molecular target) to be considered suitable as bioinsecticide leads. Notably, some of these toxins do have poorly characterised orthologs/paralogs which, given their high sequence homology, may also be suitable bioinsecticide leads. Nevertheless, there are still some promising candidates fulfilling most, or all, of the criteria defined above for an ideal bioinsecticide. Based on the information available to date, the following spider toxins would be considered as suitable candidates for insecticide leads: ω-hexatoxin-1 family, μ-agatoxin-1 family, δ-ctenitoxin-Pn1 family, μ-diguetoxin-Dc1, κ-hexatoxin-1 family, and Γ-CNTX-Pn1a (Table 1). However, more work is necessary to fully characterize these toxins in order for the suitability of these peptides can be properly assessed.

Table 1.1 Spider peptide toxins suitable as insecticidal leads

Toxin Name	Source	Insect Target	Acute toxicity test species (Order[†]: *Genus species*)	ED_{50} or PD_{50} (pmol/g)	LD_{50} (pmol/g)	paralogs/ orthologs
δ-CNTX-Pn1a	*Phoneutria nigriventer*	Na_V channel	B:*Periplaneta americana* D: *Musca domestica*	95[‡] 36		2
Γ-CNTX-Pn1a	*Phoneutria nigriventer*	GluR	B:*Periplaneta americana* D: *Musca domestica* O: *Acheta domesticus*	48[‡] 10[‡] 29[‡]		0
κ-HXTX-Hv1c	*Hadronyche versuta*	BK_{Ca} channel	D: *Musca domestica* D: *Musca domestica* D: *Lucilia cuprina* L: *Heliothis virescens* L: *Spodoptera frugiperda* O: *Acheta domesticus* O: *Acheta domesticus*		91 319[#] 117[#] 3195[#] 3070[#] 167 1022[#]	6
μ-AGTX-Aa1d	*Agelenopsis aperta*	Na_V channel	D: *Musca domestica* L: *Manduca sexta*		30 9524	11
μ-DGTX-Dc1a	*Diguetia canities*	Na_V channel	L: *Heliothis virescens*	380		3
ω-HXTX-Hv1a	*Hadronyche versuta*	Ca_V channel	D: *Musca domestica* L: *Heliothis virescens* O: *Acheta domesticus*	250[#]	77 89	27

[†] B = Blattaria; D = Diptera; L = Lepidoptera; O = Orthoptera. [‡] Not ED_{50}/PD_{50} values–Neurotoxic effects noted immediately after intrathoracic injection at this concentration. [#] Recombinant toxin.

1.11 MANUFACTURING SPIDER VENOM TOXINS AS INSECTICIDES

The high phyletic specificity and potency, and novel mode of action, of a limited range of spider toxins recommend them as lead compounds for the development of bioinsecticides. Transgenes encoding insect-specific arachnid toxins, including spider neurotoxins, have been successfully expressed in a number of crops and entomopathogens. One of the simplest ways is via the development of a recombinant baculovirus. The efficacy of insect specific baculoviruses can be significantly enhanced via the insertion of the gene encoding the toxin into the baculovirus genome. Subsequently this reduces the 'time-to-kill' in comparison to the native virus, and therefore increases the insecticidal potential of these viruses (Hughes, Wood et al., 1997; Thiem, 1997). The *Autographa californica* nuclear polyhedrosis virus (AcNPV) is the most widely used baculovirus strain for gene insertion as it infects various important lepidopteran insects pest (Elazar, Levi et al., 2001) (for a review see (Kamita, Kang et al., 2005)). To date three spider toxins have been expressed in baculovirus trials including μ-AGTX-Aa1d and two toxins with undefined targets, U_1-AGTX-Ta1a (formerly TalTX-1 from the hobo spider *Tegenaria agrestis*) and μ-DGTX-Dc1d (Tomalski, Bruce et al., 1988; Tomalski, Kutney et al., 1989; Prikhod'ko, Robson et al., 1996; Hughes, Wood et al., 1997; Prikhod'ko, Popham et al., 1998). Results have been positive with demonstrated improvements in the speed of action, causing lepidopteran larvae to die up to 50% more rapidly than those larvae infected with wild-type virus. Similarly, transgenes encoding spider toxins have been inserted into entomopathogenic funguses such as *Metarhizium anisopliae.* Insertion of the gene encoding the insect-selective scorpion toxin AaIT was found to significantly increase fungus toxicity against the tobacco hornworm *Manduca sexta* (Lepidoptera: Sphingidae) and the dengue mosquito *Aedes aegypti* (Diptera: Culicidae) without compromising its host specificity (Wang and St Leger, 2007). Unfortunately, while the construction of recombinant baculoviruses to speed the time to kill in pest insects has been validated a number of times, this technique has not become a conventional pest management strategy due to what is often dubbed the ''psychological effects of seemingly unsuccessful commercialization'' (Kamita, Kang et al., 2005).

Further transgenic approaches involve incorporating insecticidal spider toxins into plants for the control of phytophagous pests. This technique has already been

successfully employed for *Bacillus thuringiensis* Cry proteins (for a review see ref. (Bravo, Gill et al., 2007)), and further success was recently demonstrated with the orally active ω-ACTX-Hv1a following incorporation into the tobacco plant *Nicotiana tabacum*. Studies revealed that tobacco plants incorporating the spider-toxin transgene had markedly enhanced resistance to *Heliothis armigera* and *Spodoptera littoralis* (Lepidoptera: Noctuidae) (Fletcher, Smith et al., 1997; Khan, Zafar et al., 2006).

Unfortunately public reticence in some regions may limit commercial deployment of recombinant baculoviruses and transgenic plants. Nevertheless, transgenic crops are widely grown in Argentina, Australia, Brazil, Canada, China, India, Pakistan, Paraguay, South Africa, Uruguay, and the USA. These crops have been shown to markedly reduce insecticide use and increase crop yield (Qaim and Zilberman, 2003). Human insecticide poisonings have also been reduced by >75% in China since the introduction of GM crops (Huang, Rozelle et al., 2002). Despite these benefits, and reductions in risk, some people remain sceptical of the long-term safety and efficacy of GM products.

Other approaches include the development of orally active acaricidal and insecticidal agents. While ω-ACTX-Hv1a has been reported to be toxic by oral administration to the American lone star tick *Amblyomma americanum* (Mukherjee, Sollod et al., 2006) no other spider toxins have been reported to possess oral activity even in the modified gut of ticks. Nevertheless, studies have shown that the bioavailability of these peptides may be improved by coupling them to a carrier protein such as snowdrop lectin (*Galanthus nivalis* agglutinin, GNA) or garlic lectins to increase the absorption of toxins across the insect midgut (Fitches, Edwards et al., 2004; Fitches, Philip et al., 2008; Fitches, Wiles et al., 2008). The fusion of the insecticidal spider toxin U_2-SGTX-Sf1a (SFI1) to GNA was found to significantly increase its oral toxicity to the tomato moth *Laconobia oleracea* (Fitches, Edwards et al., 2004) as well as the rice brown planthopper *Nilaparvata lugens* and the peach-potato aphid *Myzus persicae* (Down, Fitches et al., 2006). Furthermore, a thioredoxin–ω-HXTX-Hv1a fusion protein was found to be insecticidal in *Helicoverpa armigera* and *Spodoptera littoralis* caterpillars by topical application (Khan, Zafar et al., 2006) (although the fusion protein was applied topically in a solution containing high levels of imidazole, a compound known to have contact insecticidal activity; (Pence, 1965)). These findings open up a variety of approaches for delivery of insecticidal peptides.

A final alternative could be to design conformationally constrained non-peptide mimetics to be used as foliar sprays. Theoretically, using a non-peptide organic scaffold, the peptide residues critical for binding to the target can be grafted onto a backbone structure to produce a peptidomimetic. Subsequently, this would overcome the bioavailability issues of peptides penetrating the insect cuticle or gut mucosa. The notion has received limited validation following attempts to develop small-molecule drug leads by 'cloning' the functional residues of peptide toxins that block vertebrate calcium or potassium channels (Menzler, Bikker et al., 2000; Baell, Duggan et al., 2006). The development of peptidomimetics presents perhaps the most promising direction for the manufacture of commericial insecticides as it overcomes both bioavailability and GMO concerns. Furthermore, as a result, the company Vesteron (www.vesteron.org.au) is presently involved in the development of a number of peptidomimetic insecticides using spider toxins, lending additional weight to the validity of this approach.

It should also be particularly interesting in the future to examine the interplay between these peptide toxins and conventional chemical insecticides. In tests with neonate *H. virescens*, the scorpion toxin AaIT was found to act synergistically with cypermethrin (McCutchen, Hoover et al., 1997). Moreover, a pyrethroid-resistant strain of *H. virescens* was found to be more susceptible than non-resistant strains to the effects of a recombinant baculovirus that expressed an AaIT transgene (McCutchen, Hoover et al., 1997). Pyrethroids and AaIT both target the insect Na_V channel and subsequently it appears that Na_V channel mutations that provide resistance to pyrethroids make the channel more susceptible to toxins that bind to other sites on the channel. This suggests that spider peptide toxins might be particularly useful for the control of insect populations that have evolved resistance to commercially available chemical insecticides.

Table 1.2 Criteria for development of competitive insecticides

Goals	OPs[†]	Carbamates	Pyrethroids	Insect-selective spider toxins
Broad pest-species specificity	+++	+++	+++	+++
Low toxicity in non-target organisms	+	+	++	+++
Remain in the environment long enough to be effective	+++	+++	++	++
Do not persist in environment to induce resistance development	+	+	++	+++
Cheap to produce	+++	+++	++	++
Easy to formulate and deliver	+++	+++	++	+
Publicly perceived as innocuous	+	+	+++	+
Accessible to small farmers and agribusinesses	+	++	+++	+

[†] Organophosphates

1.12 PROJECT AIMS AND CONCLUDING REMARKS

The overall aim of this project was to characterise the activity of isolated insecticidal spider peptide neurotoxins on the insect nervous system. This aim was pursued with the goal of identifying the lethal site and mechanism of action of selected peptide toxins and potentially also identifying novel insecticidal targets to aid in the control existing and future development of insecticide resistance. The hypothesis was; given the rapid neurotoxic activities of selected spider toxins in insects, these toxins would target voltage- or neurotransmitter-activated ion channels. These channels included: K_V, Na_V, Ca_V voltage-gated ion channels, as well as nAChR, Glu-Cl and GABA receptors. In order to characterise the effects of various toxins on ion channels the following goals were set.

Evaluation of toxin action on voltage-gated ion channels

1. Identification of the target channel subtype
2. Determination of effects on ion channel gating and kinetics
3. Determination of the dose dependency and subsequent IC_{50} / EC_{50} of the effect
4. Appraisal of the voltage-dependency of ion channel modulation

Evaluation of toxin activity on transmitter-activated ion channels

1. Identification of the target channel subtype
2. Determination of effects on channel gating and kinetics
3. Determination of the dose dependency and subsequent IC_{50} / EC_{50} of the effect
4. Appraisal of the voltage-dependency of ion channel modulation
5. Evaluation of toxin effects on agonist sensitivity

Furthermore, it was hypothesised that the target with the highest potency was most likely to be the lethal target of an insecticidal toxin. Subsequently, if a novel target was identified the aim was to prove that the target was lethal by identifying and testing toxins targeting the same channel in acute insect bioassays.

In conclusion, spider venom peptides have been highlighted as rich source of potential insecticides that seem to possess the desirable attributes for lead compounds in the development of new bioinsecticides. Moreover, the pharmacological characterisation of spider toxins is revealing novel target sites not previous exploited by conventional agrochemicals, therefore new insecticide targets have been identified for future screening programs. Importantly, research has shown that these peptides can be

delivered to insect pests via many different routes, including incorporation of transgenes encoding the peptides into entomopathogens or crop plants.

Importantly, agrochemicals still dominate the insecticide industry even though their broad spectrum of activity often means they cause significant non-target toxicity. In addition, agrochemicals act on a very limited range of targets which subsequently limits their long-term viability in the face of growing insecticide resistance (Elzen and Hardee, 2003). Therefore, it is necessary to instigate the development of new insecticides that demonstrate specificity, effectiveness against target species, minimal non-target toxicity and environmental persistence.

Chapter Two

Materials and Methods

Monique J. Windley

[2012]

2.1 SELECTION AND ISOLATION OF INSECT NEURONS: DORSAL UNPAIRED MEDIAN (DUM) NEURONS

In order to analyse the effects of various spider neurotoxins on the insect nervous system the whole-cell patch-clamp technique was employed (Hamill, Marty et al., 1981). Dorsal unpaired median (DUM) neurons isolated from the terminal abdominal ganglion (TAG) of the American cockroach (*Periplaneta americana*) ventral nerve cord, were used for experiments due to their large size (50–60 μm diameter) and characteristic 'tear drop' shaped morphology (Watson, 1984; Lapied, Malecot et al., 1989). Furthermore, a variety of ion channels within these neurons have been well characterised in the literature (Sattelle, 1992; Grolleau and Lapied, 1994; Grolleau and Lapied, 1995b; Grolleau and Lapied, 1995a; Grolleau and Lapied, 1996; Grolleau and Lapied, 2000; Raymond, Sattelle et al., 2000; Washio, 2002).

The use of whole-cell patch-clamp techniques allows voltage-clamp experiments to be performed on dissociated neurons, with low resistance recording solutions and rapid switching of solutions. Isolation of single adult DUM neurons extracted from the cockroach TAG can be achieved through mechanical and enzymatic dissociation techniques (Lapied, Malecot et al., 1989).

DUM neurons are among the most extensively characterised insect neurons (see (Grolleau and Lapied, 2000) for review). This is largely due to the unique ability of efferent DUM neurons to generate spontaneous overshooting action potentials (Grolleau and Lapied, 2000). Studies have shown that a number of voltage-gated ion channels are responsible for shaping the action potentials in DUM neurons including, voltage-gated Na^+, K^+ and Ca^{2+} channels (Grolleau and Lapied, 1994; Grolleau and Lapied, 1995b; Grolleau and Lapied, 1995a; Grolleau and Lapied, 1996; Grolleau and Lapied, 2000). Moreover, several neurotransmitter-gated ion channels including, glutamate-, γ-aminobutyric acid- and nicotine-acetylcholine-activated receptors are implicated in the modulation of electrical activity at insect neuromuscular and neuronal synapses (Grolleau, Lapied et al., 1996; Raymond, Sattelle et al., 2000; Courjaret and Lapied, 2001; Alix, Grolleau et al., 2002).

Ionic currents	Activation	Physiological role	References
Non synaptic			
I_{Na}	>-35 mV	Action potential (AP) repolarization	Lapied et al., 1990; Wicher and Penzlin, 1998Wicher *et al.*,
$I_{K(BCa)}$	>-40 mV and Ca^{2+}	AP repolarisation and afterhyperpolarisation	1994; Grolleau and Lapied, 1995b; Achenbach *et al.*, 1997
$I_{K(A)}$	>-65 mV	Regulation of AP firing frequency	Grolleau and Lapied, 1995b
$I_{K(Na)}$	>-35 mV and Na^{+}	AP repolarisation?	Grolleau and Lapied, 1994
$I_{K(DR)}$		AP repolarisation	Grolleau and Lapied, 1995b
$I_{Ca,\ tLVA}$	>-50 mV	Initial AP predepolarisation	Grolleau and Lapied, 1995a
$I_{Ca,\ mLVA}$	>-70 mV	Final AP predepolarisation	Grolleau and Lapied, 1995a
$I_{Ca,\ HVA}$	>-60 mV >-40 mV	Control of AP via $I_{K(Ca)}$	Wicher and Penzlin, 1994; 1997; Grolleau and Lapied, 1995a
$I_{K(IR)}$	<-75 mV	Inward rectification	Raymond and Lapied, 1999
$I_{Cl(Ca)}$	<-60 mV and Ca^{2+}	Limitation of AP hyperpolarization	Raymond and Lapied, 1999
Synaptic			
$I_{GABA\text{-}Cl}$	ϒ-amino-butyric acid (mM)	Inhibitory AP generation	Hue, 1998; Le Corronc *et al.*, 2002; Alix *et al.*, 2002
$I_{Glu\text{-}Cl}$	glutamate (mM)	Inhibitory AP generation	Raymond *et al.*, 2000; Washio *et al.*, 2002; Zhao *et al.*, 2004
I_{nACh}	nicotine, acetylcholine (mM)	Excitatory AP generation	Lapied *et al.*, 1990; Grolleau and Lapied, 1996; Bai and Sattelle, 1993; Buckingham *et al.*, 1993

Table 2.1 Synaptic and non-synaptic ion channels currents present in cockroach DUM neurons. These ion channels are implicated in the modulation of electrical excitability at insect neuromuscular and neuronal synapses. Further details can be found in chapters 3-6 (non-synaptic) and 7 (synaptic).

DUM neurons are named for their location (Hoyle, Dagan et al., 1974) along the dorsal midline of the ganglia of the insect ventral nerve cord. Acutely isolated DUM neurons are nearly pyriform in shape which allows for adequate space clamping of the membrane and complete diffusion of the intracellular solution (Lapied, Malecot et al., 1989), making these cell ideal candidates for patch-clamp experiments. Additionally, DUM neurons are sufficiently large in size (50–60 μm diameter) and typically have characteristic 'tear drop' morphology. While DUM neurons are found in all ganglia of the nerve cord in *P. americana* (Pollack, Ritzmann et al., 1988; Tanaka and Washio, 1988; Elia and Gardner, 1990; Sinakevitch, Geffard et al., 1996; Grolleau and Lapied, 2000), patch-clamp techniques have been developed and adapted for DUM acutely isolated from the TAG (Lapied, Malecot et al., 1989). Importantly, the electrical activity of neurons recorded *in situ* along the dorsal midline of the TAG is maintained in acutely isolated DUM neurons (Lapied, Malecot et al., 1989).

According to their position within the central nervous system, DUM neurons innervate optic lobes, mushroom bodies, and lateral cardiac nerve cords, skeletal and visceral muscles (Hoyle, Dagan et al., 1974; Orchard, Ramirez et al., 1993; Sinakevitch, Geffard et al., 1996; Bräunig and Eder, 1998; Bräunig, 1999; Grolleau and Lapied, 2000). Most efferent neurons within the DUM neuron group are neuromodulatory cells that contain and release octopamine (Morton and Evans, 1984; Achenbach, Walther et al., 1997; Roeder, 1999; Tahira, 2007).

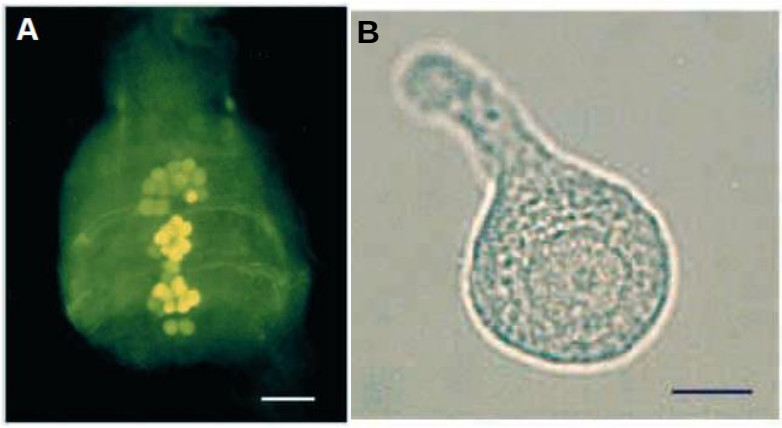


Figure 2.1: Dorsal unpaired median (DUM) neurons isolated from the terminal abdominal ganglia (TAG) of *Periplaneta americana.* Adapted from Grolleau *et al*., 2000 (Grolleau and Lapied, 2000). **(A)** A pictomicrograph of the TAG surface showing DUM neurons with octopamine-like immunoreactivity. Scale bar, 200 μm **(B)** A light micrograph of an acutely isolated DUM neuron showing the typical 'tear drop' morphology. Scale bar, 25 μm.

2.1.1 SUPPLY AND MAINTENANCE OF COCKROACHES

A breeding colony of unsexed American cockroaches, *Periplaneta americana*, were maintained in an insectory at UTS (Room CB04.07). Cockroaches were maintained in large plastic tubs (50 L capacity) sustained at 26–28°C with a relative humidity of 60–80%. Cockroaches were supplied with adequate water and dried dog food.

Healthy fully grown adult cockroaches were individually selected immediately prior to dissection. Long forceps were used to select and transfer cockroaches to a holding container with minimal damage to the specimens. Ideally specimens with all six legs and intact wings were chosen. Cockroaches were transferred in small containers and kept on ice to limit movement.

2.1.2 ACUTE ISOLATION OF DUM NEURONS

DUM neurons were isolated from unsexed adult American cockroaches (*Periplaneta americana*) as previously described (Gunning, Maggio et al., 2008). Prior to dissection cockroaches were sedated at –20 °C over a period of no greater than 5 minutes. Following the cessation of movement, individual cockroaches were secured with four pins, dorsal side up, to a 20 cm diameter glass Petri dish containing approximately 1 cm of silicone elastomer, Sylgard® 184 (Ellsworth Adhesives, Melbourne, Australia). All dissections were performed under a class I, sterile laminar flow hood. Using sterile forceps and surgical scissors the abdominal cuticle was firstly removed. The exposed digestive tract and connective tissues were then gently lifted out of the abdominal cavity and placed to the side, taking care not to perforate the digestive tract. The ventral nerve cord was identified under a dissecting microscope at a magnification of ×15. Sterile surgical forceps were used to lift the nerve cord away from the cavity just above the terminal abdominal ganglia (TAG). The TAG and a short length of connecting nerve cord were severed with sterile surgical scissors and immediately transferred to a solution of normal insect saline (NIS) containing (in mM): NaCl 180, KCl 3.1, N-hydroxyethylpiperazine-N-ethanesulfonic acid (HEPES) 10 and D-glucose 20. The ganglionic sheath was removed under 40× magnification with fine tweezers and a pair of forceps.

10–15 ganglia were transferred to a 15 ml centrifuge tube (BD Biosciences, North Ryde, Australia) containing 1 mg/ml collagenase (type IA) dissolved in 3–5 ml NIS. The ganglia were incubated in the collagenase solution for 40 minutes at 29°C. Gentle agitation of the solution was applied at 15 minute intervals to ensure adequate mixing. Following enzymatic treatment, the tube was centrifuged at 1,000 rpm for 30 seconds and the supernatant containing collagenase was replaced with NIS. The ganglia were washed three times with 2 ml of NIS. Finally, the ganglia were suspended in NIS supplemented with 5% v/v foetal bovine serum (FBS), 0.1% penicillin (50 IU/ml) and streptomycin (50 μg/ml) (Life Technologies, Victoria, Australia).

To dissociate individual neurons, ganglia were triturated through a fire-polished Pasteur pipette. Ganglia were passed through a pipette (with a diameter equal in size to a single TAG), up to 15 times in order to ensure adequate dissociation. The cell suspension was then distributed into 8 wells of a sterile 24-well plate. Each well contained a 12-mm diameter glass coverslips (Deckgläser, Germany) pre-coated with 1 mg/ml concanavalin A (type IV). The cell suspension was aliquoted evenly between 8 coverslips, with ~1–2 ganglia per coverslip. Cells were maintained for no longer than 24 hrs in NIS supplemented with 5 mM $CaCl_2$, 4 mM $MgCl_2$, 5% FBS and 0.1% penicillin and streptomycin, and maintained at 30°C, 100% humidity.

2.1.3 COVERSLIP PREPARATION

Single, autoclaved 12 mm diameter glass coverslips (Deckgläser, Germany) were flamed after being dipped in 100% ethanol and transferred aseptically to a sterile 24-well plate (16 mm diameter, flat bottom wells, Iwaki, Tokyo, Japan). Coverslips were coated with 1 mg/ml concanavalin A (type IV) for 2–3 hours. Prior to use, the solution was removed through aspiration, the coverslips washed with sterile water and allowed to air dry in the Class I hood. Concanavalin A was employed to assist cell adherence to the coverslip and subsequently aid stable patch-clamp recordings.

2.2 TOXIN SOURCE AND STORAGE

2.2.1 *HADRONYCHE VERSUTA* VENOM

All toxins originally isolated from the venom of the Blue Mountains funnel-web spider, *Hadronyche versuta* were generously provided by Prof. Glenn King (Institute for Molecular Bioscience, University of Queensland, Australia). Toxins were synthesized using the methods described below. Freeze-dried toxins were maintained at –20°C and stock solutions of 1–10 µM were made up ddH_2O. Immediately prior to usage, stock toxin solutions were diluted in either electrophysiological external solution for patch-clamp experiments and normal insect saline for insect bioassays.

2.2.1.1 PRODUCTION OF RECOMBINANT κ-HXTX-HV1C, ω-HXTX-HV1A AND HYBRID-HXTX-HV1A

κ-HXTX-Hv1c, ω-HXTX-Hv1a and hybrid-HXTX-Hv1a were obtained through a combination of overexpression and purification (Tedford, Fletcher et al., 2001; Maggio and King, 2002b; Chong, Hayes et al., 2007; Gunning, Maggio et al., 2008). Briefly, *E. coli* were transformed with pFM1, pHWT1 and pBLS1 encoding for κ-HXTX-Hv1c, ω-HXTX-Hv1a and hybrid-HXTX-Hv1a, respectively. Each plasmid encodes for the toxin gene as an in-frame fusion of the C-terminus of *Schistosoma japonicum* glutathione *S*-transferase with an incorporated thrombin cleavage site. The recombinant toxins were purified from the soluble cell fraction using affinity chromatography followed by thrombin cleavage of the mature toxin peptide. The correctly folded toxins were purified with C18 reverse-phase high performance liquid chromatography (rp-HPLC) and masses were verified with electrospray ionization quadrupoletime-of-flight mass spectrometry (ESI-QTOF).

2.2.1.2 PRODUCTION OF CYCLIC ω-HXTX-HV1A

Cyclic ω-HXTX-Hv1a was built by solid-phase peptide synthesis using standard Boc chemistry. The peptides were synthesized on a 0.5 mmol scale on a 100-200 mesh PAM-glycine resin. For the cyclic ω-HXTX-Hv1a, the resin was coupled to s-trityl-β-mercaptopropionic acid. The N- and C-terminal residues were linked with an eight-residue linker (ASGSAGAS). Cyclisation of the peptide backbone was achieved by employing a native chemical ligation strategy (Dawson, Muir et al., 1994). Peptides were oxidized and cyclized overnight at 0.1 mg/ml, in 0.1 M 3-morpholinopropane-1-

sulfonic acid (MOPS), 0.2 M KCl, 1 mM 2,2',2",2"'-(Ethane-1,2-diyldinitrilo)tetraacetic acid (EDTA), 2 mM reduced glutathione and 0.5 mM oxidized glutathione, pH 7.3. The reaction was quenched in 0.1% 2,2,2-trifluoroacetic acid (TFA). The toxin was purified using rp-HPLC to > 95% purity and the mass was confirmed with ESI-QTOF mass spectrometry.

2.2.1.3 PRODUCTION OF DISELENIDE MUTANT κ-HXTX-HV1C

Native and diselenide κ-HXTX-Hv1c were also prepared by Boc-mediated solid-phase peptide synthesis (Mobli, de Araújo et al., 2009). Selenocysteine (Sec) was incorporated at positions 13 and 14 of the diselenide toxin during assembly. Oxidation of the toxin in glutathione buffer resulted in the fully folded diselenide toxin. Folding conditions were the same as for the cyclic ω-HXTX-Hv1a.

2.3 INSECT LETHALITY ASSAYS

2.3.1 SUPPLY OF CRICKETS

Juvenile 3rd–4th instar house crickets (*Acheta domestica*) were purchased from Pets on Broadway (Sydney, Australia). Crickets were maintained in small containers with carrots and water. Specimens were only maintained for up to one week prior to experiments.

2.3.2 ACUTE TOXICITY TESTING

3rd–4th instar juvenile house crickets (sex not determined) of mass 80–120 mg were injected intrathoracically with 2–6 μl of solution using a 0.5 ml precision syringe. An Arnold microapplicator (Burkhard Scientific Supply, Rickmansworth, England) was used to inject toxin into the upper dorsal region of the thorax between the 2nd and 3rd set of limbs using a 29 gauge Insulin syringe.

Purified toxin was dissolved in insect saline containing (in mM): NaCl 200, KCl 3.1, $CaCl_2$ 5.4, $MgCl_2$ 4, $NaHCO_3$ 2, Na_2HPO_4 0.1 and 0.1% (w/v) bovine albumin serum (BSA), pH adjusted to 7.4 with 1 M NaOH. Concentrated toxin solutions were made up in > 90% NIS with final concentrations between 1 and 5 nmol/g for spider and scorpion toxins, while paxilline and 4-aminopyridine (4-AP) were tested at concentrations up to 5 μmol/g.

Following injection crickets were placed in sealed 20 cm diameter Petri dishes with a small piece of carrot for sustenance. The Petri dish was lined with 20 cm diameter filter paper dampened with several drops of water. Each dish contained no more than 3 crickets. Various behavioral aspects such as: twitching of limbs and antennae, abdominal contractions, paralysis and any other abnormal activity were observed up to 72 hours post injection.

10 to 30 crickets were injected at each toxin concentration. For each toxin, a control group of 10 were injected with saline only. Control experiments were also undertaken with a 1:10 volume of ethanol, as ethanol was used to dissolve paxilline in NIS, especially at high concentrations.

Percentage lethality was noted at 12, 24, 48 and 72 hrs following injection. Knockdown, defined as the loss of the righting reflex or the inability to remain upright, was also recorded at the same time points. Median knockdown (KD_{50}) and lethal (LD_{50}) doses were calculated from data fitted by a Logistic equation (See Eq. 2, section 2.5).

2.4 WHOLE-CELL PATCH-CLAMP PROTOCOLS

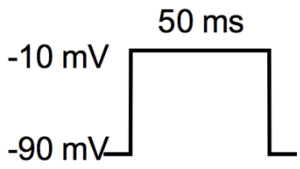
2.4.1 VOLTAGE-ACTIVATED CURRENTS

Whole-cell currents were recorded in voltage-clamp mode using the whole-cell patch-clamp technique, employing version 9 of the pCLAMP data acquisition system (Molecular Devices, Sunnyvale, CA, USA). Data were filtered at 5 kHz with a low-pass Bessel filter with leakage and capacitative currents subtracted using P-P/4 procedures. Digital sampling rates were set between 15 and 25 kHz depending on the length of the voltage protocol. Using a Flaming-Brown micropipette puller (Sutter Instruments Co., USA), single use electrodes were pulled from borosilicate glass to d.c. resistances of *ca*. 1, 1.5 and 2.5 MΩ for Na_V, Ca_V and K_V channel current recordings, respectively. Liquid junction potentials for the various combinations of internal pipette and external bath solutions were calculated using JPCALC (Barry, 1994), and all data were compensated for these values. Series resistance compensation was >80% for all cells. Cells were bathed in external solution through a continuous pressurised perfusion system at 1 ml/min, while toxin solutions were introduced via direct pressurised application via a perfusion needle at *ca*. 50 µl/min/PSI (Automate Scientific, San Francisco, CA). All experiments were performed at ambient room temperature (20–23ºC).

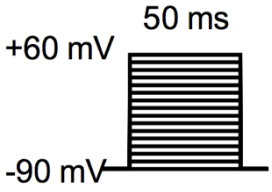
2.4.1.1. Na_V CHANNEL CURRENTS

To record I_{Na}, the external bath solution contained (in mM): NaCl 130, CsCl 5, $CaCl_2$ 1.8, tetraethylammonium chloride (TEA-Cl) 20, 4-aminopyridine (4-AP) 5, HEPES 10, $NiCl_2$ 0.1, and $CdCl_2$ 1, adjusted to pH 7.4 with 1 M NaOH. The pipette solution contained (in mM): NaCl 20, CsF 135, $MgCl_2$ 1, HEPES 10, ethylene glycol-bis(2-aminoethylether)-N,N,N′ ,N′ -tetraacetic acid (EGTA) 5, ATP-Na_2 2 and D-glucose 10, adjusted to pH 7.4 with 1 M CsOH.

Whole-cell Na_V channel currents were evoked by 50-ms depolarising voltage pulses applied at 0.1 Hz. To evoke rapidly inactivating I_{Na} in DUM neurons the membrane potential was stepped to –10 mV from a holding potential (V_h) of –90 mV.



To determine the voltage-dependence of Na_V channel activation, families of I_{Na} were evoked by 50-ms depolarising test pulses from –90 to +60 mV in 10-mV increments, at 0.1 Hz.



2.4.1.2 Ca_V (BARIUM) CHANNEL CURRENTS.

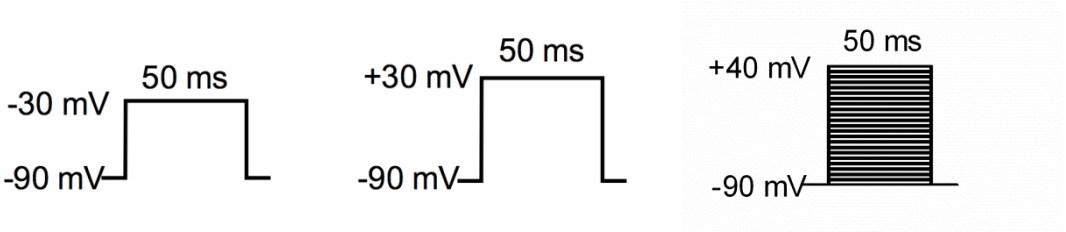
In order to record Ca_V channel currents, the external bath solution for barium current (I_{Ba}) recordings contained (in mM): Na acetate 140, TEA-Br 30, $BaCl_2$ 3, HEPES 10 and 300 nM TTX, adjusted to pH 7.4 with 1 M TEA-OH. Pipette solutions contained (in mM): Na acetate 10, CsCl 110, TEA-Br 50, ATP-Na_2 2, $CaCl_2$ 0.5, EGTA 10 and HEPES 10, adjusted to pH 7.4 with 1 M CsOH. Due to the report of I_{Ca} rundown with calcium as a charge carrier (Grolleau and Lapied, 1996), as well as reports of greater

success when barium was used as the charge carrier (Wicher and Penzlin, 1997), $BaCl_2$ replaced $CaCl_2$ in all experiments.

Voltage-activated calcium channel (Ca_V) currents in DUM neurons can be separated into two components, mid-to-low voltage-activated (M-LVA) and high-voltage-activated (HVA), based on activation threshold, kinetics and pharmacologies (Wicher and Penzlin, 1994; Wicher and Penzlin, 1997). M-LVA Ca_V channel currents activate at thresholds more depolarised than –50 mV with peak amplitudes at –10 mV. These currents inactivate rapidly and exhibit sensitivity to low concentrations of Ni^{2+} (Wicher and Penzlin, 1997). Alternately, HVA I_{Ca} are activated at potentials above –30 mV with maximal peak current elicited at +10 mV. HVA currents are largely sustained, displaying minimal signs of inactivation. These currents are resistant to depolarising membrane potentials up to –50 mV and are less dependent on Ca^{2+} as a charge carrier.

Previous studies have determined that the classical mammalian Ca_V channel blockers ω-conotoxin-MVIIC, $NiCl_2$ and SKF-96365 failed to separate insect M-LVA and HVA Ca_V channel current components (Y. Chong, PhD Thesis 2012: Characterisation of novel insecticidal ion channel toxins from araneomorph and mygalomorph spider venoms). This confirms the observations of Wicher and Penzlin (Wicher and Penzlin, 1997) that the subtype profile of insect Ca_V channels is inconsistent with vertebrates and there is no single peptide, organic or inorganic blocker that can exclusively block one type of Ca_V channel subtype. Therefore as previously reported by Wicher and Penzlin (Wicher and Penzlin, 1994; Wicher and Penzlin, 1997), the most appropriate method for delineating Ca_V channel current subtypes for patch-clamp experiments is to program the voltage protocol to elicit M-LVA and HVA dominating currents using two-step depolarising pulses. Therefore, in order to characterise the effects of spider peptide toxins on DUM neuron I_{Ba} a dual-pulse protocol has been designed to selectively activate M-LVA and HVA Ca_V current components (Chong, Hayes et al., 2007). M-LVA I_{Ba} were evoked by 50-ms depolarising steps to –30 mV from a holding potential of –90 mV. HVA I_{Ba} were subsequently evoked by stepping the potential to +30 mV for durations of 50-ms. Currents were recorded at 0.1 Hz, with –30 mV and +30 mV voltage steps alternating.

To determine the voltage-dependence of Ca_V channel activation, families of I_{Ca} were evoked by 50-ms depolarising test pulses from –90 to +40 mV in 5- or 10-mV increments, at 0.1 Hz.

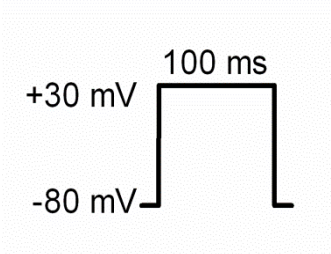


2.4.1.3 K_V CHANNEL CURRENTS

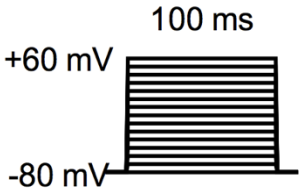
Global I_K

To record global I_K the external bath solution for recording macroscopic K_V channel currents contained (in mM): NaCl 150, KCl 30, $CaCl_2$ 5, $MgCl_2$ 4, HEPES 10, D-glucose 10 and 300 nM TTX, adjusted to pH 7.4 with 1 M NaOH. The pipette solution consisted of (in mM): KCl 135, KF 25, NaCl 9, $CaCl_2$ 0.1, $MgCl_2$ 1, EGTA 1, HEPES 10 and ATP-Na_2 3, adjusted to pH 7.4 with 1 M KOH.

Whole-cell global K_V channels currents (I_K) were evoked by 100-ms voltage pulses applied at 0.2 Hz. Voltage steps to +30 mV from a holding potential of –80 mV were used to elicit large outward I_K.



To determine the voltage-dependence of K_V channel activation, families of I_K were evoked by 100-ms depolarising test pulses from –80 to +60 mV in 10-mV increments, at 0.1 Hz.

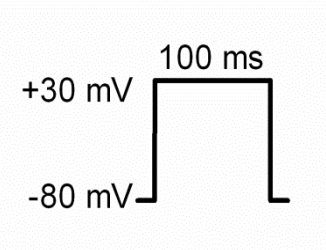


Multiple K_V channel subtypes have been identified in cockroach neurons. These are identified according to their current kinetics and pharmacological properties and include slowly activating, non-inactivating delayed-rectifier ($I_{K(DR)}$), transient 'A-type' ($I_{K(A)}$), transient Na^+-activated ($I_{K(Na)}$), as well as large-conductance 'late-sustained' and 'fast-transient' Ca^{2+}-activated ($I_{BK(Ca)}$) K^+ channel currents (Grolleau and Lapied, 1995b). A combination of selective channel toxins and inactivation protocols were used to identify and record various K_V channel subtypes.

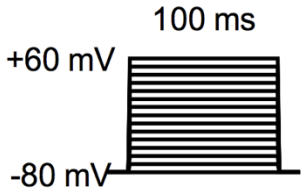
Delayed rectifier (K_{DR})

In order to isolate outward $I_{K(DR)}$ in DUM neurons, $I_{K(A)}$ were blocked with 5 mM 4-aminopyridine (4-AP) (Grolleau and Lapied, 1995b; Gunning, Maggio et al., 2008), K_{Na} (and Na_V) were blocked with the inclusion of 300 nM TTX and BK_{Ca} channels were blocked with 1 mM $CdCl_2$ (also blocking Ca_V) and 30 nM iberiotoxin (IbTx) (Grolleau and Lapied, 1995b).

As for global K_V currents, +30 mV test pulses were used to evoke outward non-inactivating K_{DR} channel currents. 100-ms pulse protocols were employed at 5-ms intervals from a holding potential of –80 mV.



To determine the voltage-dependence of K_{DR} channel activation, families of $I_{K(DR)}$ were evoked by 100-ms depolarising test pulses from –80 to +60 mV in 10-mV increments, at 0.1 Hz.

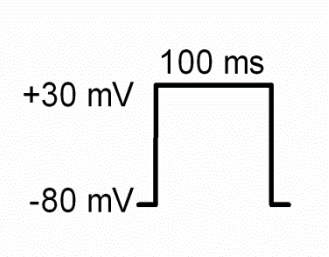


Calcium-activated (K_{Ca})

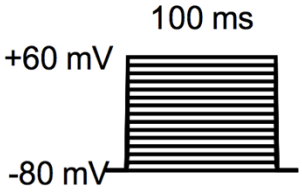
The K_{Ca} current in cockroach DUM neurons is of the large-conductance (BK_{Ca}) subtype since the current is voltage-activated, whereas SK_{Ca} and IK_{Ca} channel currents are voltage-insensitive, and no apamin-sensitive SK_{Ca} channels have been found in isolated cockroach DUM neurons (Grolleau and Lapied, 1995b). Outward K_{Ca} channel currents consist of both a transient and a sustained component. Specific $I_{K(Ca)}$ blockers such as IbTx and charybdotoxin (ChTx) do not appear to discriminate between these components, however evidence does suggest that these components can be isolated on the basis of holding potential (Grolleau and Lapied, 1995b). Nevertheless, for the experiments detailed in further chapters, transient and sustained components were not recorded individually. Subsequently, to record large-conductance calcium-activated K_V channel currents ($I_{BK(Ca)}$) 1 mM $CdCl_2$ and 5 mM 4-AP (Grolleau and Lapied, 1995b; Gunning, Maggio et al., 2008) were included in the external solutions to block Ca_V channel currents and $I_{K(A)}$, respectively.

BK_{Ca} channel currents ($I_{BK(Ca)}$) cannot be recorded in isolation from $I_{K(DR)}$ because there are no selective blockers of insect $I_{K(DR)}$. Subsequently, channel current isolation was achieved using the following steps. Initially both $I_{K(DR)}$ and $I_{K(Ca)}$ are recorded concurrently (i). Secondly, the test substance is applied and recordings are made until the channel current has stabilised (ii). Finally, 100 nM IbTx is perfused at the conclusion of current recordings to eliminate $I_{BK(Ca)}$ (iii). The subsequent isolation of $I_{K(Ca)}$ is achieved through offline subtraction of the remaining $I_{K(DR)}$, i.e. control $I_{K(Ca)}$ is calculated as (i) minus (iii) while toxin $I_{K(Ca)}$ is calculated as (ii) minus (iii).

Again, as for global I_K, +30 mV test pulses were used to evoke $I_{K(Ca)}$. 100-ms pulse protocols were employed at 5-ms intervals from a holding potential of –80 mV.



To determine the voltage-dependence of K_{Ca} channel activation, families of $I_{K(Ca)}$ were evoked by 100-ms depolarising test pulses from –80 to +60 mV in 10-mV increments, at 0.1 Hz.



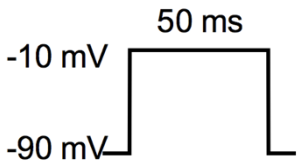
Sodium-activated (K_{Na})

To record outward transient sodium-activated K_V channel currents ($I_{K(Na)}$), recording solutions adapted from Grolleau and Lapied (1994) were utilised (Grolleau and Lapied, 1994). External solutions contained (in mM): NaCl 100, Tris-Cl 70, KCl 3.1, $CaCl_2$ 1.8, $MgCl_2$ 4, HEPES 10, $CdCl_2$ 1, and 30 nM IbTx, adjusted to pH 7.4 with NaOH. 4-AP was not included in the external solution as it blocks $I_{K(Na)}$. The pipette solution contained (in mM): KCl 135, KF 25, NaCl 9, $CaCl_2$ 0.1, $MgCl_2$ 1, HEPES 10, EGTA 1 and ATP-Na_2 3, adjusted to pH 7.4 with 2 M KOH.

In order to isolate $I_{K(Na)}$, channel currents were initially recorded in the absence of TTX. The outward $I_{K(Na)}$ is activated by Na^+ and as such channel activation is closely correlated to the inward Na_V current and subsequently both are blocked by TTX (Grolleau and Lapied, 1994). In order to record $I_{K(Na)}$ in isolation a similar technique to $I_{K(Ca)}$ isolation was employed. Initially $I_{K(Na)}$ and I_{Na} were recorded in unison (i). Secondly, the test substance was applied and recordings were made until the current had stabilised (ii). Finally, 300 nM TTX was added in order to block $I_{K(Na)}$ and I_{Na} (iii). Subsequently, through employing offline subtraction the portion of the outward current sensitive to TTX could be identified. To clarify, control $I_{K(Na)}$ were isolated from (i)

minus (iii) and $I_{K(Na)}$ in the presence of the test substance were obtained from (ii) minus (iii). Furthermore, I_{Na} are inward currents and therefore did not affect the isolation of the outward transient $I_{K(Na)}$.

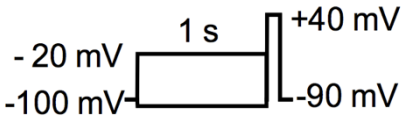
Subsequently, $I_{K(Na)}$ were evoked by 50-ms, –10 mV test pulses from a holding potential of –90 mV. Test potentials of –10 mV were used due to requirement of a large inward sodium current in order to record adequate K_{Na} currents (Grolleau and Lapied, 1994). Voltage pulses were applied at 10-second intervals.



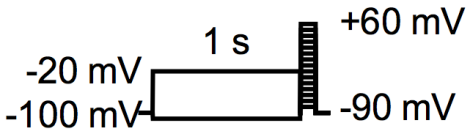
A-Type, transient (K_A)

To record isolated transient $I_{K(A)}$, 1 mM $CdCl_2$ and 30 nM iberiotoxin (IbTx) were introduced to the external solution block BK_{Ca} channel currents. Furthermore, 7 mM KCl was used in the external solution, rather than 30 mM, in order to increase the K^+ driving force and obtain larger currents.

A stated previously, $I_{K(A)}$ cannot be recorded in isolation from $I_{K(DR)}$ because there are no selective blockers of insect $I_{K(DR)}$. $I_{K(A)}$ were isolated using a 1-sec prepulse followed by a 100 ms test pulse to +40 mV. Voltage protocols were designed to exploit the sensitivity of $I_{K(A)}$ to depolarised test potentials ((Grolleau and Lapied, 1995b) see chapter 6 for experimental details). Initially $I_{K(A)}$ and $I_{K(DR)}$ were elicited by test pulses with prepulse potentials of –100 mV (i). On every alternate pulse $I_{K(DR)}$ were recorded in isolation with a prepulse to –20 mV (ii) whereby all $I_{K(A)}$ were rendered inactive for the following +40 mV test pulse. In order to isolate $I_{K(A)}$, the currents resulting from test pulse (ii) (–20 mV prepulse) were digitally subtracted offline from the currents resulting from pulse (i) (–100 mV prepulse). Each $I_{K(A)}$ trace was isolated by subtracting only the immediately following trace current. Pulse protocols were delivered at 0.33 Hz.



To determine the voltage-dependence of K_A current activation, families of $I_{K(A)}$ were evoked. Families of test potentials were also used to evoke $I_{K(A)}$ by using prepulse protocols. Initial test pulses including 1-second –100 mV prepulses followed by 100-ms test pulses from –80 to +60 mV in 10-mV increments were used to evoke K_A and K_{DR} channel currents. Secondly, depolarising test pulses from –80 to +60 mV in 10-mV increments, preceded by 1-sec prepulse potentials to –20 mV, were used to inactivate $I_{K(A)}$. In order to isolate $I_{K(A)}$ families, currents resulting from prepulse –20 mV protocols were subtracted from those evoked in the presence of the –100 mV prepulse. Pulse protocols were applied at 0.33 Hz.



2.4.2 TRANSMITTER-ACTIVATED CURRENTS

Transmitter-activated currents were recorded under slightly altered conditions from voltage-activated channel currents. Whole-cell currents were recorded using version 10 of the PCLAMP data acquisition system (Molecular Devices, Sunnyvale, CA, USA). Data were filtered at 5 kHz with a low-pass Bessel filter. Digital sampling rates were set between 2 and 5 kHz depending on the length of the voltage protocol. Single use electrodes were pulled from borosilicate glass to d.c. resistances of *ca*. 1–2 MΩ for glutamate- , γ-aminobutyric acid- (GABA) and nicotinic-acetylcholine-activated (nACh) channel current recordings. Channel currents were activated by neurotransmitters delivered through a pressurised Picospritzer system (Parker, Castle Hill, Australia). Solutions under pressure (2–5 PSI) were introduced through a glass micropipette (resistance <0.5 MΩ when filled with agonist) positioned within 50 μm of the DUM neuron. This system allowed for controlled and direct application of agonist while minimising channel desensitisation, which occurs from bath application. With a

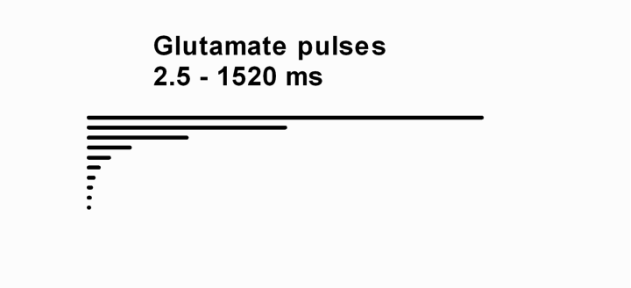
constant bath flow of 1 ml/min the agonist was rapidly removed from the vicinity surrounding the cell. In no experiment did the pressurised injection of normal saline with the same protocol result in any deviation from the baseline current. Steady-state recordings were made at least 15 minutes following the initiation of the whole-cell configuration.

pCLAMP digital inputs were employed to program agonist pulse protocols of varying durations. Droplets injected under oil immersion were measured by an ocular micrometer to confirm a linear relationship between the volume delivered and the pulse duration. From this data the logarithmic concentration of the applied agonist at any point on the cell membrane will be approximately proportional to the pulse duration, assuming a constant pressure is employed (McCaman, McKenna et al., 1977). This finding has been reported from a number of previous studies on the same preparation (Lapied, Corronc et al., 1990; Courjaret, Grolleau et al., 2003). Therefore, by increasing the injection time at a constant pressure an agonist dose-response relationship may be established.

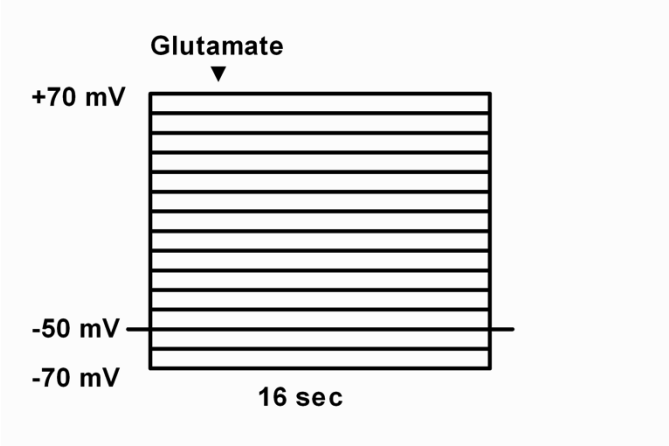
2.4.2.1 Glutamate-activated chloride (Glu-Cl) channel currents

To record whole-cell inward glutamate-activated chloride channel currents ($I_{Glu\text{-}Cl}$) the external bath solution consisted of (in mM): NaCl 167, K gluconate 33, KCl 3.1, $MgCl_2$ 4, $CaCl_2$ 5, HEPES 10, pH adjusted with 1 M NaOH. The internal pipette solution contained (in mM): NaCl 15, KCl 170, $MgCl_2$ 1, $CaCl_2$ 0.5, EGTA 10, HEPES 20, phosphocreatine-diTris 10 and 3 ATP-Mg_2, pH adjusted with 2 M KOH. 100 µM L-glutamate was employed to evoke Glu-Cl channel currents.

To determine channel sensitivity to glutamate digital inputs encoding increasing pulse durations at 2.5, 5, 10, 20, 40, 80, 160, 380, 760, 1520 ms were used. Inward glutamate-activated chloride currents ($I_{Glu\text{-}Cl}$) were evoked at 1-min intervals. Recorded at a frequency of 2 kHz, protocol lengths were set at 10 seconds in order to make optimal current recordings. 10-ms pulses of 100 µM glutamate were used for single pulse protocols.



In order to assess any changes in the ionic conductance, digitally programmed 10-ms glutamate pulses were used to evoke $I_{Glu\text{-}Cl}$. The membrane voltage was stepped in 10-mV intervals from –70 mV to +70 mV for durations of 16.25 sec from a holding potential of –50 mV. Glutamate pulses were applied 3.75 seconds into the voltage step and currents were allowed to resolve before stepping back to the holding potential. The membrane potential was held at –50 mV between pulses and during the first and last 5 ms of the pulse protocol. Currents were evoked at 1-min intervals.

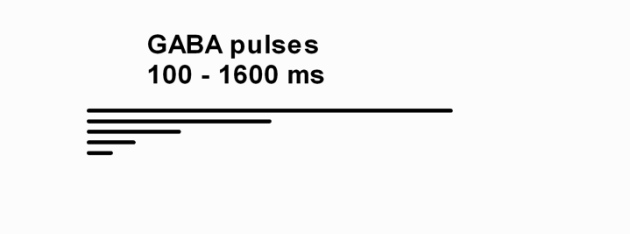


2.4.2.2 γ-Aminobutyric Acid-activated Chloride (GABA-Cl) Channel Currents

Whole-cell inward GABA-activated chloride channel currents (I_{GABACl}) were evoked by 100 μM GABA pulses. The external bath solution for recording I_{GABACl} included (in mM): NaCl 167, K gluconate 33, KCl 3.1, $MgCl_2$ 4, $CaCl_2$ 5 and HEPES 10, pH adjusted with 1 M NaOH. The internal pipette solution consisted of (in mM): NaCl 15, KCl 170, $MgCl_2$ 1, $CaCl_2$ 0.5, EGTA 10, HEPES 20, phosphocreatine-diTris 10, ATP-Mg_2 3, pH adjusted with 2 M KOH.

To assess channel sensitivity to GABA digital inputs encoding pulse durations of 100, 200, 400, 800, 1600 ms were designed. Inward GABA activated chloride (GABA-Cl) channel currents were evoked at 1-minute intervals. Recorded at a frequency of 2 kHz,

GABA pulse durations were set at 400-ms in order to record currents of optimal amplitudes.

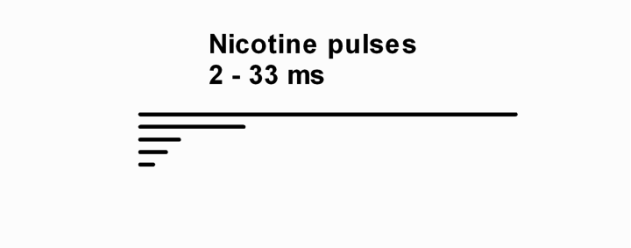


In order to assess potential changes in ionic conductance digitally programmed 400-ms GABA pulses were used to evoke GABA-Cl channel currents. The membrane potential was stepped in 10-mV intervals from –70 mV to +70 mV for durations of 16.25 sec from a holding potential of –50 mV. GABA pulses were applied 3.75 seconds into the voltage step and currents were allowed to resolve before stepping back to the holding potential. The membrane potential was held at –50 mV between pulses and during the first and last 5 ms of the pulse protocol.

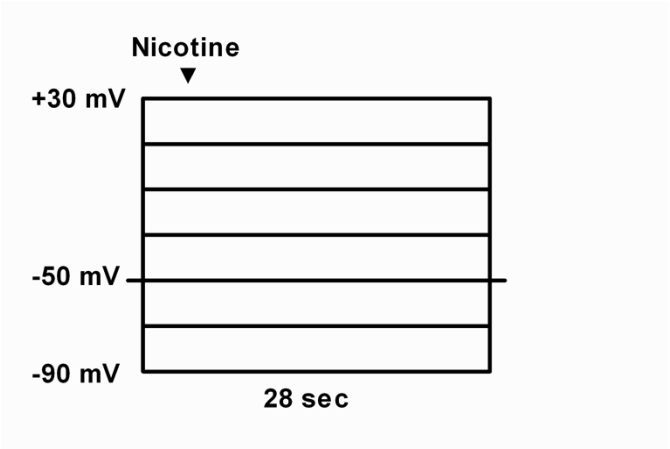
2.4.2.3 NICOTINIC-ACETYLCHOLINE RECEPTOR (NACHR) CHANNEL CURRENTS

The external bath solution for recording nAChR channel currents (I_{nAChR}) included (in mM): NaCl 200, KCl 3.1, $CaCl_2$ 5, $MgCl_2$ 4, HEPES 10 and 300 nM TTX, pH adjusted with 1 M NaOH. The internal pipette solution contained (in mM): NaCl 10, KCl 170, $CaCl_2$ 0.5, $MgCl_2$ 1, HEPES 20, EGTA 10**,** ATP-Mg_2 3, pH adjusted with 2 M KOH. 10 μM nicotine pulses were employed to evoke I_{nAChR} (see below for pulse protocols).

In order to channel sensitivity to nicotine digitally programmed pulse durations of 4, 5, 6, 7, 12, 33 ms were used to evoke nicotinic acetylcholine receptor (nAChR) associated currents at 1-minute intervals. Channel currents evoked by 10-ms nicotine pulses were recorded at a frequency of 2 kHz for durations of 40-ms.



To assess any changes in the ionic conductance of I_{nAChR} digital inputs programming 10 ms nicotine pulses were employed. At 1-min intervals the membrane potential was stepped in 20-mV intervals from –90 mV to +30 mV from a holding potential of –50 mV. Nicotine pulses were applied 3.75 seconds into the voltage step and currents were allowed to resolve before stepping back to the holding potential. Pulses were 16.25 sec in duration and the membrane potential was held at –50 mV between pulses and during the first and last 5 ms of the pulse protocol.



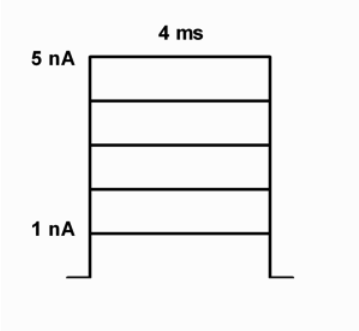
In order to assess the ability of a toxin to reverse nAChR desensitisation to nicotine 5-minute nicotine pulses were applied at low pressure (2–3 psi) via the Picospritzer pressure ejection system. Whole-cell nAChR channel currents were recorded at 2 kHz. Toxin was only applied when channels were fully desensitised (currents returned to baseline).

2.4.3 CURRENT-CLAMP: ACTION POTENTIAL PROTOCOLS

To record action potentials under current-clamp conditions the external solution contained (in mM) NaCl 190, KCl 3.1, $CaCl_2$ 5, $MgCl_2$ 4 and HEPES 10. The pH was adjusted to 7.4 with 1 M NaOH and the osmolarity was adjusted to 400 ± 5 mOsmol/L with sucrose. Internal pipette solutions for recording action potentials included (in mM): K gluconate 160, KF 10, $CaCl_2$ 0.5, NaCl 15, $MgCl_2$ 1, HEPES 10 and EGTA 10. The pH was adjusted to 7.4 with 2 M KOH and osmolarity was also adjusted to 400 ± 5 mOsmol/L with sucrose.

Under current-clamp conditions, the voltage threshold of action potential generation was determined by increasing current amplitude. 4-ms duration pulses from 1 nA to 5

nA at 1-nA intervals were applied at 0.1 Hz. The minimum current at which an action potential was evident was regarded as the threshold of activation. Current-clamp data were rejected if the initial resting membrane potential was more depolarized than –40 mV.



DUM neurons are spontaneously active and at resting membrane potentials, most DUM neurons are capable of generating repetitive action potentials with firing frequencies of around 6–7 Hz (Grolleau and Lapied, 2000). Gap-free recordings for 5 seconds were made at one-minute intervals in order to record any spontaneous overshooting action potentials. No current stimuli were applied under these recording conditions.

To eliminate any influence of differences in osmotic pressure, all internal and external solutions were adjusted to 400 ± 5 mOsmol/l with sucrose. Experiments were rejected if there were large leak currents or currents showed signs of poor space clamping.

2.4.4 EXPERIMENTAL PROCEDURES

2.4.4.1 PATCH-CLAMP SET UP

The electrophysiological characterization of nervous system toxins can be achieved through the whole-cell patch-clamp technique. A Huxley-style micromanipulator was positioned to the right of an inverted phase-contrast microscope for visualization of isolated DUM neurons. On the stage of the microscope a glass bottom perfusion chamber (1 ml volume, RC series, Warner Instruments, Hamden, CT, USA) was mounted to house isolated neurons. The coverslip, with adherent cells, was placed within the chamber and continuously bathed in an external solution delivered by a pressurized fast-perfusion system (Automate Scientific, Berkeley, CA, USA) at a rate of 1 ml/min at 2 PSI. To the left of the microscope a multibarrel 4–8 perfusion pencil was fixed to an additional manipulator. The pencil was attached to an eight-way pressurized

fast-perfusion system allowing rapid exchange between varying solutions. Attached to the end of the pencil a 100 μm (3.8 cm length, polyimide needle, World precision instruments, USA) needle was placed within 100 μm of the DUM neuron whereby the cell was directly bathed in solution.

The microscope, perfusion system and associated equipment were positioned on a vibration isolation work table (Newport, Irvine, CA, USA) in order to minimize vibrations above 5 Hz. All equipment was also situated inside a Faraday cage (Newport series 3036) to minimise any 50 Hz mains and radio frequency noise that may have disrupted recording.

To the right of the microscope the recording electrode was secured to the micromanipulator. A glass pipette filled with internal solution was fitted over the recording electrode, which consisted of a Teflon coated silver wire (A-M systems Inc., Carlsborg, WA, USA). The tip of the recording electrode was coated with $Ag/AgCl_2$ acting as a conducting interface between the internal solution and the CV201A headstage input (Axon instruments Inc., Foster City, CA, USA).

Single-use glass pipettes were prepared prior to each experiment. Pipettes were pulled using a Flaming/Brown micropipette electrode puller (model P-87, Sutter Instruments Co., Novato, CA, USA) from glass capillary tubes (1.5 mm o.d., 1.16 mm i.d., 7052 glass, Harvard apparatus Ltd, Kent, England). The electrode puller was programmed according to the pipette resistance required. Further refinement of electrode tips was achieved by means of fire polishing with a MF-83 microforge (Narashige Scientific Instruments Inc., Tokyo, Japan). Glass pipettes were filled with internal solution prior to recording. The fine tip of the pipette was filled by means of capillary action while the remaining tubing was backfilled using a long tipped 30 G syringe needle. All bubbles were expelled through gentle tapping.

To the rear of the main bath housing the coverslip, a secondary bath was positioned from where the bath level was maintained. A fitted suction tube was positioned to remove solution without disrupting the patch-clamp recording set up. The bath electrode holder was positioned to the left of the bath and fitted with a KCl salt bridge. The salt bridge consisted of a short length of polyethylene tubing (1.5 mm outer diameter, Microtube extrusions, North Rocks, Sydney) filled with 3% agar in 3 M KCl. The bath

electrode encases an $Ag/AgCl_2$ which operates to interface with the external solution. Both recording and bath electrodes where re-chlorided with a weak (~20%) sodium hypochlorite solution, as needed, in order to further minimise noise.

2.4.4.2 PATCH-CLAMP TECHNIQUE

Single 12 mm coverslips containing adherent *P. americana* DUM neurons were placed within the microscope perfusion chamber. Single, isolated DUM neurons of healthy appearance were selected for recordings. Only cells that had a clearly visible, intact membrane and were not in contact with surrounding cells were selected for experiments. Round, light cells with diameters in excess of 40 μm and the characteristic teardrop shape were chosen. Cells with extensive neurite or axon growth were not selected to avoid issues related to poor space clamping of the membrane potential. Cells with poor space clamping display large activation of channel currents upon small depolarisations. Selected cells were positioned centrally in the field of view under high power magnification (× 320).

The fast perfusion needle was then positioned just outside the field of view, at approximately 100 μm from the left-hand side of the cell. The needle was positioned at a 45-degree angle from the base of the coverslip however the tip of the needle was never allowed to touch the coverslip. The function of the perfusion needle was to directly perfuse the cell with either control or toxin solution at all times.

On the right-hand side of the cell, the recording electrode fitted with the glass micropipette was positioned at a 45-degree angle. The recording electrode was lowered into the bath solution using the course movement control of the Huxley style micromanipulator.

Additionally, a third pipette was positioned from the rear of the bath for application of agonists for the recording of ligand-activated channel currents. The ligand delivery pipette was positioned at a 45-degree angle from the coverslip and positioned between the patch pipette and perfusion needle. The ligand pipette tip was lowered until it was positioned just above the coverslip and at a distance of 100 μm from the cell. Movement was similarly controlled by a Sutter MM-33 micromanipulator (SDR, Middlecove, Sydney).

Once the patch pipette was lowered into the bath solution the liquid junction potential and pipette offset were adjusted on the Axopatch 200A integrating patch-clamp amplifier (Axon Instruments Inc., Foster City, CA, USA). The pipette offset control was adjusted to 0 pA, prior to patching onto the cell. The liquid junction potential takes into account the differences in ion mobility between internal and external solutions. This value was calculated using the program JPCalc (Barry, 1994). The liquid junction potential (LJP) between the external bath and internal pipette solutions for recording $Na_{V,}$ Ca_V and K_V channel currents were adjusted prior to recording. All current recordings were compensated for this difference by zeroing-off the holding current at a membrane potential of –8.7 mV (I_{Na}), –14.6 mV (I_{Ba}) and –4.7 mV (I_K, $I_{K(Ca)}$ and $I_{K(DR)}$), –5.0 mV ($I_{K(A)}$), –8.0 mV ($I_{K(Na)}$), –14.2 mV (action potentials), –4.3 mV (I_{nAChR}) and –2.5 mV ($I_{Glu\text{-}Cl}$ and $I_{GABA\text{-}Cl}$).

Prior to lowering the patch pipette onto the cell, the response to a continuous 5-mV line-frequency square wave test pulse was recorded on an oscilloscope (Tektronix, TDS 420 Series) as a square current wave varying in amplitude according the tip resistance of the recording electrode. The tip diameter was altered according to the type of channel current to be recorded (see section 2.4.1.1).

Patching onto a cell involves two discrete steps, the formation of the gigaohm seal and formation of the whole-cell patch clamp configuration. Firstly, in order to form a gigaohm seal the pipette was positioned directly above the centre of the cell with the micromanipulator fine control under the microscope. The initial contact between the cell and the pipette was visualised as a sharp dip in the 5-mV square wave on the oscilloscope. Immediately following the dip, gentle suction was applied through fine tubing attached to the side of the electrode holder. Manual suction was continued until no current was visible, indicating formation of the gigaohm seal. Following the formation of a gigaohm seal the holding potential was set manually on the patch-clamp amplifier (Axopatch 200A integrating patch-clamp amplifier, Axon Instruments, Foster City, CA, USA). Again, gentle suction was applied until the broadening of the capacitive transient currents was observed on the oscilloscope. This broadening was indicative of the pipette rupturing the cell membrane and the internal solution becoming contiguous with the cell contents—the whole-cell configuration.

Cell recordings were only undertaken if a stable seal between the cell membrane and the pipette was maintained. Experiments with high leakage currents (>1 nA) and poor space clamping were rejected. These characteristics are obvious signs of poor seals and inadequate control of membrane potential. Current recordings from cells with stable leakage currents throughout the experiment were used for analysis.

The series resistance and capacitance were adjusted manually on the patch-clamp amplifier. Controls were adjusted until capacitive currents were small and fast, with >80% compensation. Transient capacitive currents were compensated to minimise their influence on channel current recordings.

2.5 CURVE-FITTING AND STATISTICAL ANALYSIS

Data analyses, using AXOGRAPH X version 1.1 (Molecular Devices), were completed off-line at the conclusion of experiments. Mathematical curve fitting was accomplished using PRISM version 5.00b for Macintosh (GraphPad Software, San Diego, CA, USA). All curve-fitting routines were performed using non-linear regression analysis employing a least squares method. Comparisons of two sample means were made using a paired Student's *t*-test. Multiple comparisons were assessed by repeated measures analysis of variance (ANOVA) with a Bonferroni's multiple comparison post-hoc test; differences were considered to be significant if $p < 0.05$. All data are presented as mean ± standard error of the mean (SEM) of *n* independent experiments, unless stated otherwise.

The following equation was employed to fit current-voltage (*I-V*) curves:

$$I = g_{max}\left(1 - \frac{1}{1 + \exp\left[\left(V - V_{1/2}\right)/s\right]}\right)\left(V - V_{rev}\right) \qquad \textit{Equation 1}$$

Where I is the amplitude of the peak current (either I_{Ba}, I_{Na}, I_K, $I_{Glu\text{-}Cl}$, I_{GABA} or I_{nAChR}) at a given test potential V, g_{max} is the maximal conductance, $V_{1/2}$ is the voltage at half-maximal activation, s is the slope factor, and V_{rev} is the reversal potential.

Concentration-response curves were fitted using the following Logistic equation:

$$y = \frac{1}{1 + \left([x]/IC_{50}\right)^{n_H}} \qquad \textit{Equation 2}$$

Where x is the toxin dose, n_H is the Hill coefficient (slope parameter), and IC_{50} is the concentration at which 50% block of channel current is evident. In the case of concentration response curves to neurotransmitters the IC_{50} values was substituted by the EC_{50}.

2.6 SOURCES OF CHEMICALS

All chemicals were of analytical grade. $CaCl_2$, $MgCl_2$, KH_2PO_4 and Na_2HPO_4 were obtained from Merck Chemicals (Kilsyth, Victoria). Charybdotoxin, iberiotoxin and paxilline were purchased from Alomone Labs (Jerusalem, Israel). All remaining chemicals were obtained from Sigma Aldrich (Castle Hill, NSW).

Chapter Three: Windley et al., 2011

A novel family of insect-selective peptide toxins targeting insect large-conductance calcium-activated K^+ channels isolated from the venom of the theraphosid spider *Eucratoscelus longiceps*

Declaration of Contribution

The following chapter consists of a paper published in the Journal of Molecular Pharmacology. The article is based on research pursued within my PhD candidature. All electrophysiological experimentation and related experimental design and analysis were performed by me. Furthermore, I was the principal author of this paper and chief contributor to the initiation, design, development and writing.

I also acknowledge the contribution of Dr Pierre Escoubas for the isolation, purification, sequencing and biochemical characterisation of the peptide toxins.

X	X
Monique J. Windley Candidate	Prof. Graham M. Nicholson Supervisor

J. Windley

[2012]

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Windley, MJ et al.. 2011 "A Novel Family of Insect-Selective Peptide Neurotoxins Targeting Insect Large-Conductance Calcium-Activated K^+ Channels Isolated from the Venom of the Theraphosid Spider Eucratoscelus constrictus."
Molecular Pharmacology Vol 80. No 1. p1-13
http://molpharm.aspetjournals.org/content/80/1/1.full

Chapter Four

Proteolytically stable structural modifications of κ-HXTX-Hv1c and ω-HXTX-Hv1a

Monique J. Windley

[2012]

4.1 INTRODUCTION

The susceptibility of peptides to enzymatic degradation and ensuing poor stability is a challenge that has somewhat limited the development of peptides for use in insecticide and pharmaceutical drug design. Although spider peptide toxins are considered quite stable due to presence of cross bracing disulfide bonds, in many cases forming an inhibitory cystine-knot, there is still progress that can be made to improve peptide stability.

One method employed to reduce proteolytic degradation by exoproteases has been adapted from a family of plant-based peptides. Cyclotides consist of a topologically unusual motif known as the cyclic cysteine knot (Craik, Daly et al., 1999; Rosengren, Daly et al., 2003; Craik, Mylne et al., 2010; Craik, 2010). These small peptides of ca. 30 amino acids possess not only the typical interlinking disulfide bonding patterns of spider toxins but also consist of a cyclic peptide backbone (Craik, Daly et al., 1999; Craik, 2001; Gruber, Cemazar et al., 2007; Craik, Mylne et al., 2010; Craik, 2010). The practice of joining the C- and N- terminus of these molecules affords exceptional stability to these peptides which allows the molecules to retain biological activity subsequent to boiling and provides significant resistance to enzymatic attack (Colgrave and Craik, 2004; Cemazar and Craik, 2006; Halai, Callaghan et al., 2011).

In practice, the insertion of a peptide linker has been successfully utilised to join the N- and C- terminus of linear peptides (Clark, Fischer et al., 2005; Armishaw, Jensen et al., 2011). The use of cyclisation to improve peptide stability has been effectively reproduced in a number of peptide toxins isolated from cone snails (Clark, Fischer et al., 2005; Lovelace, Armishaw et al., 2006; Armishaw, Dutton et al., 2010; Clark, Jensen et al., 2010; Armishaw, Jensen et al., 2011; Halai, Callaghan et al., 2011; Lovelace, Gunasekera et al., 2011). Cyclic peptides formed from α-conotoxin MII (Clark, Fischer et al., 2005) and χ-conotoxin MrIA (Lovelace, Armishaw et al., 2006) demonstrated improved stability towards enzymatic degradation while maintaining both tertiary structure and biological activity.

Another avenue for improving peptide stability lies with the strengthening of the cross bracing disulfide bonds. Disulfide bonds exist as covalent bonds between the thiol groups of cysteine residues with a function to direct three-dimensional folding and

therefore stabilise the resulting structure (Norton and Pallaghy, 1998; Craik, Daly et al., 2001). Disulfide bonds are critical for peptide function (Wang, Connor et al., 2000) and are found in a diverse range of secretory peptides including peptide hormones, growth factors and peptide toxins found in animal venoms (Escoubas and King, 2009).

Furthermore, nuclear magnetic resonance (NMR) is the major technique employed to elucidate peptide structure provides insufficient information on disulfide bonding patterns (Mobli, de Araújo et al., 2009). The inability to determine bonding patterns lies with the unfavourable NMR properties of sulphur. The solution is to replace the NMR inactive S^{32} with a similar chemical. Selenocysteine is a replacement that occurs within nature (Kryukov, Castellano et al., 2003), and involves replacement by a residue almost identical to Cys, where the sulphur molecules are replaced by selenium (Se). Importantly, this replacement can be achieved both chemically and recombinantly without any changes to the overall structure or function of the peptide (Gettins and Wardlaw, 1991; Müller, Senn et al., 1994; Mobli, de Araújo et al., 2009).

Moreover, not only does selenocysteine incorporation afford the observation of native proteins in solution, benefits are also applied to the stability of the peptide structure. In comparison to the disulfide bond, the diselenide bond is highly resistant to air oxidation and is not attacked by non-oxidising acids (Müller, Senn et al., 1994; Mobli, de Araújo et al., 2009). Therefore, while a portion of disulfide bound peptide toxins may remain in a reduced state in solution, the selenocysteine incorporation insures this scenario does not occur.

However, for the purpose of this study diselenide replacement was predominantly employed to explore the function of the critical vicinal disulfide ring (VDR) in κ-HXTX-Hv1c. The vicinal disulfide bond is formed between two adjacent Cys residues at positions 13 and 14 of this 37 residue peptide (Wang, Connor et al., 2000). Only 11 unrelated proteins in the protein data base (PDB) possess vicinal disulfide bonds (Hudáky, Gáspári et al., 2004); however they remain functionally important in each case (Table 4.1). For example, the VDR at the agonist binding site of the nicotinic acetylcholine receptor (nAChR) is critical for ligand binding (Craik, 2010) and reduction of the VDR in methanol dehydrogenase abolishes enzymatic activity (Craik, 2001). A VDR is also present in transient peptide intermediates that occur during

oxidative folding of a cystine knot protein, where the constraints imposed by the VDR presumably assist the folding process (Clark, Fischer et al., 2005).

Furthermore, the VDR of κ-HXTX-Hv1c plays a role other than merely stabilising the structure as revealed by the complete loss of bioactivity when Cys was isosterically replaced by serine (Wang, Connor et al., 2000; Gunning, Maggio et al., 2008). Due to the importance of the VDR role in toxin activity it has been theorised that the structure may act as a redox-reaction switch (Carugo, Cemazar et al., 2003) or the VDR constitutes a special binding recognition site. A series of experiments were designed to distinguish between these two scenarios in which the native vicinal (S–S) was replaced by the more stable diselenide (Se–Se) linkage. The modification was designed to minimise possible redox reactions while still maintaining the ring scaffold structure of the linkage.

Table 4.1 Some proteins that posses the VDR motif and its function.

Protein	VDR importance
nAChR	Binding of acetylcholine is sensitive to the oxidation state of the vicinal cysteines (Kao and Karlin, 1986)
Methanol dehydrogenase	Reduction inactivates enzyme; VDR assists electron transfer or protein conformation (Avezoux, Goodwin et al., 1995)
RNase H1	Redox-conformational switch: Significant change in protein
hSH3 domain of ADAP	conformation from reduced to oxidised vicinal cysteines (Lima, Wu et al., 2003; Zimmermann, Kuhne et al., 2007)
Mercuric ion reductase	Oxidation inactivates enzyme (Moore, Miller et al., 1992)
ArsD repressor	Vicinal cysteines important for binding of arsenium (Li, Chen et al., 2001)
Human glyoxalase 1	VDR involved in glutathionylation (Birkenmeier, Stegemann et al., 2010)

This study aimed to assess the effects of cyclisation and selenocysteine (Sec) replacement on toxins isolated from the venom of the Blue Mountains funnel-web spider, *Hadronyche versuta*. As potential insecticidal compound leads, the stability of these toxins is paramount to their success. In this study we assessed the bioactivity of cyclised ω-HXTX-Hv1a and diselenide (Cys13, Cys14) κ-HXTX-Hv1c on their established ion channel targets in insects.

4.2 RESULTS

4.2.1 DISELENIDE κ-HXTX-HV1C

The selenocysteine-modified κ-HXTX-Hv1c was generously provided by Prof. Glenn F. King (Institute for Molecular Bioscience, University of Queensland, Australia). The toxin was built using Boc-mediated solid phase peptide synthesis (SPPS) incorporating Sec residues at positions 13 and 14 (Figure 4.1), as detailed in Mobli *et al*. (Mobli, de Araújo et al., 2009). Both native and diselenide toxin structures were found to overlay well with a backbone root mean squared deviation (rmsd) of 0.91 Å (Mobli, de Araújo et al., 2009).

κ-HXTX-Hv1c has been electrophysiologically characterised as a high affinity blocker of insect BK_{Ca} channels (Gunning, Maggio et al., 2008). Importantly, the vicinal disulfide is known to play a crucial role in toxin function (Wang, Connor et al., 2000). Studies have established that while Cys replacement by Ser does not significantly alter toxin structure, insecticidal activity is completely absent (Wang, Connor et al., 2000). Having clearly established the functional significance of the vicinal disulfide linkage for toxin function, the next step was to further understand the nature of the interaction with insect BK_{Ca} channel.

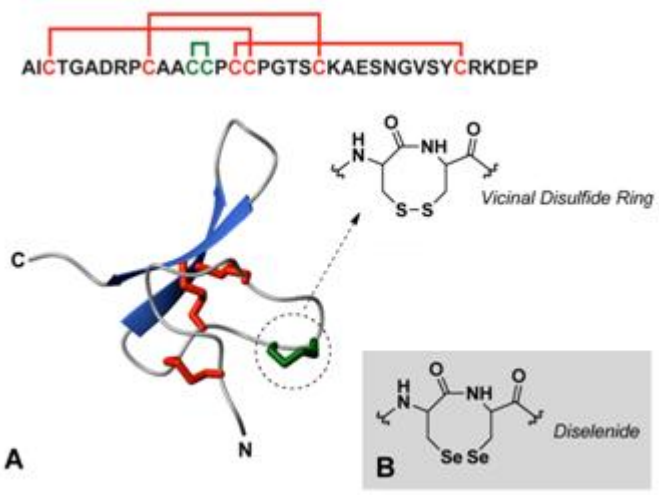

Figure 4.1: Primary and tertiary structure of κ-HXTX-Hv1c. (A) The three disulfide bonds that form the inhibitory cysteine knot (ICK) motif commonly found in spider peptides are indicated by red lines. The vicinal disulfide bond between Cys13 and Cys14 is highlighted in green, and the structure of the eight-membered vicinal disulfide

ring is shown in the inset. **(B)** The structure of the eight-membered diselenide ring replacing the vicinal disulfide ring in the diselenide derivative.

4.2.2 Effects of native and diselenide κ-HXTX-Hv1c on BK_{Ca} channels

The biological activities of both native- and diselenide-modified κ-HXTX-Hv1c were assessed by comparing their ability to block cockroach BK_{Ca} channels. Electrophysiological recordings were elicited from isolated cockroach DUM neurons. The IC_{50} for block of cockroach BK_{Ca} channels was previously determined to be 3 nM (Gunning, Maggio et al., 2008). Accordingly, the diselenide toxin was tested at the same concentration to determine if diselenide incorporation altered toxin interaction with the target channel.

Whole-cell BK_{Ca} currents were evoked by 100-ms voltage pulses to +30 mV from a holding potential of –80 mV at 0.2 Hz. In order to record BK_{Ca} currents in isolation, current offline subtraction routines were employed following perfusion with BK_{Ca} blockers. Control $I_{BK(Ca)}$ and $I_{K(DR)}$ were recorded in the presence of 4-aminopyridine to block $I_{K(A)}$, TTX to block Na_V and $CdCl_2$ to block Ca_V. Neuron currents were subsequently recorded in the presence of 3 nM diselenide toxin for a period of 10 min or until equilibrium was reached. At the conclusion of experiments, isolated $I_{K(DR)}$ were recorded in the presence of 100 nM iberiotoxin. The remaining $I_{K(DR)}$ was then digitally subtracted. In parallel experiments, whole-cell $I_{BK(Ca)}$ were also recorded in the presence of the synthetically produced native κ-HXTX-Hv1c.

At 3 nM both toxins were shown to result in a similar block of insect BK_{Ca} channels. Native and diselenide toxins reduced peak $I_{BK(Ca)}$ by 49 ± 4% (n = 4) and 60 ± 4% (n = 4), respectively (Figure 2 A and B). Sustained $I_{BK(Ca)}$ were also reduced correspondingly. The concentration-response relationship was established by plotting the percentage block of maximal current amplitude in the presence of varying concentrations of toxin and fitted with Eq. 2 in Chapter 2, section 5. The IC_{50} values were calculated as 3.5 ± 0.9 nM (n = 3–4) and 1.5 ± 0.4 nM (n = 3–4) for native and diselenide toxin, respectively (Figure 4.2C). However, a one-way ANOVA followed by Bonferroni's post-hoc test revealed no significant differences between the dose-response curves ($p > 0.05$).

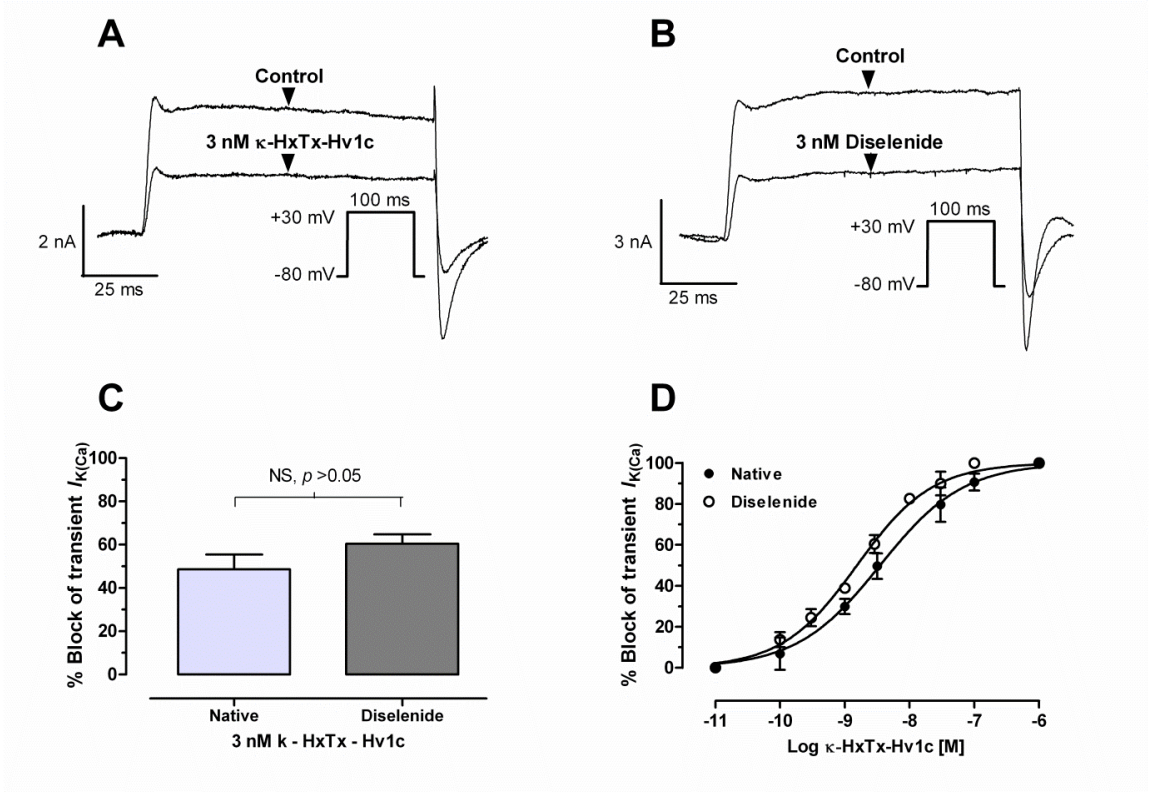

Figure 4.2: Effects of native and diselenide κ-HXTX-Hv1c on DUM neuron K_{Ca} channels. **(A**–**B)** Superimposed traces of $I_{BK(Ca)}$ representing the typical effect of 3 nM native **(A)** and 3 nM diselenide modified κ-HXTX-Hv1c. **(C)** A comparison of $I_{BK(Ca)}$ inhibition in the presence of 3 nM native and diselenide κ-HXTX-Hv1c with values of 49 ±7 and 60 ± 4%, respectively (n = 4). NS: not significant (p > 0.05), one-way ANOVA. **(D)** Dose-response curve of transient $I_{BK(Ca)}$ inhibition in the presence of native (closed circles) or diselenide (open circles) κ-HXTX-Hv1c, yielding respective IC_{50} values of 3.5 and 1.5 nM (n = 3–4). All $I_{BK(Ca)}$ were evoke by the test pulse protocol illustrated in the insets of traces (A) and (B).

The voltage-dependence of channel block was also determined in the presence of both native and diselenide toxins. Families of $I_{BK(Ca)}$ were evoked by 100-ms test pulses from a holding potential of –80 mV to a maximum of +50 mV. $I_{BK(Ca)}$ were elicited at 10-mV increments at 0.2 Hz. Gunning *et al.* (Gunning, Maggio et al., 2008) previously reported channel block occurring in the presence of κ-HXTX-Hv1c in the absence of any alterations in the voltage dependence of BK_{Ca} channel activation. Similarly, Figure 4.3 shows comparable results for both native and diselenide toxins. In the presence of either

toxin, neither the voltage at half maximal activation ($V_{1/2}$) nor the threshold of channel activation were significantly shifted (n = 4–5, p > 0.05) for peak or sustained $I_{BK(Ca)}$.

Table 4.2 $V_{1/2}$ values for native and diselenide κ-HXTX-Hv1c on $I_{K(Ca)}$.

	$V_{1/2}$ (mV)			
$I_{K(Ca)}$	Control	native	Control	diselenide
Transient	17.3± 12.7	12.3 ± 2.7	19.7 ± 1.0	22.0 ± 4.7
Sustained	12.1 ±4.1	35.3 ± 4.6	18.2 ± 9.6	24.7 ± 6.8

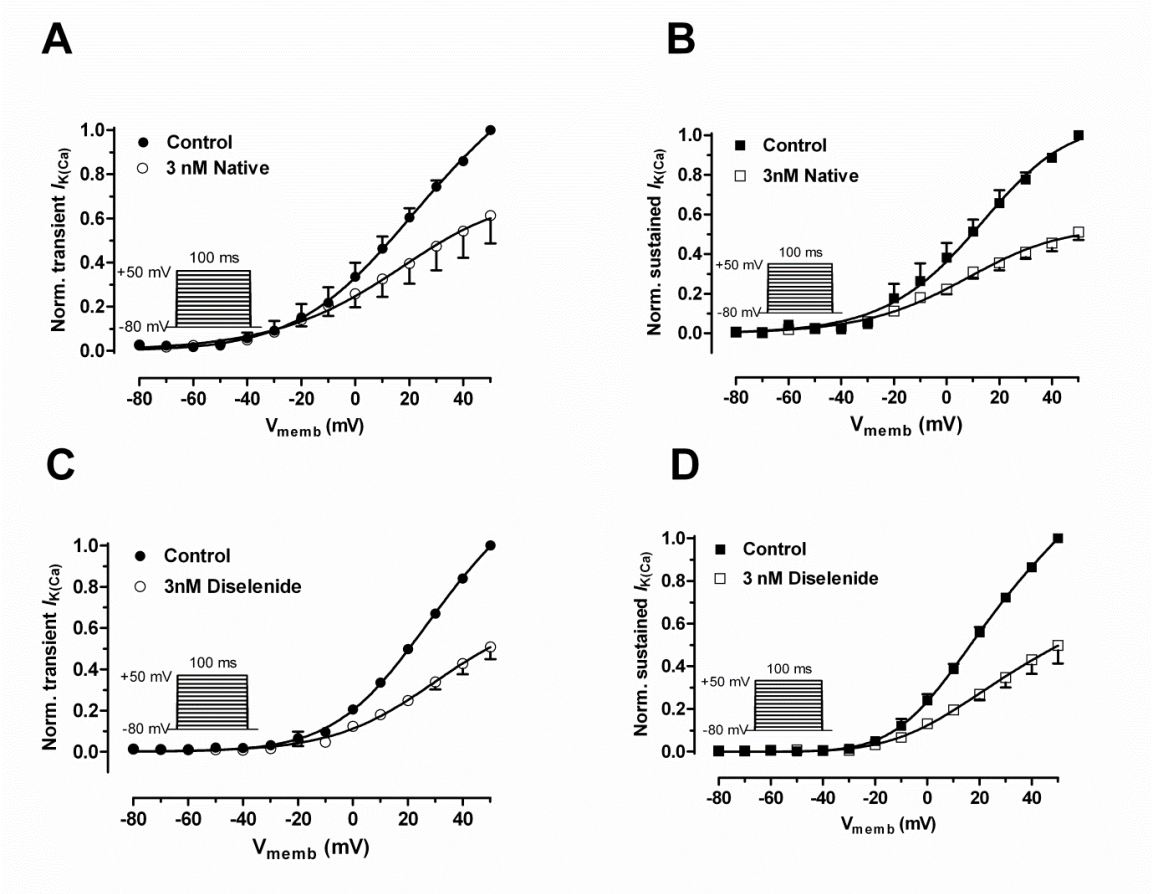

Figure 4.3: Effects of native and diselenide κ-HXTX-Hv1c on the voltage-dependence of BK_{Ca} channel activation. $I_{BK(Ca)}/V$, fitted by Eq. 1 (see Chapter 2, section 5), n = 3–6. Normalised $I_{BK(Ca)}/V$ for control (closed symbols) and in the presence of 3 nM native **(A**–**B)** and 3 nM diselenide **(C**–**D)** toxin (open symbols). Both transient **(A and C)** and sustained **(B and D)** $I_{BK(Ca)}$ were measured. All test pulse protocols used to evoke channel currents are shown in the insets of each panel.

4.2.3 CYCLIC ω-HXTX-HV1A

The second strategy aimed at improving toxin stability was the N- to C- terminal cyclisation of the peptide backbone. This approach was particularly appropriate for ω-HXTX-Hv1a due to the relatively close proximity of the N- and C- termini (Figure 4.4A). Similarly to κ-HXTX-Hv1c, the cyclic toxin was built using Boc-mediated SPPS with the addition of an eight-residue peptide linker (ASGSAGAS) and supplied by Profs Paul Alewood and Glenn F King. Cyclisation of the backbone was achieved by means of a native chemical ligation strategy (Dawson, Muir et al., 1994). Peptides were oxidized and cyclised at 0.1 mg/ml overnight in 0.1 M 3-morpholinopropane-1-sulfonic acid (MOPS), 0.2 M KCl, 1 mM ethane-1,2-diyldinitrilo tetraacetic acid (EDTA), 2 mM reduced glutathione, 0.5 M oxidized glutathione, pH 7.3. The reaction was quenched in 0.1% 2,2,2-trifluoroacetic acid (TFA). Peptides were purified by rp-HPLC to > 95% purity and the mass confirmed by electrospray mass spectrometry.

All the main structural features of native ω-HXTX-Hv1a were reproduced in the oxidised cyclic form (V. Herzig, A. Dantas de Araujo, K. P. Greenwood, M. J. Windley, Y. Chong, D. Wilson, M. Muttenthaler, M. Mobli, N. Audsley, G. M. Nicholson, P. F. Alewood, G. F. King, unpublished data). The antiparallel β-sheets, C-terminal β-hairpin and disulphide linkages were all maintained following toxin cyclisation. As the overall structure of the cyclic toxin was maintained it was important to also characterise the biological activity.

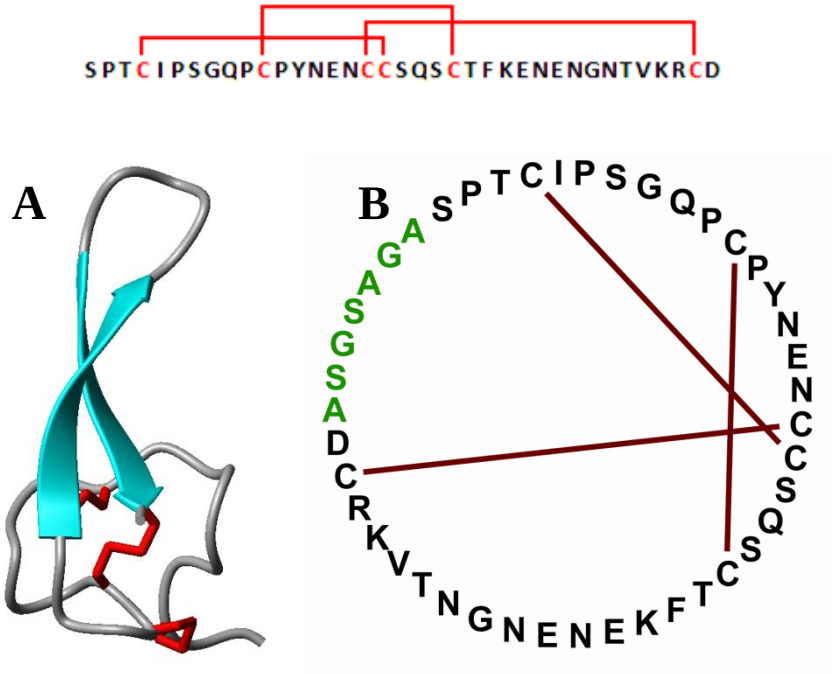

Figure 4.4: Primary and tertiary structures of ω-HXTX-Hv1a. The three disulfide bonds that form the inhibitory cysteine knot (ICK) motif commonly found in spider peptides are indicated by red lines. **(A)** NMR structure illustrated as a ribbon schematic of ω-HXTX-Hv1a (PDB 1AXH) highlighting the β-strands as cyan arrows. **(B)** A bracelet representation of cyclic ω-HXTX-Hv1a. The eight-residue linker is indicated in green with the sequence running in the clockwise direction, N- to C- terminus.

4.2.4 Effects of cyclic ω-HXTX-Hv1a on Ca_V channels

The insecticidal toxin ω-HXTX-Hv1a has been characterised as a relatively potent blocker of the ω-HXTX-Hv1a insect voltage-activated calcium (Ca_V) channel (Chong, Hayes et al., 2007). ω-HXTX-Hv1a has been shown to block cockroach M-LVA Ca_V channels in isolated DUM neurons with an IC_{50} of 278 nM (Chong, Hayes et al., 2007). High voltage activated (HVA) Ca_V channels were blocked to a lesser extent with an IC_{50} of 1080 nM, however 20–30% block was still observed at concentrations around 300 nM (Chong, Hayes et al., 2007). Subsequently, concentrations of 278 nM cyclic ω-HXTX-Hv1a were employed to assess the activity of the toxin on DUM neuron Ca_V channels.

Due to reports of significant I_{Ca} rundown associated with using calcium as the charge carrier and greater success with using barium (Wicher and Penzlin, 1997), $CaCl_2$ was replaced with $BaCl_2$ in order to record stable currents. Isolated I_{Ba} were recorded in the presence of K_V and Na_V channel blockers; tetraethyl-ammonium (TEA) and tetrodotoxin (TTX). Whole-cell currents were evoked by dual pulse 50-ms voltage steps from a holding potential of –90 mV. M-LVA and HVA currents were elicited alternately at 10-ms intervals by stepping the membrane potential to –30 and +30 mV, respectively. Following the establishment of stable control currents toxin was perfused for up to 10 min or until equilibrium was reached.

Similarly to native ω-HXTX-Hv1a, 278 nM cyclic toxin resulted in 37 ± 4% (M-LVA) and 19 ± 3% (HVA) block of I_{Ba} in DUM neurons (Figure 4.5). A one-way ANOVA followed by Bonferroni's post-hoc test further demonstrated that the activities of native and cyclic toxin were equipotent in relation to both M-LVA and HVA I_{Ba} block (n = 3–5, p > 0.05; Figure 4.5D). Similar to native ω-HXTX-Hv1a, the cyclic toxin was also found to induce a voltage independent block of M-LVA and HVA Ca_V channels. Families of I_{Ba} were evoked by 50-ms test pulses from a holding potential of –90 mV, every 10 seconds. The membrane potential was stepped to a maximum of +40 mV at 5-mV increments. The *I–V* relationship was determined by plotting the maximal I_{Ba} at each potential. The data was normalised against the peak maximal response of control data. Both native and cyclic toxins failed to significantly alter the $V_{1/2}$ (control –38.3 ± 1.5 mV vs. toxin –39.0 ± 2.6 mV; n = 5, p > 0.05) or the reversal potential (V_{rev} control 58.6 ± 2.9 mV vs. toxin 61.8 ± 7.8 mV; n = 6, p > 0.05; Figure 4.5C).

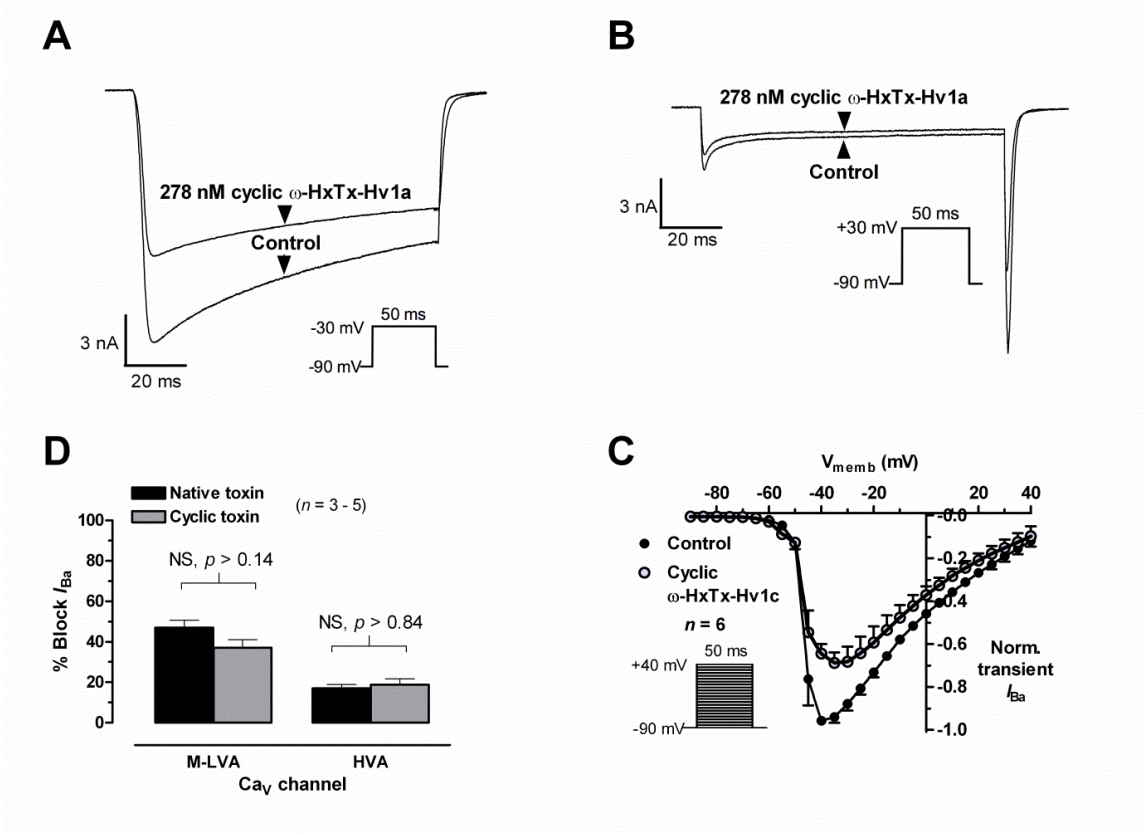

Figure 4.5: Effects of native and cyclic ω-HXTX-Hv1a on DUM neuron Ca_V channels. (A) Representative traces illustrating the typical effect of 278 nM cyclic ω-HXTX-Hv1a on HVA Ca_V channel currents. **(B)** Superimposed traces showing the typical effect of 278 nM ω-HXTX-Hv1a on M-LVA Ca_V channel currents. **(C)** A comparison of native ω-HXTX-Hv1a (Chong, Hayes et al., 2007) (black) and cyclic ω-HXTX-Hv1a (grey) block of M-LVA and HVA-Ca_V channels, with values of 47 ± 4% , 37 ± 4%, 17 ± 2% and 19 ± 3% (left to right). NS: not significant, one-way ANOVA ($p > 0.05$). **(D)** I_{Ba}/V for control (closed circles) and in the presence of 278 nM cyclic ω-HXTX-Hv1a (open circles) fitted with Eq. 1 (chapter 2, section 5). All I_{Ba} were evoked by voltage test pulses illustrated in the panel insets.

4.3 DISCUSSION

The aim of the present study was twofold: firstly, to utilise peptide cyclisation and diselenide bond formation as means to improve toxin stability, and secondly to explore the role of the vicinal disulfide ring in κ-HXTX-Hv1c function. Results indicate that cyclisation of ω-HXTX-Hv1a does not significantly alter toxin structure (V. Herzig, A. Dantas de Araujo, K. P. Greenwood, M. J. Windley, Y. Chong, D. Wilson, M.

Muttenthaler, M. Mobli, N. Audsley, G. M. Nicholson, P. F. Alewood, G. F. King, unpublished data) and toxin affinity for the Ca_V channel is maintained. As for the native toxin (Chong, Hayes et al., 2007), cyclic ω-HXTX-Hv1a inhibits both M-LVA and HVA at 278 nM. The results for native and cyclic toxin block of M-LVA and HVA I_{Ba} were not found to be significantly different (Figure 4.5).

Secondly, diselenide replacement of the vicinal disulfide bond in κ-HXTX-Hv1c was found not to affect the biological activity of the toxin. In this study the diselenide toxin was found to block insect K_{Ca} with an IC_{50} of 1.5 nM in comparison to 3.5 nM for the native toxin (Figure 4.2). A comparison between the dose-response relationships of the toxins found the results not significantly different ($p > 0.05$).

4.3.1 TOXIN CYCLISATION

Due to the oral toxicity demonstrated by ω-HXTX-Hv1a against insects (Fletcher, Smith et al., 1997; Mukherjee, Sollod et al., 2006), one aim of this study was to explore various techniques to improve toxin stability. Previous studies have revealed that ω-HXTX-Hv1a exhibits oral activity against ticks (*Amblyomma americanum*) but at a LD_{50} value 109-fold less than by injection (Mukherjee, Sollod et al., 2006), however this is better than the 714-fold loss of toxicity via the oral route for the scorpion toxin AaIT in sarcophagi flies (Zlotkin, Fishman et al., 2000; Mukherjee, Sollod et al., 2006). Moreover, ω-HXTX-Hv1a is also orally active against the agricultural pests *Helicoverpa armigera* and *Spodoptera littoralis* (Khan, Zafar et al., 2006) therefore indicating the potential of this toxin to be developed as a orally active insecticide.

Cyclisation of ω-HXTX-Hv1a has been shown to improve neurotoxic activity when injected into blowflies, however, at detriment to the oral activity of the toxin (V. Herzig, *et al.*, unpublished data). Recently, Volker *et al.* (unpublished data) obtained results indicating that the permeation of the cyclic toxin from the insect gut into the haemolypmh was substantially reduced when compared to the native ω-HXTX-Hv1a. Subsequently, it is possible that the slowed diffusion of the cyclic toxin into the insect haemolymph exposes the toxin to enzymes within the insect mid-gut for longer and a reduced amount of the intact toxin reaches the target in the insect CNS.

4.3.2 VICINAL DISULFIDE BOND

One of the aims of this study was to determine whether the functionally important vicinal disulfide acts as a redox-activated switch. In most cases the formation of a vicinal disulphide 8 membered ring results in a high degree of dihedral strain (DSE) and the peptide bond between the two covalently linked cysteine side chains is therefore non planar (Wang, Connor et al., 2000). Subsequently, vicinal disulphide bonds have been proposed to act as redox conformational switches (Carugo, Cemazar et al., 2003). Dantas de Araujo *et al.* (Dantas de Araujo, Herzig et al., 2012) reports that the vicinal disulfide of κ-HXTX-Hv1c assumes a distorted *trans* conformation without a particularly high calculated DSE. The relatively low dihedral strain and the similar activity of diselenide-modified toxin lend support to the vicinal disulphide acting as a special recognition site and not a redox switch. Furthermore, replacement of the vicinal disulphide with a non-reducible ethylene was shown to block insect BK_{Ca} to a similar degree as the native toxin (A. Dantas de Araujo, V. Herzig, M. J. Windley, M. Dziemborowicz S., Mobli, G.M. Nicholson, P. F. Alewood and G. F. King, unpublished data). Both diselenide and ethylene modifications were designed to maintain the ring structure and to minimise possible redox reactions. As replacement of the vicinal disulphide with both diselenide and ethylene linkages was not found to alter the bioactivity of the toxin, κ-HXTX-Hv1c's vicinal disulfide is unlikely to act as a redox switch.

The ring motif is therefore believed to be essential as it is involved in a direct non-covalent interaction with the channel. The slightly higher IC_{50} values for diselenide κ-HXTX-Hv1c action on cockroach BK_{Ca} channels support the theory of a precisely configured molecular interaction. Furthermore, the vicinal diselenide is more hydrophobic than the native disulfide potentially explaining the increase in affinity to the insect BK_{Ca} channel. Importantly, the vicinal disulfide is a strong and specific binding site; therefore it may be useful in the design of small insecticidal molecules.

4.3.3 FURTHER APPLICATIONS OF DISELENIDE REPLACEMENT

Determination of disulfide bonding is poorly established with NMR. Other methods involve determining dipolar interactions between β-methylene portions of a covalent disulfide (Walewska, Skalicky et al., 2008) however the results become ambiguous

when multiple bonds are involved (Boisbouvier, Albrand et al., 1998; Jordan, Poppe et al., 2009). A solution to this problem has been to replace the NMR inactive ^{32}S with a similar chemical (Mobli, de Araújo et al., 2009). Selenocysteine is a replacement which can occur naturally (Kryukov, Castellano et al., 2003). The residue almost identical to Cys however the sulphur is replaced by selenium (Mobli, de Araújo et al., 2009). Importantly, this modification can be introduced synthetically and does not appear to alter the structure or function of the molecule (Mobli, de Araújo et al., 2009). Diselenide replacement is also viable for larger proteins via recombinant methods (Müller, Senn et al., 1994; Strub, Hoh et al., 2003).

4.3.4 APPLICATIONS IN INSECTICIDE AND DRUG DESIGN

In conclusion, ω-HXTX-Hv1a and κ-HXTX-Hv1c remain promising leads for the design and development of insecticidal compounds. While the permeability of cyclic ω-HXTX-Hv1a through the insect gut has been reported to be reduced in insect bioassays resulting in the subsequent reduction in insect oral activity (V. Herzig *et al*., unpublished data), cyclised toxin was more toxic by injection (V. Herzig *et al*., unpublished data) and the toxin retained its activity on the Ca_V channel target. Consequently, this study validates the cyclisation of peptide toxins to improve stability, assuming toxin permeability through the insect gut can be improved. Furthermore, we believe the unique vicinal disulfide bond found in the structure κ-HXTX-Hv1c acts as a highly specific structural motif that is crucial for toxin interaction with the insect K_{Ca} channel target. The unique structural motif may in fact have positive implications for the development of small (Craik, Cemazar et al., 2006a; Craik, Cemazar et al., 2006b) non-peptide mimetic structures applicable for the production foliar sprays.

Chapter Five

The effects of κ-HXTX-Hv1c and hybrid-HXTX-Hv1a on neurotransmission in the insect nervous system

Monique J. Windley

[2012]

5.1 INTRODUCTION

In insects, κ-HXTX-Hv1c has been previously shown to exhibit an excitatory phenotype, resulting in death (Wang, Connor et al., 2000). The present study aimed to identify the changes in neurotransmission that may underlie these excitatory effects in insects. Specifically the study assessed changes in the spontaneous firing frequency, threshold and amplitude of action potentials, timecourse of repolarisation, amplitude of the afterhyperpolarisation (AHP) and changes in the resting membrane potential in DUM neurons. The study specifically aimed to correlate such changes with the known ability of κ-HXTX-Hv1c to block BK_{Ca} channels (Gunning, Maggio et al., 2008).

5.1.1 HYBRID-HXTX-HV1A

This study also sought to examine the effects of a related spider ICK toxin, hybrid-HXTX-Hv1a, originally isolated from a cDNA library of *Hadronyche versuta* (Brie Sollod, Glenn F. King, unpublished results). The hybrid toxin has potent insecticidal activity against a range of insect species with an LD_{50} of 38 ± 3 pmol/g against *Musca domestica*, which makes this toxin the most potent toxin isolated from Australian funnel-web spiders to date (Brie Sollod, Glenn F. King, unpublished results). This toxin family was not isolated via normal venom screening methods due to low expression levels. In order to obtain sufficient levels of material for structural and function characterisation a bacterial expression system was developed for overproduction of the toxin (see chapter 2 for more details).

Hybrid-HXTX-Hv1a is a prototypic member of a family of up to nine homologous peptides which share little homology to other hexatoxins. Although the hybrid toxin sequence does contain some elements from both κ-HXTX-Hv1c and ω-HXTX-Hv1a, they share little overall homology. On the other hand, the pharmacophores of all three of these hexatoxins overlay with a high degree of superposition of key functional residues (Figure 5.1). Moreover, the hybrid toxin has been recently found to block the known targets of both ω- and κ-hexatoxins (Simon Gunning, Suping Wen, Glenn F. King and Graham Nicholson, unpublished results). Whole-cell patch clamp on cockroach DUM neurons has revealed that the hybrid toxin potently blocks Ca_V and BK_{Ca} (pSlo) channel currents in cockroach DUM neurons, similarly to ω- and κ-HXTX-1 toxins (Wang, Smith et al., 1999; Chong, Hayes et al., 2007; Gunning, Maggio et al., 2008),

respectively. Furthermore, hybrid-HXTX-Hv1a has been shown to have no significant effects on DUM neuron I_{Na}, or delayed-rectifier or A-type transient K_V channel currents (Simon Gunning, Suping Wen and Graham Nicholson, unpublished results).

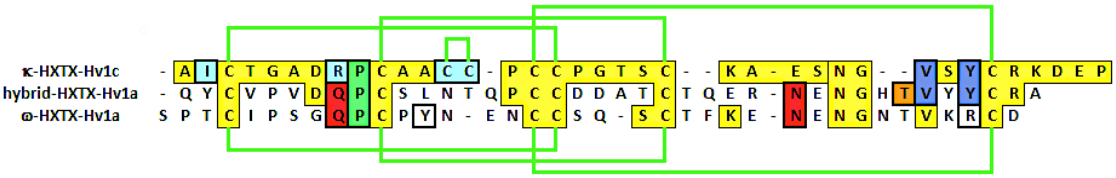

Figure 5.1: Comparison of mature toxin sequences of κ-HXTX-Hv1c, ω-HXTX-Hv1c and hybrid-HXTX-Hv1a. Homologies are show relative to hybrid-HXTX-Hv1a; identities are boxed in yellow. The experimentally determined disulfide bonding pattern for κ-HXTX-Hv1c (Wang, Connor et al., 2000) and ω-HXTX-Hv1c (Wang, Smith et al., 1999) are represented in green above and below the alignment, respectively. Red shading shows the pharmacophore of ω-HXTX-Hv1c (Tedford, Gilles et al., 2004) that are also found in hybrid-HXTX-Hv1a, while the pink shading indicates residues that are unique to ω-HXTX-Hv1c pharmacophore. Blue shading highlights the key functional residues that are common to κ-HXTX-Hv1c (Maggio and King, 2002b) and hybrid-HXTX-Hv1a. Additional key residues that are part of the κ-HXTX-Hv1c pharmacophore are indicated by pale blue shading. Residues highlighted in green are part of the pharmacophore for all three toxins. Residues highlighted in orange are part of the pharmacophore of hybrid-HXTX-Hv1a only.

Subsequently, the aim of this chapter was to assess the effects of κ-HXTX-Hv1c and hybrid-HXTX-Hv1a on action potential generation and propagation. In order to achieve this goal, concentrations of each toxin were selected based on previous studies of their effects on BK_{Ca} (κ-HXTX-Hv1c and hybrid-HXTX-Hv1a) and Ca_V channel currents (hybrid-HXTX-Hv1a) in DUM neurons. The concentrations chosen for κ-HXTX-Hv1c, were 3 nM, the IC_{50} for block of native DUM neuron BK_{Ca} channels, and 100 nM, a concentration blocking 100% of channels. As hybrid-HXTX-Hv1a has not been tested on native DUM neuron $I_{BK(Ca)}$, the results from expressed pSlo channels were examined. The IC_{50} value for hybrid activity on pSlo channel currents was recorded as 671 ± 7 nM (Simon Gunning and Graham Nicholson, unpublished data). As studies have clearly indicated that the effects of toxins on expressed pSlo BK_{Ca} are not equally reflected on native channels (MacKinnon, Heginbotham et al., 1990; Hanner, Schmalhofer et al.,

1997; Gunning, Maggio et al., 2008) a direct comparison could not be made. For example, the IC_{50} for block of DUM neuron $I_{BK(Ca)}$ by κ-HXTX-Hv1c was 3 nM, while the IC_{50} for block of pSlo channels was 240 nM (an 80-fold loss of potency; (Gunning, Maggio et al., 2008)). In support, the vertebrate BK_{Ca} channel blocker charybdotoxin was also found to have a 83-fold loss in potency on expressed mammalian BK_{Ca} (mSlo) that was improved by 50-fold in the presence of the modulatory β-subunit for hSlo (human BK_{Ca}) (Hanner, Schmalhofer et al., 1997). Unfortunately, insect homologs for the mammalian β-subunit remain elusive and channel currents are not functionally modified by coexpression with mammalian subunits. Thus the IC_{50} of hybrid-HXTX-Hv1a for block of native $I_{BK(Ca)}$ was estimated at around 10–20 nM, hence a concentration of 20 nM was chosen. In addition, to the block of BK_{Ca} channels, hybrid-HXTX-Hv1a also blocks Ca_V channels with an IC_{50} of around 400 nM (Simon Gunning and Graham Nicholson, unpublished data). Therefore hybrid-HXTX-Hv1a was tested at both 20 nM and 400 nM reflecting approx. IC_{50} concentrations on BK_{Ca} and Ca_V channels in DUM neurons.

5.1.2 DUM NEURONS GENERATE SPONTANEOUS OVERSHOOTING ACTION POTENTIALS

In the insect nervous system a distinct population of neurons are capable of generating spontaneous overshooting action potentials (AP). Modulatory efferent dorsal unpaired median (DUM) neurons are a distinct group of cells that exhibit pacemaker-like activity (Kerkut, Pitman et al., 1968; Crossman, Kerkut et al., 1971; Hoyle and Dagan, 1978). While DUM neurons exhibit serotonin- (Orchard, Lange et al., 1989), myomodulin- (Swales and Evans, 1994), GABA- (Distler, 1989), glutamate- (Bicker, Schafer et al., 1988), proctolin- (Yasuyama, Kimura et al., 1992) and taurine-like (Bicker, 1991) immunoreactivity, it has been well established that these neurons are neuromodulatory cells that both produce and secrete octopamine (Achenbach, Walther et al., 1997; Roeder, 1999). Modulation of DUM neuron activity by the synthesis and release of octopamine is considered essential to the control of various peripheral muscles and organs (Hoyle and Dagan, 1978; Morton and Evans, 1984; Orchard, Ramirez et al., 1993; Roeder, 1999; Tahira, 2007). In particular, the insect octopaminergic system is equated to the noradrenergic system in vertebrates due to its role in stress.

It is assumed that the release of neurosecretory products is related to the temporal distribution of the electrical pattern in the insect nervous system. In fact, the regulation

of the large conductance calcium-activated potassium (BK_{Ca}) channel is considered to be particularly important in the control of neurotransmitter release due to its role in AP generation and firing frequency (Grolleau and Lapied, 1994; Reid, Bekkers et al., 2003; Raffaelli, Saviane et al., 2004; Cheron, Sausbier et al., 2009). Similarly, modulation of voltage-gated sodium (Na_V) and calcium (Ca_V) channel significantly influence spike activity in DUM neurons (for a review see ref. (Grolleau and Lapied, 2000)).

Spontaneous spiking activity occurs in DUM neurons in the absence of a circuit and on the basis of intrinsic membrane properties. It is assumed that a driving input is needed such as background currents or adjustments in specialised currents around subthreshold potentials (Grolleau and Lapied, 2000). While little more is known about the mechanism behind spontaneous spike generation, it is known that the Na_V channel is an essential component. Studies have shown that TTX application completely abolished spontaneous activity both *in situ* and in isolated neurons (Lapied, Malecot et al., 1989). Furthermore, the addition of Co^{2+} or La^{3+} was also found to abolish spike activity indicating Ca_V channels were also essential to the generation of spontaneous APs (Goodman and Heitler, 1979; Lapied, Malecot et al., 1989). Finally, the introduction of the K_V blocker TEA-Cl was found to substantially increase AP duration, abolish AHP and induce repetitive firing (Goodman and Heitler, 1979).

These channels are believed to play such a fundamental role in the generation of spontaneous activity due to their individual functions in shaping the AP. Firstly, Na_V channel activation is responsible for the depolarisation phase of the AP (Lapied, Malecot et al., 1989; Lapied, Malecot et al., 1990) while Ca_V channels are involved in the predepolarisation phase and control of the $I_{K(Ca)}$ (Wicher and Penzlin, 1994; Grolleau, Lapied et al., 1996). Furthermore, K_V channels are known to play roles in the repolarisation (K_{DR} and K_{Ca}), AHP (K_{Ca}) and the regulation of firing frequency (K_A) (Grolleau and Lapied, 1995b).

In order to gain further insights into the effects of κ-HXTX-Hv1c and hybrid-HXTX-Hv1a on neurotransmission, the actions of these toxins on the generation of spontaneous and evoked APs was assessed in cockroach DUM neurons. Furthermore, due to the important role of K_V channels in modulating DUM neuron pacemaker activity (Thomas, 1984; Wicher, Walther et al., 1994; Grolleau and Lapied, 1995b) a comparison was made with the archetypal BK_{Ca} and ‘A-type’ transient K_V channel blockers iberiotoxin

(IbTx) and 4-aminopyridine (4-AP), respectively, as well as the less-selective K_V channel blocker, tetraethylammonium (TEA) (Grolleau and Lapied, 1995b; Grolleau and Lapied, 2000; Wicher, Walther et al., 2001).

5.2 RESULTS

5.2.1 SPONTANEOUS ENDOGENOUS NEURONAL ACTIVITY

DUM neurons isolated from the terminal abdominal ganglia (TAG) of *Periplaneta americana* are capable of generating spontaneously overshooting APs. As shown in Figure 5.2, these neurons generate APs at a frequency 4 ± 0.6 Hz (n = 52) under control conditions. Measured from maximum peak potential to the resting membrane potential, spontaneous APs are around 120 mV in amplitude, with an overshoot of around +50 mV. Although little is known about the mechanisms underlying the generation of spontaneous APs, voltage-gated ion channels are believed to play an intrinsic role in both shaping the AP and modulating firing frequency (Grolleau and Lapied, 2000).

5.2.1.1 EFFECTS OF κ-HXTX-HV1C ON DUM NEURON SPONTANEOUS ACTIVITY

Under, current-clamp conditions, spontaneous APs were recorded from the majority of DUM neurons. In the absence of all channel blockers, cells exhibited resting membrane potentials of –49.9 ± 1.0 mV and overshooting potentials of approx. +120 mV. Spontaneous activity was recorded for a period of 5 s at 1 min intervals until a stable firing frequency could be ascertained. In the presence of toxin spontaneous currents were recorded for up to 10 min.

In the presence of 3 nM κ-HXTX-Hv1c, spontaneous firing frequency showed a trend towards increasing with a frequency of 6.1 ± 1.6 Hz (n = 3, p > 0.5; Figure 5.3), while similar results were also seen following exposure to 100 nM κ-HXTX-Hv1c (6.0 ± 3.0 Hz, n = 3, p >0.05; Figure 5.3A). This trend towards an increase of DUM neuron firing frequency was evident within 1–2 min. The resting membrane potential remained unaltered in the presence of 3 (–51.6 ± 2.9 Hz, n = 12, p > 0.05) or 100 nM (–54.1 ± 1.5 Hz, n = 4, p > 0.05) κ-HXTX-Hv1c. As expected, similar results were observed with the classical vertebrate BK_{Ca} channel blocker IbTx. At saturating concentrations of 100 nM IbTx spontaneous activity showed a trend towards increasing with a frequency of 4.4 ±

1.1 Hz (n = 3, p > 0.05; Figure 5.3), in addition, the resting membrane potential was not significantly modified (–48.8 ± 2.0 Hz, n = 7, p > 0.05).

A magnification of the AHP and pre-depolarisation phase is represented in Figure 5.2B. In order to influence firing frequency κ-HXTX-Hv1c must somehow alter the firing threshold of the AP. As indicated by Figure 5.2B the AP threshold of activation is attained more rapidly in the presence of toxin, however this does not seem to correlate with the spontaneous activity data. This may suggest, however, that the influence of K_V channels (such as BK_{Ca} and $K_{(A)}$ channels) on the AHP is just more evident in non spontaneously active DUM neurons.

In agreement with previous reports (Grolleau and Lapied, 1995b), it was evident that the A-type transient K_V channel blocker 4-AP more significantly influenced AP firing frequency (Figure 5.2D). Almost immediately following 5 mM 4-AP exposure, firing frequency was enhanced to 12.1 ± 4.7 Hz (n = 3, p > 0.05; Figures 5.2 and 5.3). The ability of 4-AP to modify firing frequency is believed to be a consequence of enhanced sensitivity to stimulus evidenced by a reduction in the minimum refractory period (Grolleau and Lapied, 1995b).

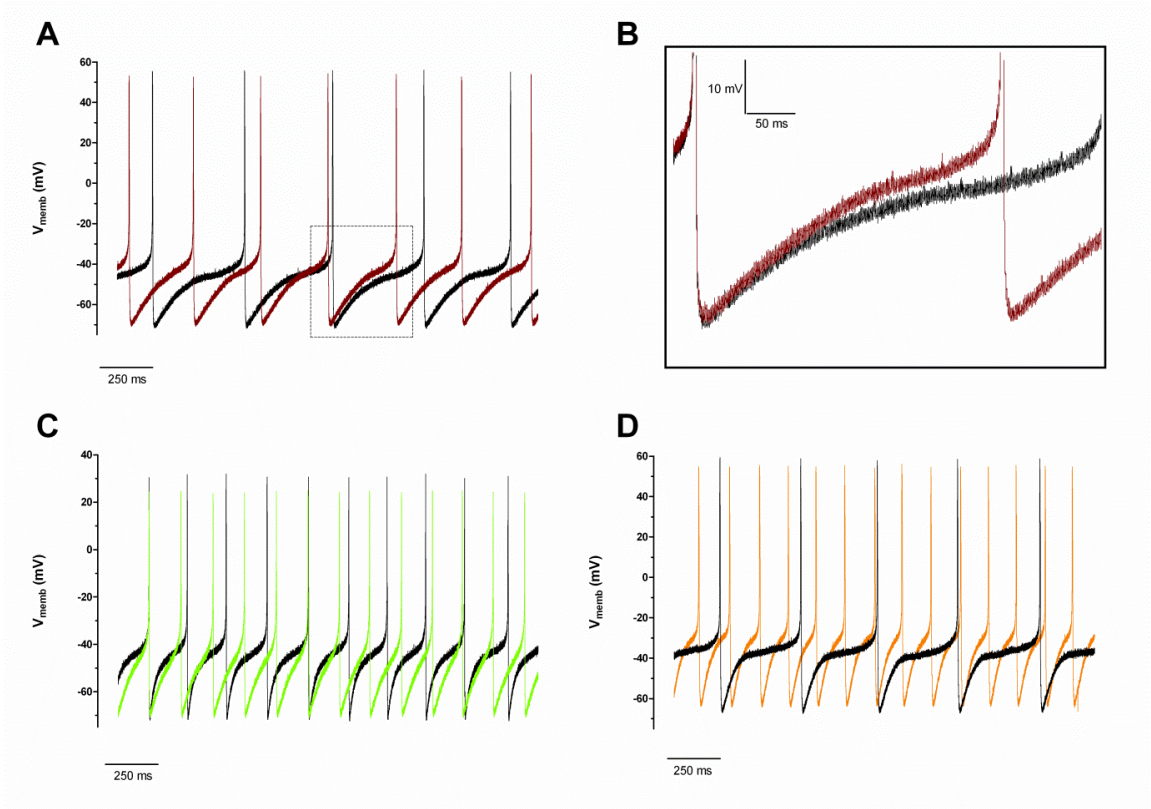

Figure 5.2: Effects of κ-HXTX-Hv1c and classical vertebrate K_V channel toxins on spontaneously generated APs in DUM neurons. Spontaneous overshooting APs were recorded in DUM neurons under current-clamp conditions. Resting membrane potentials were measured at between –60 and –40 mV. Traces represent spontaneous activity in controls (black) compared with **(A)** 100 nM κ-HXTX-Hv1c (red), **(C)** 100 nM iberiotoxin (green), and **(D)** 5 mM 4-AP (orange). Figure **(B)** represents a magnification of the boxed area in panel (A), highlighting AHP and pre-depolarisation phases of the AP.

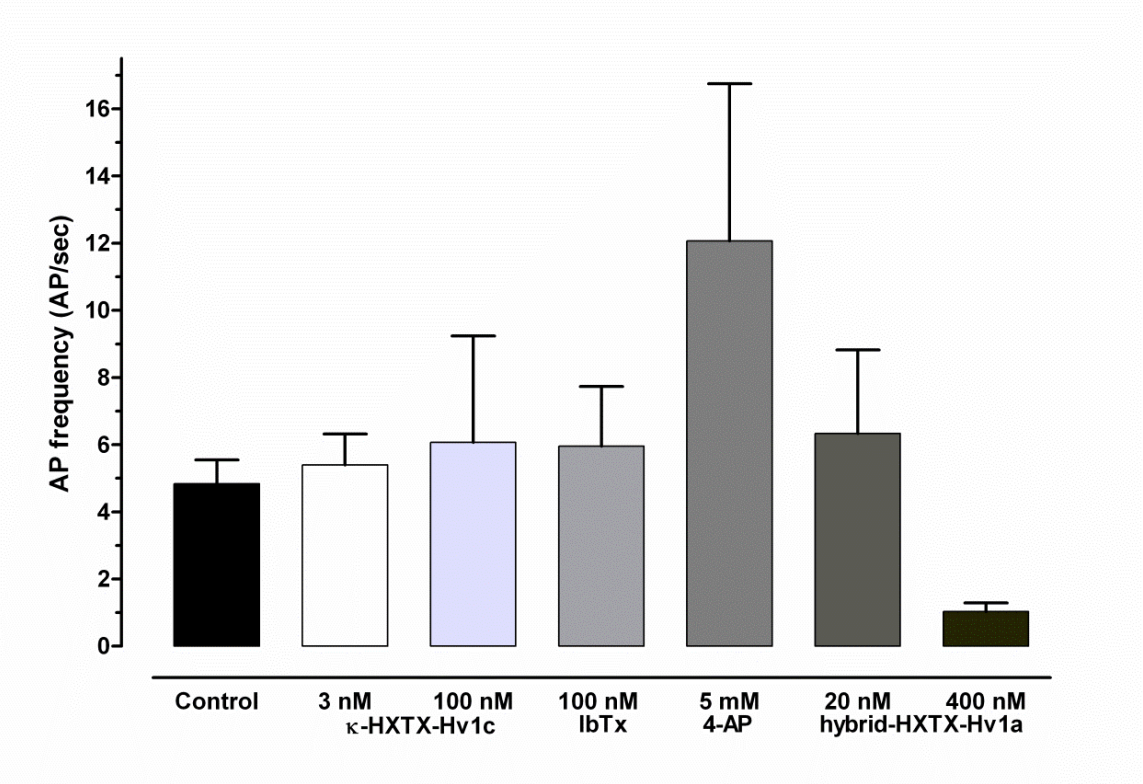

Figure 5.3: Effects of K_V channel toxins on spontaneous activity in DUM neurons. A comparison of spontaneous AP firing frequency in the presence of hybrid- and κ-hexatoxin-1 toxins, the K_A channel blocker 4-AP and the K_{Ca} channel blocker IbTx. Spontaneous firing frequency in the presence of each toxin was calculated as a percentage of the control firing frequency recorded in the absence of toxin. Comparisons of two means were made using a paired one-way ANOVA; changes from control frequency were considered significant if $p < 0.05$.

5.2.2 EFFECTS OF κ-HXTX-HV1C ON EVOKED ACTION POTENTIALS

In this study not all DUM neurons were found to have or maintain stable spontaneous activity. The loss of spontaneous activity is potentially a result of (i) extended time in culture, (ii) depolarisation of the membrane potential due to patching onto the cell or (iii) a depletion or dilution of intracellular second messengers that modulate ion channel activity such as inorganic phosphate, intracellular Ca^{2+} or intracellular enzymes such as kinases or phosphatases (see (Wicher, Walther et al., 2001) for review).

To further examine the effects of κ-HXTX-Hv1c on AP generation in non-spiking DUM neurons, single current pulses were employed to generate APs. Changes in the threshold and amplitude of depolarisation, timecourse of repolarisation, amplitude of the AHP and the resting membrane potential were then assessed. Under current-clamp conditions 4-ms, 2 nA current pulses were employed to elicit APs of ~120 mV. Action potentials were evoked at 10-s intervals at a sampling rate of 2 kHz. Following application of 3 nM and 100 nM κ-HXTX-Hv1c, gradual trends towards slowing the repolarising and reducing the hyperpolarising phases were evident (Figure 5.4A and B). Similar effects were also observed in the presence of saturating concentrations of both 4-AP and IbTx.

However, similarly to $I_{BK(Ca)}$ inhibition, the effects of $I_{K(A)}$ block on the AHP do not correlate well with spontaneously inactive DUM neurons. As little is still known about the involvement of various voltage-gated ion channels generation of spontaneous activity in DUM neurons (Grolleau and Lapied, 2000) these differences cannot be easily explained.

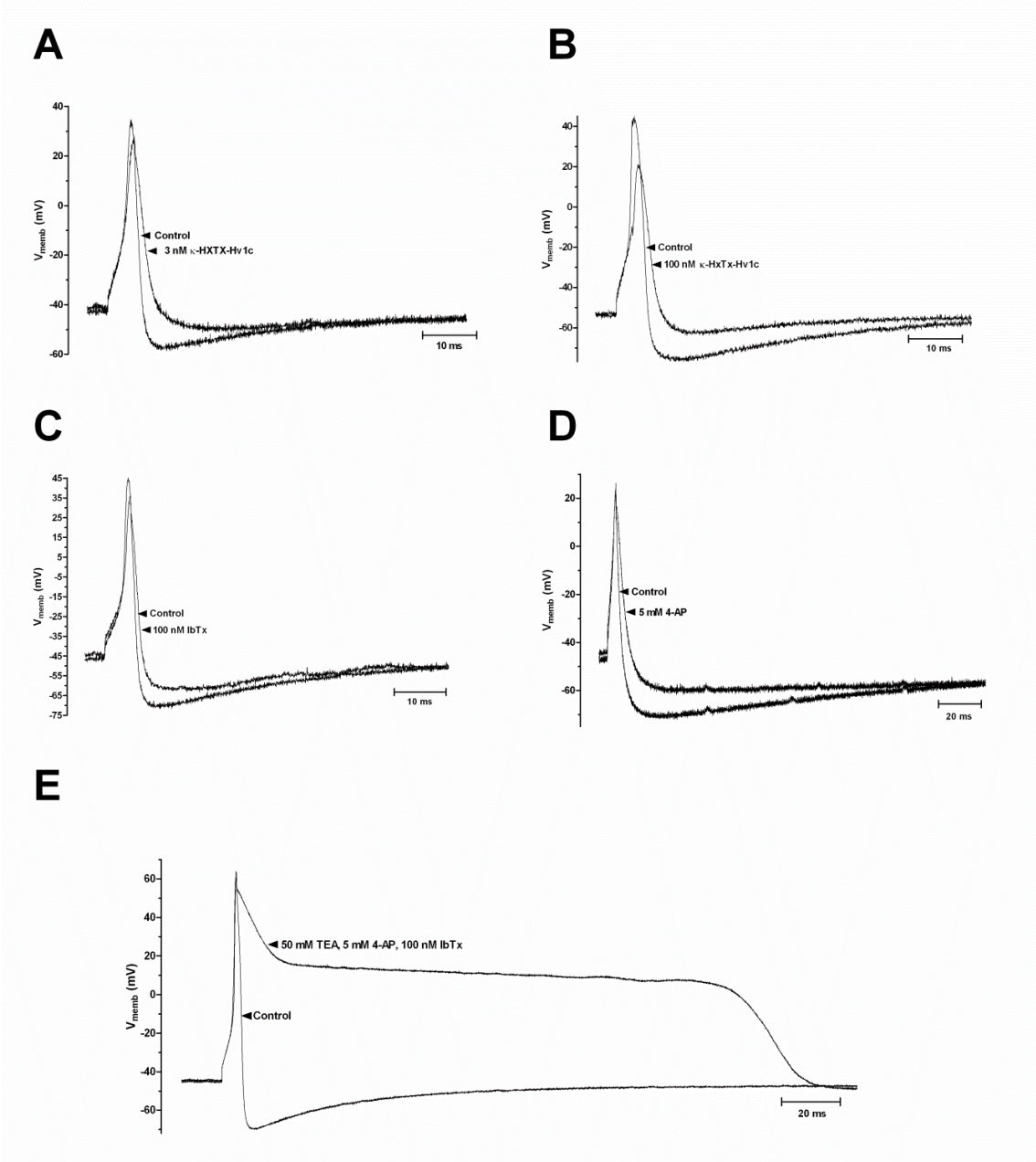

Figure: 5.4 Effects of κ-HXTX-Hv1c and other K_V channel blockers on DUM neuron APs. APs were generated in response to 4 ms, 2 nA stimuli recorded at 10-s intervals. Traces show the effects of **(A)** 3 nM κ-HXTX-Hv1c, **(B)** 100 nM κ-HXTX-Hv1c, **(C)** 100 nM iberiotoxin, **(D)** 5 mM 4-AP and **(E)** the combined effects of 50 mM TEA, 5 mM 4-AP and 100 nM IbTx on APs.

The change in AP duration was quantitated by measuring AP duration at 25% ($duration_{25}$) and 50% ($duration_{50}$) of peak AP amplitude (Figure 5.5C). AP $duration_{25}$ and $duration_{50}$ were measured at 4.8 ± 0.4 and 2.7 ± 0.2 ms under control conditions. 100 nM κ-HXTX-Hv1c demonstrated a trend towards increasing AP duration with

values of 6.1 ± 0.2 and 4.0 ± 1.1 ms at $duration_{25}$ and $duration_{50}$, respectively (n = 4, p > 0.05; Figure 5.5). In addition, 100 nM IbTx demonstrated a trend towards increasing AP duration with values of 5.9 ± 0.3 and 3.0 ± 1.0 ms at $duration_{25}$ and $duration_{50}$, respectively (n = 7, p > 0.05; Figure 5.5). Interestingly, 4-AP had a similar effect on AP duration to κ-HXTX-Hv1c, exhibiting AP durations of 6.7 ± 0.4 ms ($duration_{25}$) and 4.0 ± 0.2 ms ($duration_{50}$) (n = 5, p > 0.05; Figure 5.6). These results suggest that both BK_{Ca} and A-type transient K_V channel currents play an important role in the repolarisation phase of the AP.

Finally, complete inhibition of outward I_K results in extensive prolongation of repolarisation whilst completely abolishing AHP. In the presence of 50 mM TEA, 5 mM 4-AP and 100 nM IbTx, blocking delayed-rectifier, A-type transient, K_{Na} and BK_{Ca} channel currents, plateau potentials of up to 500 ms $duration_{50}$ were evident (Figure 5.4E; (Grolleau and Lapied, 1995b)). Consequently, complete block of I_K was shown to increase AP duration with $duration_{50}$ increased to 92.1 ± 10.4 ms and $duration_{25}$ to 298.3 ± 80.7 (n = 10 p < 0.05; Figure 5.6). Therefore, these results further validate the crucial role of K_V channels in the modulation of AP shape and specifically the control of repolarisation and after-hyperpolarisation.

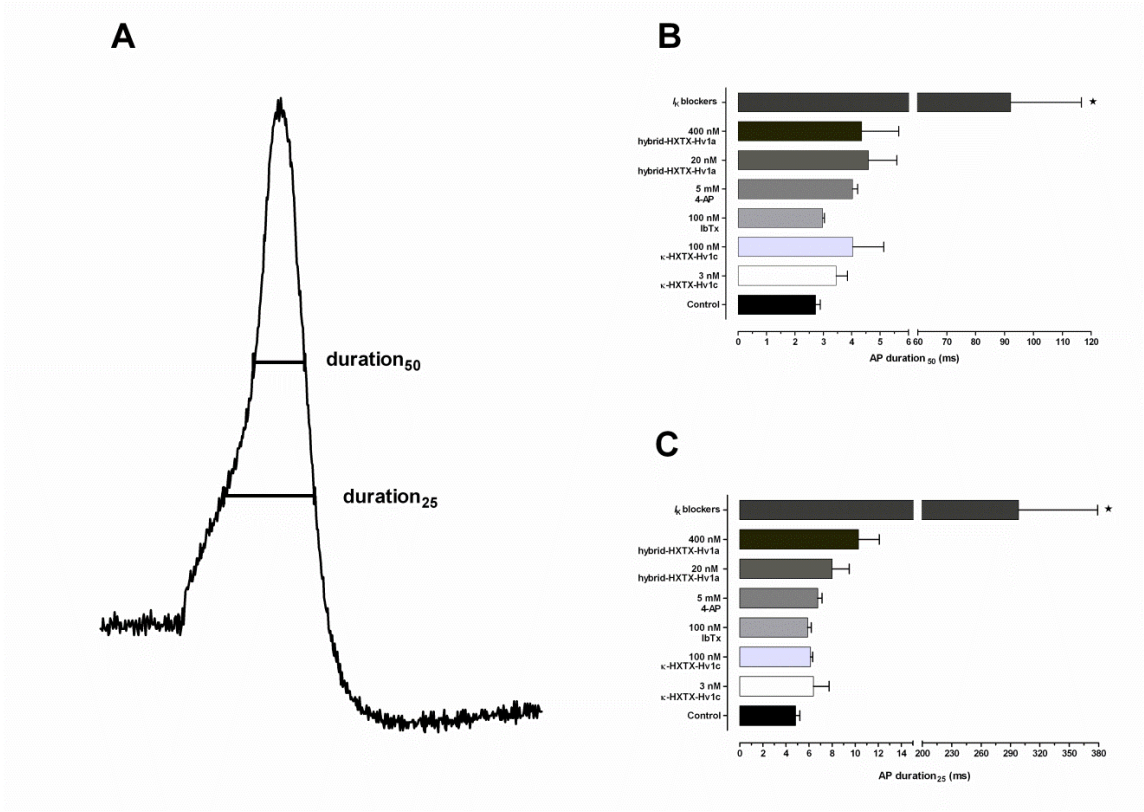

Figure 5.5: Effect of κ- and hybrid-hexatoxin-1 toxins on AP repolarisation. Measurements of DUM neuron AP duration were taken at 25% ($duration_{25}$) and 50% ($duration_{50}$) of maximum overshoot amplitude **(A)**. Single APs were stimulated by 4-ms, 2 nA current pulses under current-clamp conditions. Values were expressed as a percentage of control duration at $duration_{50}$ **(B)** and $duration_{25}$ **(C)**. Control data was recorded on the same cell prior to the addition of toxin. Each column represents data from at least 4 cells. Comparisons of two means were made using a one-way ANOVA; changes from AP control duration were considered significant if $p < 0.05$ (★).

Analysis of additional AP parameters also revealed various effects on the amplitude and decay rate of the AHP phase of the AP. The AHP amplitude was measured at 19.2 ± 1.0 mV under control conditions (n = 52). Exposure to κ-HXTX-Hv1c was found to result in a dose-dependent trend towards the attenuation of AHP amplitude. In the presence of 3 nM κ-HXTX-Hv1c the AHP amplitude was recorded as 14.1 ± 3.4 mV (n = 12, p > 0.05), whereas application of 100 nM κ-HXTX-Hv1c resulted in AHP amplitudes of 12.6 ± 1.8 mV (n = 4, p > 0.05; Figure 5.6). Furthermore, saturating concentrations of IbTx and 4-AP indicated a trend towards the reduction of AHP amplitudes with values of 19.1 ± 0.9 mV (n = 7) and 11.1± 2.7 mV (n = 7), respectively.

Despite the trend towards increasing firing frequency, duration and the AHP 100 nM κ-HXTX-Hv1c failed to alter the resting membrane potential, which did not vary more than 5 mV (n = 4; p>0.05, Figure 5.6A). The spike amplitude, taken as the maximum potential in the depolarising direction (Figure 5.6B), remained relatively unaffected in the presence of κ-HXTX-Hv1c (control 93.5 ± 2.0 mV vs. 100 nM toxin 85.8 ± 4.4 mV; n = 4, p > 0.05). Consistent with the absence of activity on DUM neuron Na_V channel currents (Gunning, Maggio et al., 2008), κ-HXTX-Hv1c also exhibited no effect on the depolarising phase of the AP (p > 0.05).

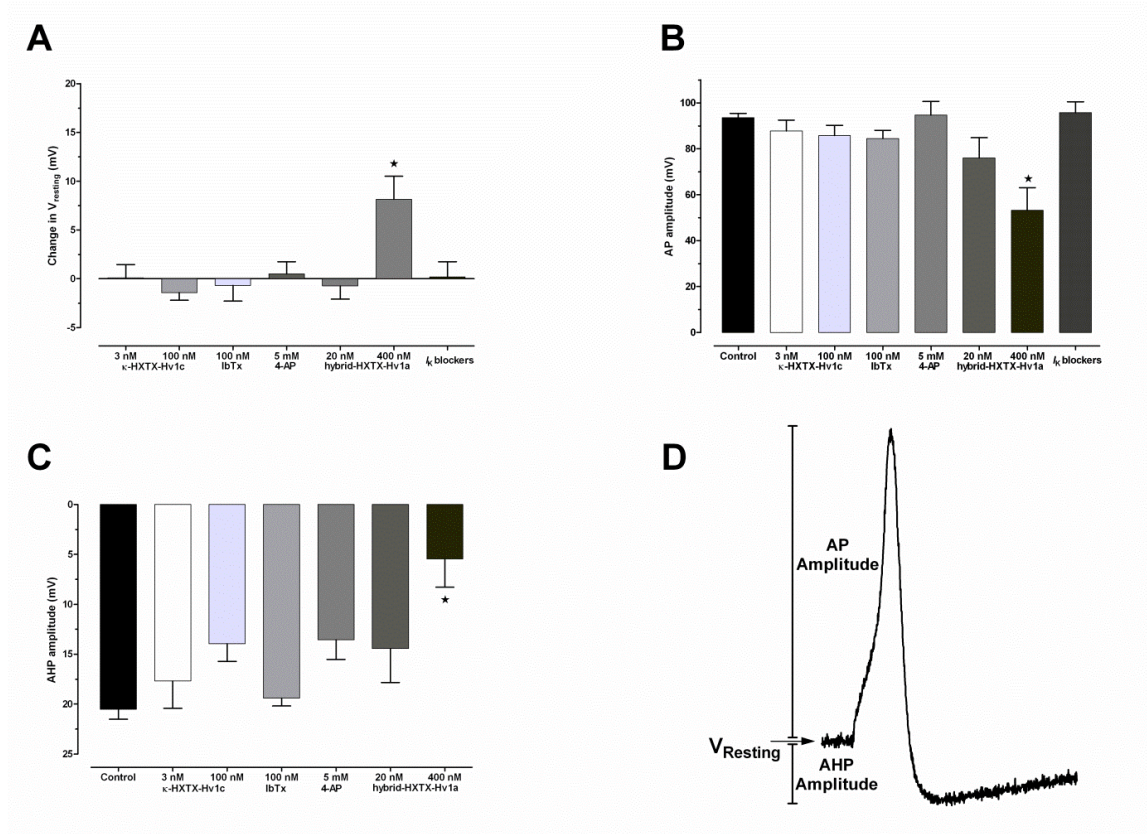

Figure 5.6: Changes in AP parameters in the presence of hybrid-HXTX-Hv1a, κ-HXTX-Hv1c and other classical vertebrate K_V channel toxins. Action potentials were generated in response to 4 ms, 2 nA stimuli recorded at 10-s intervals. **(A)** Resting membrane potential ($V_{resting}$) was measured as shown in panel D. Values were recorded before, and after, the application of toxins. Values are expressed as the change in control $V_{resting}$ (in mV). **(B)** AP amplitude was measured as shown in panel D. AP amplitude in the presence of various toxins was calculated as a percentage of control AP amplitude. **(C)** Afterhyperpolarisation (AHP) amplitude was measured as shown in panel D. Values are expressed as a percentage of control AHP amplitude. Each column in panels A–C represents data from n = 3–5 cells. Comparisons of two means were made using a one-way ANOVA; changes were considered significant if $p < 0.05$ (★). Trace **(D)** is a visual representation of the AP measurement parameters of the preceding graphs.

5.2.3 HYBRID-HXTX-HV1A ON DUM NEURON SPONTANEOUS ACTIVITY

In the presence of 20 nM hybrid-HXTX-Hv1a, spontaneously generated DUM neuron activity (Figure 5.7). Similarly to κ-HXTX-Hv1c, spike frequency was recorded as 6.3 ± 2.5 Hz (n = 3, p > 0.05; Table 5.1) in the presence of the 20 nM hybrid toxin. In contrast, higher concentrations of hybrid toxin caused an opposite trend on firing frequency (Figure 5.7B and D). Following the application of 400 nM hybrid-HXTX-Hv1a, DUM neuron spike frequency exhibited a trend toward decreasing within 1 min of toxin application. After a period of 5 min, spike frequency was recorded as 1.0 ± 0.3 Hz (n = 3; Table 5.1).

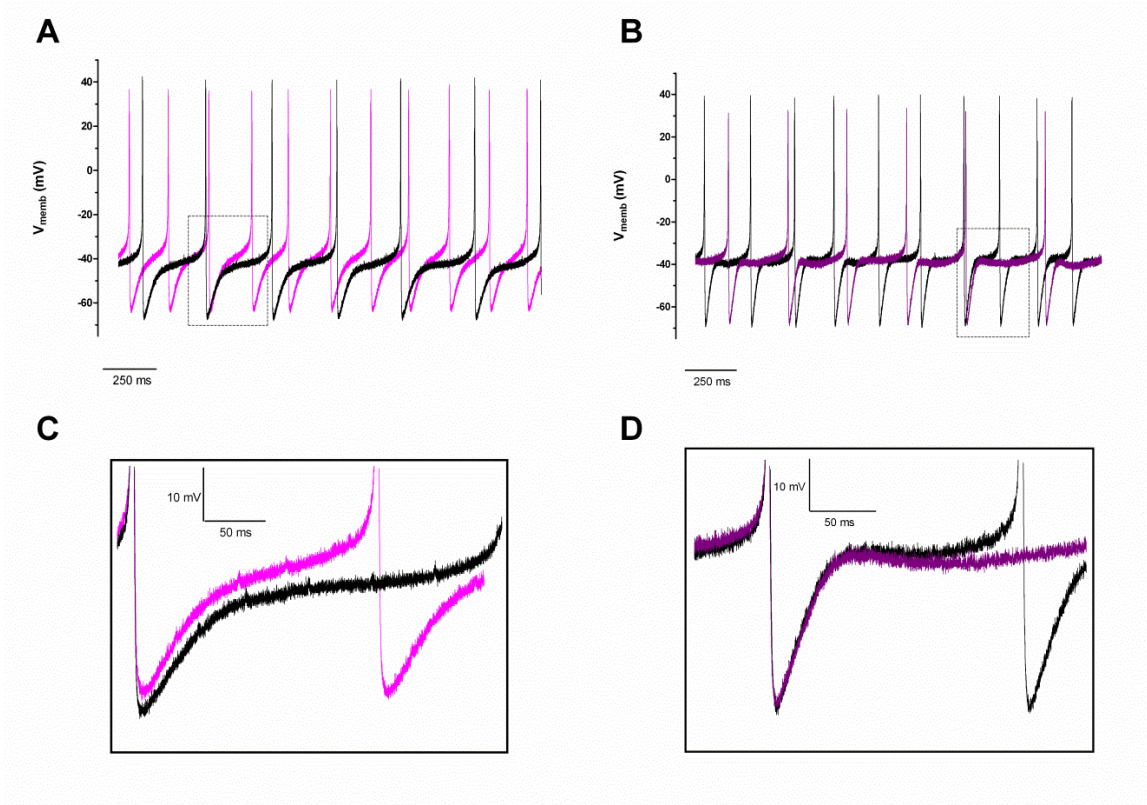

Figure 5.7: Effects of hybrid-HXTX-Hv1a on DUM neuron spontaneous activity. Spontaneous overshooting APs were recorded in DUM neurons under current-clamp conditions. Resting membrane potentials were recorded approx. –50 mV. The traces represent spontaneous activity in the absence (black traces) and in the presence of **(A)** 20 nM hybrid-HXTX-Hv1a (purple) and **(B)** 400 nM hybrid-HXTX-Hv1a (dark purple). Traces **(C)** and **(D)** represent magnifications of (A) and (B), respectively, highlighting the AHP and predepolarisation phases of AP generation.

5.2.4 Effects of hybrid-HXTX-Hv1a on evoked action potentials

To further assess the effects of the hybrid toxin on AP generation, APs were evoked by single 1–2 nA current pulses at 0.2 Hz permitting the analysis of various aspects of the AP shape including changes in the threshold and amplitude of depolarisation, timecourse of repolarisation, amplitude of the AHP and the resting membrane potential.

At low concentrations, the effects of hybrid-HXTX-Hv1a were similar to κ-HXTX-Hv1c, described above. In the presence of 20 nM hybrid AP amplitude exhibited a trend towards decreasing (control 93.5 ± 2.0 mV vs. toxin 76.0 ± 8.8 mV, $n = 8$, $p > 0.05$; Table 5.1). Trends in AP repolarisation were also similar to κ-HXTX-Hv1c, whereby duration was recorded as 4.6 ± 1.0 ms at duration_{50} and 8.0 ± 1.4 ms at duration_{25} in the presence of the hybrid toxin ($n = 8$, $p > 0.05$; Table 5.1). Additionally, application of 20 nM hybrid toxin resulted in a trend towards reduced AP AHP amplitude (11.6 ± 3.7 mV, $n = 8$, $p > 0.05$; Table 5.1) similar to the effects of κ-HXTX-Hv1c. No significant changes or trends in the membrane resting potential were observed ($p > 0.05$; Table 5.1).

At 400 nM hybrid toxin exhibited more consequential effects on single evoked APs. From initial experiments it was noted that 400 nM toxin considerably influenced the resting membrane potential. Shortly following perfusion with 400 nM hybrid toxin the resting membrane potential depolarised and reached a steady-state level of −37.5 ± 6.5 mV ($n = 6$, $p < 0.05$; Table 5.1). Due to these depolarising effects, the membrane potential was manually hyperpolarised to control levels to measure the effects on AP amplitude and kinetics. 400 nM hybrid toxin was found to cause a trend towards increasing the AP duration with values of 4.3 ± 1.3 ms at duration_{50} and 10.3 ± 1.8 ms at duration_{25}, resulting in a trend towards the slowing of AP repolarisation ($n = 6$, $p > 0.05$; Table 5.1). Additionally, at this concentration the hybrid toxin displayed significant effects on AP AHP, which was reduced to 3.5 ± 2.6 mV in the presence of toxin ($n = 6$, $p < 0.05$; Table 5.1). Alternately, the AP amplitude (control 93.5 ± 2.0 mV; $n = 52$) was diminished to 33.2 ±9.9 mV ($n = 6$, $p < 0.05$; Table 5.1).

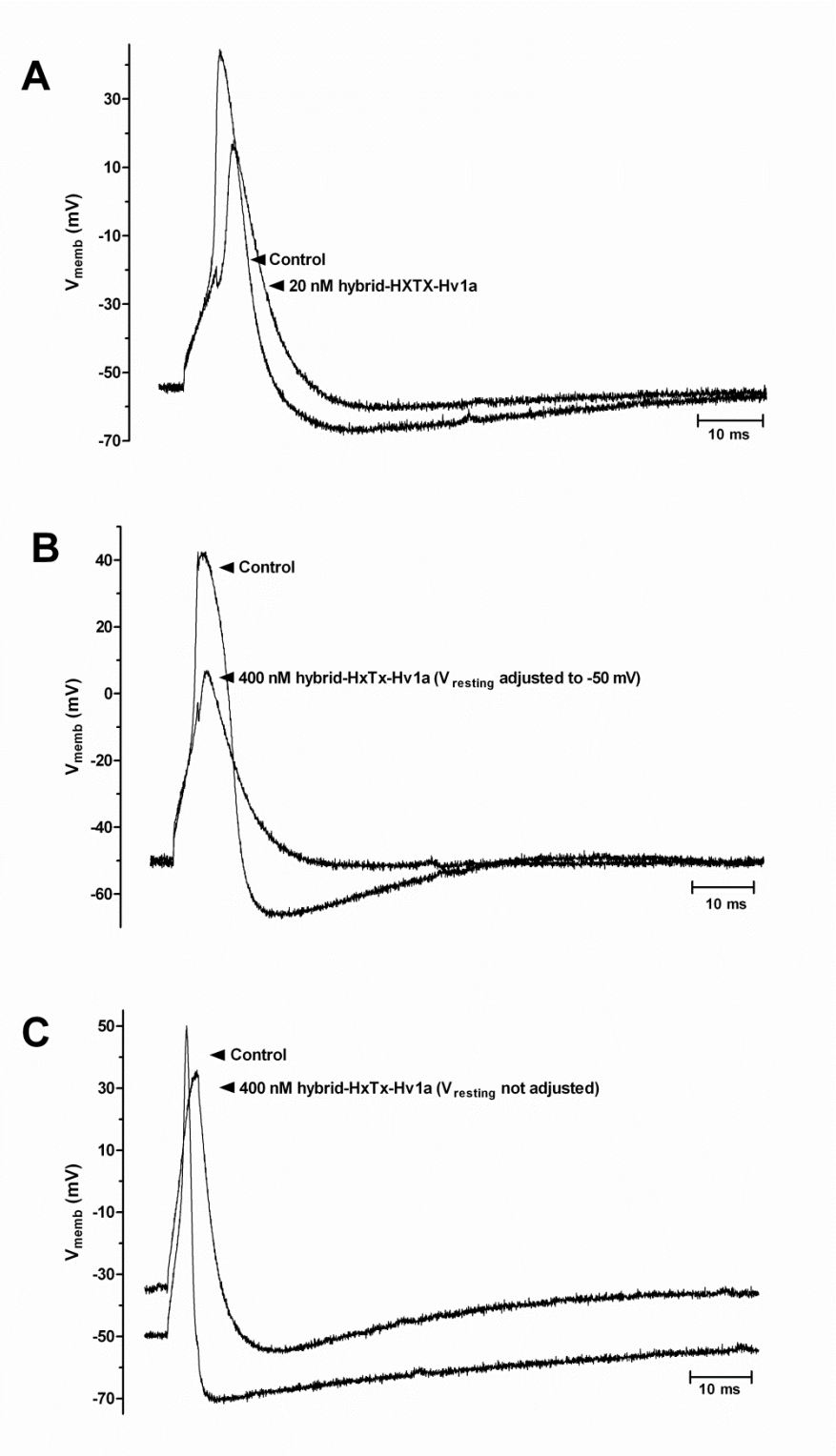

Figure 5.8: Effects of hybrid-HXTX-Hv1a on evoked APs in DUM neurons. Traces show representative APs generated in response to 4 ms, 2 nA stimuli recorded at 10-s intervals. Traces show the effects of **(A)** 20 nM hybrid-HXTX-Hv1c, (B–C) 400 nM hybrid-HXTX-Hv1c. **(B)** Represents the typical effect of 400 nM hybrid-HXTX-Hv1c where the resting membrane potential has been adjusted to control levels. This has been

done to allow the comparison of AP shape without the influence of the changing membrane potential. **(C)** Represents the typical effect of 400 nM hybrid-HXTX-Hv1c where the resting membrane potential has not been adjusted.

In comparison to κ-HXTX-Hv1c, the hybrid toxin was found to have additional effects on both firing frequency and AP shape. At low concentrations the effects of hybrid-HXTX-Hv1a were similar to κ-HXTX-Hv1c including a trend towards; increasing firing frequency, diminished AHP amplitude and prolonged AP repolarisation. However, at higher concentrations, hybrid toxin application resulted in a trend towards *decreasing* firing frequency, a shifting of membrane potential in the depolarising direction, a more substantial trend towards decreasing the AHP and a significant reduction in AP amplitude (Table 5.1). The additional and contrasting effects at a higher concentration of hybrid toxin are likely to be a result of the Ca_V channel block.

Table 5.1. Summary AP of results. AP; action potential, AP duration$_{50}$; duration at 50% of maximal AP amplitude, AHP; AHP *$p < 0.05$ using one way ANOVA followed by a Tukey's HSD post-hoc test.

Toxin	Concentration (nM)	Spontaneous AP firing frequency (AP/sec)	Resting membrane potential (mV)	AP amplitude (mV)	AHP amplitude (mV)	AP duration$_{50}$ (ms)	AP duration$_{25}$ (ms)
Control (n = 52)	0	4.0 ± 0.6	–49.9 ± 1.0	93.5 ± 2.0	19.2 ± 1.0	2.7 ± 0.2	4.8 ± 0.4
κ-HXTX-Hv1c (n = 12) (n = 4)	3 100	6.1 ± 1.6 6.0 ± 3.0	–51.6 ± 2.9 –54.1 ± 1.5	87.7 ± 4.8 85.8 ± 4.4	14.1 ± 3.4 12.6 ± 1.7	3.5 ± 0.4 4.0 ± 1.1	6.4 ± 1.3 6.1 ± 0.2
Iberiotoxin (n = 7)	100	4.4 ± 1.1	–48.8 ± 2.0	84.5 ± 3.6	18.0 ± 1.3	3.0 ± 0.1	5.9 ± 0.3
4-AP (n = 5)	5 (mM)	12.1 ± 4.7*	–48.5 ± 1.1	94.7 ± 6.0	12.2 ± 2.0	4.0 ± 0.2	6.7 ± 0.4
hybrid-HXTX-Hv1a (n = 8) (n = 6)	20 400	6.3 ± 2.5 1.0 ± 0.3	–49.2 ± 2.3 –37.5 ± 6.5*	76.0 ± 8.8 53.2 ± 9.9*	11.6 ± 3.4 3.5 ± 2.8*	4.6 ± 1.0 4.3 ± 1.3	8.0 ± 1.4 10.3 ± 1.8
TEA, 4-AP & IbTx (n = 10)	20 (mM), 5 (mM), 100	-	–46.9 ± 2.0	95.8 ± 4.7	< 0*	92.1 ± 24.5*	298.3 ± 80.7*

5.3 DISCUSSION

The aim of this study was to determine the neurotoxic effects of the BK_{Ca} channel blocker κ-HXTX-Hv1c and hybrid-HXTX-Hv1a on spike electrogenesis in the insect nervous system. In order to achieve this goal, the effects of these toxins on resting membrane potential, evoked AP duration, amplitude, and AHP, and spontaneous firing frequency were analysed in cockroach DUM neurons. Cockroach DUM neurons possess complex membrane properties which enable spontaneous generation of repetitive APs in the absence of a rhythmic somatic input. The unique pacemaker-like properties of these neurons helped facilitate the analysis of toxin activity on spontaneous spike frequency. The ultimate aim of this study was to assess the effects of two neurotoxins from the funnel-web spider, *Hadronyche versuta*, on various aspects of AP generation in order to provide an enhanced understanding of how insect lethality is achieved.

The present study found that κ-HXTX-Hv1c exhibited a trend towards prolonging the repolarisation phase of single evoked APs. This was evidenced by a tendency of AP durations to increase in response to toxin application. Whole cell current-clamp experiments also revealed that κ-HXTX-Hv1c trends towards reducing AP AHP, while failing to influence spike depolarisation or resting membrane potential in any particular direction. In terms of firing frequency, κ-HXTX-Hv1c moves towards increasing the frequency of spontaneously generated APs in cockroach DUM neurons. This trend towards increasing firing frequency is likely explained by a shorter after-hyperpolarisation phase whereby the membrane potential returns to resting levels more rapidly.

The effects of hybrid-HXTX-Hv1a DUM neuron electrical activities were found to be somewhat more complex than those of κ-HXTX-Hv1c. At low concentrations the effects of hybrid-HXTX-Hv1a closely mimicked those of κ-HXTX-Hv1c. At 20 nM the hybrid toxin exhibited a tendency towards reducing AP AHP amplitude, in addition to slowing spike repolarisation. Repetitive spike frequency was also augmented in a comparable manner to κ-HXTX-Hv1c. Conversely, high concentrations of hybrid-HXTX-Hv1a led to a significant shift of the resting membrane potential to more depolarised potentials. Independent of the depolarised resting potential, AP repolarisation demonstrated a trend towards slowing and AHP amplitudes were

significantly reduced in the presence of toxin. The major difference at higher concentrations was that 400 nM the hybrid-HXTX-Hv1a caused trend towards *slowing* the repetitive spontaneous firing frequency. This additional activity at higher concentrations no doubt arises from the ability of hybrid-HXTX-Hv1a to block Ca_V channels in addition to its activity on BK_{Ca} channels.

5.3.1 THE ROLE OF BK_{CA} CHANNELS IN ACTION POTENTIAL GENERATION

BK_{Ca} channel currents are believed to play an important role in the modulation of electrical activity in neurons. In vertebrate dorsal root ganglia neurons, BK_{Ca} channels were found to shorten AP duration, increase the rate of repolarization and contribute to fast afterhyperpolarisation. As a consequence, BK_{Ca} channels reduce the amount of calcium entering a neuron during an AP (Raffaelli, Saviane et al., 2004). BK_{Ca} channel currents also prolong the refractory period, leading to a reduced repetitive activity (Scholz, Gru et al., 1998). The role of BK_{Ca} in spike activity is often likened to an "emergency brake" under conditions of increasing levels of internal Ca^{2+} or excessive depolarisation (Hu, Shao et al., 2001; Derst, Messutat et al., 2003; Raffaelli, Saviane et al., 2004).

Previous studies in DUM neurons and DRG neurons have shown that blockers of BK_{Ca} channels such as iberiotoxin and charybdotoxin prolong action potential duration, suppress the AHP and promote repetitive firing (Lapied, Malecot et al., 1989). This is consistent with the trends towards the broadening of the AP, reduction in the AHP and increasing in spontaneous firing seen in the presence of the insect-selective BK_{Ca} channel blockers κ-HXTX-Hv1c and low concentrations of hybrid-HXTX-Hv1a.

A number of previous studies have also analysed the consequences of BK_{Ca} channel block in vertebrate nervous tissue preparations. Changes in vertebrate BK_{Ca} channel conductance have been linked to changes in neurotransmitter release. At the frog neuromuscular junction, block of BK_{Ca} channels by the scorpion toxin charybdotoxin was shown to produce a two-fold increase in transmitter release (Robitaille and Charlton, 1992; Robitaille, Adler et al., 1993). Similarly, the BK_{Ca} channel blockers paxilline and iberiotoxin have also been shown to result in enhanced transmitter release from rat hippocampal pyramidal neurons (Raffaelli, Saviane et al., 2004). Although the effects of BK_{Ca} channel block on neurotransmitter release have not been assessed in

insects it is reasonable to suggest that κ-HXTX-Hv1c could potentially lead to increases in neurotransmitter release and subsequent excitatory neurotoxicity. It would be interesting to look at changes in neurotransmitter release in response to κ-HXTX-Hv1c particularly at central synapses and flight muscles. Therefore the increased spontaneous spike frequency and increased duration of the terminal action potential produced by κ-HXTX-Hv1c and low concentrations of hybrid-HXTX-Hv1a would be hypothesised to result in an increase in calcium entry into the nerve terminal and an increase in neurotransmitter release leading to an excitatory phenotype.

5.3.2 THE ROLE OF Ca_V CHANNELS IN ACTION POTENTIAL GENERATION

While the excitotoxic activity of κ-HXTX-Hv1c on insects appears to result from a selective block of BK_{Ca} channels, hybrid-HXTX-Hv1a is known to induce a depressant phenotype in insects, seemingly at odds with a block of BK_{Ca} channels. Importantly however, hybrid toxins have been previously shown to inhibit Ca_V channels in DUM neurons at higher concentrations (Simon Gunning, Suping Wen, Glenn King and Graham Nicholson, unpublished results).

To date, two classes of Ca_V channels have been identified in cockroach DUM neurons. The mid-to-low-voltage-activated (M-LVA) and high-voltage-activated (HVA) Ca_V channel classification is based on the pharmacological profiles, gating kinetics and voltage sensitivities of these currents. Both channel types are believed to play important, but distinct, roles in shaping AP generation. While the subtype specificity of hybrid-HXTX-Hv1a has not been investigated on these channel subtypes, the highly homologous hybrid-HXTX-Ar1a targets both M-LVA and HVA Ca_V channels, consistent with the conservation of all residues within the pharmacophore of ω-HXTX-Hv1a.

LVA Ca_V channels play two distinct roles in the generation of spontaneous activity. It has been demonstrated that LVA Ca_V channel currents in DUM neurons contribute to the pre-depolarisation phase (Grolleau, Lapied et al., 1996) of the AP. As LVA Ca_V channels are inactive at hyperpolarised potentials and activate around resting potentials (–50 mV) they are ideal candidates to initiate this pre-depolarisation phase. In addition, LVA Ca_V channel currents are involved in the regulation of firing frequency. It is possible that the slowing of firing frequency in response to 400 nM hybrid-HXTX-Hv1a

may be as a consequence of its effect to block LVA Ca_V channels. In support, the hybrid toxin greatly appears to prolongs the pre-depolarisation phase of spontaneously generated APs (Figure 5.4D). Of course this may result from a depolarising shift in the voltage-dependence of Ca_V channel activation, however no shifts in the threshold of activation were observed for either hybrid-HXTX-Hv1a or hybrid-HXTX-Ar1a (Simon Gunning, Suping Wen, Glenn King and Graham Nicholson, unpublished results).

Ca^{2+} conductance through Ca_V channels also plays an important role in the modulation of neuronal electrical activity. The role of internal Ca^{2+} is particularly important in the control of BK_{Ca} channel conductance as well as the inactivation of the Ca_V channel itself. Ca_V channels control the rising phase of AP and AP duration, with the ability to prolong or shorten the AP in relation to K_{Ca} channel activation (Achenbach, Walther et al., 1997; Wicher, 2001). Specifically, M-LVA Ca_V channels are believed to play an important role in BK_{Ca} channel activation in terms of controlling duration and AHP of the AP (Wicher and Penzlin, 1994; Wicher, Walther et al., 1994; Wicher and Penzlin, 1997; Derst, Messutat et al., 2003).

Thus, at low concentrations (20 nM) hybrid-HXTX-Hv1a appears to act in a similar manner to κ-HXTX-Hv1c by directly blocking the BK_{Ca} channel to increase tonic AP firing. It would appear that hybrid-HXTX-Hv1a enhances this effect by a synergistic, albeit minor, block of Ca_V channels at low concentrations. At higher concentrations (400 nM) hybrid-HXTX-Hv1a produces a depolarisation of the resting membrane potential and a reduction in spontaneous firing frequency consistent with a depressant phenotype. This appears to be due to a strong inhibition of Ca_V channels at this concentration.

Importantly, this study not only looks at the effects BK_{Ca} channel block, but also examines the impact of simultaneous Ca_V channel block on DUM neuron electrical activity. Reflected in these results we see the enhanced prolongation of AP duration, reduction in hyperpolarisation amplitude as well as a reduction spike frequency, relating directly to Ca_V channel block.

5.3.3 Conclusions and future directions

This study illustrates that there is still much that is unknown about the insect nervous system. While we are beginning to understand the roles of various voltage-gated ion

channels in the shaping of APs, it is still difficult to make any conclusions as far as their role in overall neurotoxicity. From this study we further validate the effects of BK_{Ca} channel block on AP shape and firing frequency. However, it is demonstrated the the action of κ-HXTX-Hv1c on insect AP only exhibits trends towards slowing repolarisation and reducing AHP, and subsequently many of the results are not statistically significant. On the other hand, we were able to look at the significant effects evoked by simultaneous block of BK_{Ca} and Ca_V channels by a hybrid toxin, on DUM neuron electrical activity. While we can make certain attempts to explain the insecticidal activity in terms of the effects seen on APs there remain many unanswered questions.

Furthermore, the results of this study seem to indicate that the effects of κ-HXTX-Hv1c on AP repolarisation and AHP are more substantial than those seen under complete block of the BK_{Ca} in the presence of IbTx. In fact, the results seem to more closely resemble the effects of $I_{K(A)}$ inhibition by 4-AP. As previous studies (Gunning, Maggio et al., 2008) have revealed a moderate effect of κ-HXTX-Hv1c on $I_{K(A)}$ (albeit at high concentrations) the results of this study seem to indicate that block of this channel may play a supporting role in the insecticidal toxicity of κ-HXTX-Hv1c. In fact, it should be considered that $I_{K(A)}$ could potentially be the sole lethal target of κ-HXTX-Hv1c. Moreover, it is also imperative to recognise that the effects of κ-HXTX-Hv1c on insect AP generation are not statistically significant and further implicate the involvement of an additional lethal target.

Chapter Six

The effects of κ-HXTX-Hv1c on voltage-gated potassium channels

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[2012]

6.1 INTRODUCTION

The κ-HXTX-Hv1 toxins are a family of excitatory insecticidal neurotoxins isolated from the venom of the Blue Mountains funnel-web spider, *Hadronyche versuta* (Wang, Connor et al., 2000). These toxins are lethal against a number of agriculturally important arthropod pests including those from the orders Coleoptera, Dictyoptera, Diptera, Lepidoptera and Orthoptera (Wang, Connor et al., 2000; Maggio and King, 2002a; Tedford, Maggio et al., 2007). Direct application of κ-HXTX-Hv1c to the metathoracic ganglia of *Periplaneta americana* results in the rapid development of excitatory symptoms including spontaneous uncoordinated movement of all legs and limb fasciculations (Wang, Connor et al., 2000). Secondly, the κ-HXTX-Hv1 toxins exhibit insect selectivity with no activity against a number of vertebrates including new born mice and adult rabbits, preparations of rat vas deferens and chick biventer cervicis (Wang, Connor et al., 2000).

Recent patch-clamp studies on *Periplaneta americana* DUM neurons identified the large conductance calcium-activated potassium (BK_{Ca}) channel as a target of the prototypic member, κ-HXTX-Hv1c (Gunning, Maggio et al., 2008). The study details that although κ-HXTX-Hv1c failed to affect voltage-gated sodium (Na_V) and calcium (Ca_V) channel currents, the toxin blocks a significant proportion of the global voltage-gated potassium current (I_K) which is attributed to a potent block of BK_{Ca} channel currents. Channel block was also confirmed on expressed insect BK_{Ca} (pSlo) channels albeit at higher concentrations (Gunning, Maggio et al., 2008). However, this reduced potency has also been observed with the classical BK_{Ca} channel toxin charybdotoxin (ChTx) (Derst, Messutat et al., 2003; Gunning, Maggio et al., 2008). This discrepancy was believed to be due to the absence of the auxiliary β-subunit which is believed to be important for optimal channel activity (Hanner, Schmalhofer et al., 1997).

This chapter describes the use of several selective BK_{Ca} channel blockers to assess the validity of this channel as the lethal insecticidal target of κ-HXTX-Hv1c. The blockers selected included the well characterised peptidic toxins charybdotoxin and iberiotoxin and the non-peptidic paxilline a tremorgenic indole alkaloid isolated from the fungus *Penicillium Paxilli* (Cole, Kirksey et al., 1974). Prior to this study paxilline had only been recognised for its activity against mammalian BK_{Ca} channels. Furthermore, this

toxin was selected for this study due to its selective block of mammalian BK_{Ca} channels. For example, paxilline fails to modify a variety of other mammalian channels, including; hSK1 (human small conductance K_{Ca}), mouse K_{DR} (minor effect), L-type Ca_V and Na_V (Knaus, McManus et al., 1994).

In terms of neurotoxicity, the contractile response elicited by paxilline and other tremorgenic indole alkaloids in vertebrates has been attributed to increases in neurotransmitter release (Knaus, McManus et al., 1994). Due to the role of BK_{Ca} channels in the regulation of action potential duration and subsequently neurotransmitter release, the effects of paxilline have been extensively analysed on a number of vertebrate channels (DeFarias, Carvalho et al., 1996; Sanchez and McManus, 1996; Strobaek, Christophersen et al., 1996; Li and Cheung, 1999; Longland, Dyer et al., 2000; Molinari, Sullivan et al., 2000). Furthermore, paxilline has also been found to enhance ChTx and IbTx binding through an allosteric mechanism (DeFarias, Carvalho et al., 1996; Sanchez and McManus, 1996; Li and Cheung, 1999; Molinari, Sullivan et al., 2000).

In this study the electrophysiological characterisation of the fungal metabolite paxilline on cockroach DUM neurons is described. Due to κ-HXTX-Hv1c's high potency against the BK_{Ca} channel, this channel was initially perceived to be the insecticidal target; however the failure to evoke lethality through isolated block of this channel using a variety of other non-phylum selective BK_{Ca} blockers such as paxilline, charybdotoxin and iberiotoxin highlights the need to identify additional insect targets. Subsequently, we used whole-cell patch-clamp analysis of cockroach dorsal unpaired median (DUM) neurons to study the effects of κ-HXTX-Hv1c on sodium-activated K_V (K_{Na}) channels and A-type transient K_V (K_A) channel currents. This work indicates that although κ-HXTX-Hv1c targets the BK_{Ca} channel with very high potency, it is unlikely to be the lethal target and moreover highlights a Shal-like K_A channel as a potential lethal target.

6.2 RESULTS

6.2.1 PAXILLINE BLOCKS INSECT BK_{CA} CHANNELS

Previous studies allude to the BK_{Ca} channel as the lethal target of κ-HXTX-Hv1c; however it still remains to be proven whether block of this channel is in fact

insecticidal. Therefore, we assayed a number of BK_{Ca} channel blockers for neurotoxic activity in the house cricket, *Acheta domestica*, including phylum-selective (κ-HXTX-Hv1c and hybrid-HXTX-Hv1c) and non-selective (charybdotoxin, iberiotoxin and paxilline) BK_{Ca} channel blockers.

Paxilline is one of the most potent non-peptidic blockers of BK_{Ca} channels in vertebrates (Sanchez and McManus, 1996) and was of great interest in the present study as it has been shown to display insecticidal properties in insects (Belofsky, Gloer et al., 1995). Although the activities of paxilline have been assessed on a number of mammalian BK_{Ca} channels, little is understood concerning its acute toxicity in invertebrates. Therefore, prior to assessing the neurotoxic effects of paxilline it was imperative to first confirm BK_{Ca} channel activity in insect neurons.

Whole-cell patch-clamp techniques were used to assess the effects of paxilline on isolated DUM neurons from the cockroach *Periplaneta americana*. Paxilline was tested on a number of insect voltage-gated channel currents likely to be associated with neurotoxicity. In order to confirm the activity of paxilline on BK_{Ca} channels, $I_{BK(Ca)}$ were recorded in isolation from other voltage-gated ion channels in the presence of 300 nM TTX (blocking Na_V channels), 1 mM $CdCl_2$ and internal fluoride (blocking Ca_V channels). Due to the absence of selective blockers for $I_{K(DR)}$ a combination of channel blockers followed by offline subtractions where employed to isolate $I_{BK(Ca)}$ from other $I_{K.}$ In the presence of 5 mM 4-aminopyridine (4-AP) to block $I_{K(A)}$, +30 mV test pulses from a holding potential (V_h) of –80 mV were used to elicit $I_{BK(Ca)}$ and $I_{K(DR)}$. At the conclusion of experiments 30 nM IbTx was applied to block $I_{BK(Ca)}$, therefore enabling the offline subtraction of the remaining $I_{K(DR)}$ (see chapter 2 for further details). In the presence of 1 μM paxilline, $I_{BK(Ca)}$ were completely abolished within five minutes of initial exposure (n = 4). At lower concentrations, paxilline demonstrated a concentration-dependent inhibition of both transient and sustained $I_{BK(Ca)}$ elicited in DUM neurons (Figure 6.1B). The toxin was shown to have potent actions on cockroach BK_{Ca} channels with an IC_{50} of 13.6 ± 4 nM on the transient $I_{BK(Ca)}$ and 12.5 ± 0.6 nM on the sustained $I_{BK(Ca)}$ (n = 4), which is comparable with its effects on mammalian $I_{BK(Ca)}$ (mSlo K_i 2.2 nM (Sanchez and McManus, 1996), hSlo IC_{50} 30–50 nM (Strobaek, Christophersen et al., 1996; McMillan, Carr et al., 2003) and rat mesenteric arterial cells K_i 35.7 nM (Li and Cheung, 1999)).

To determine the mode of action of $I_{BK(Ca)}$ block, the effects of paxilline on the voltage-dependence of channel activation were assessed. Families of $I_{BK(Ca)}$ were elicited by100-ms voltage pulses from a holding potential of –80 mV in 10-mV increments. No significant shift in the $V_{1/2}$ was observed ($p > 0.05$, $n = 5$; Table 6.1). Therefore indicating that paxilline is likely to block the pore of the insect BK_{Ca} channel.

Table 6.1 $V_{1/2}$ values for 10 μM paxilline on $I_{K(Ca)}$.

	Transient $I_{K(Ca)}$		Sustained $I_{K(Ca)}$	
	Control	**paxilline**	**Control**	**paxilline**
$V_{1/2}$ (mV)	19.1 ± 3.2	10.0 ± 1.7	6.0 ± 2.3	10.0 ± 5.3

Due to ability of some toxins to simultaneously target a number of K_V channel subtypes, the effects on A-type fast transient ($I_{K(A)}$), sodium-activated ($I_{K(Na)}$) and delayed-rectifier ($I_{K(DR)}$) currents in cockroach DUM neurons were assessed. $I_{K(A)}$ were recorded in the presence of 300 nM TTX, internal fluoride and 100 nM iberiotoxin in order to block Na_V, Ca_V and K_{Ca} channel currents, respectively. $I_{K(DR)}$ were recorded in the presence of the same blockers with the addition of 4-AP to block $I_{K(A)}$. Alternately, $I_{K(Na)}$ were initially recorded in the presence of only internal fluoride and iberiotoxin with TTX applied only at the conclusion of experiments. Due to the absence of selective blockers for $I_{K(DR)}$ that do not also block $I_{K(Na)}$ or $I_{K(A)}$ these currents were recorded in the presence of $I_{K(DR)}$. Subsequently, in order to isolate these currents from $I_{K(DR)}$, selective blockers (TTX or 4-AP) for the channels of interest ($I_{K(Na)}$ or $I_{K(A)}$, respectively) were applied at the conclusion of recordings so the remaining $I_{K(DR)}$ could be manually subtracted offline (see section 2.4.1.3 for further details). 10 μM paxilline failed to alter DUM neuron $I_{K(A)}$ (0.6 ± 7.7% inhibition; $n = 6$, $p > 0.05$, 6.1G), $I_{K(DR)}$ (0.1 ± 3.6% inhibition; $n = 6$, $p > 0.05$, 6.1E) or $I_{K(Na)}$ (7.0 ± 4.1% inhibition; $n = 3$, $p > 0.05$, 6.2F). Additionally, paxilline failed to modify the voltage-dependence of activation or $V_{1/2}$ for $I_{K(A)}$ ($V_{1/2}$ control –5.7 ± 2.2 mV vs. paxilline –12.2 ± 2.7 mV; $n = 6$, $p > 0.05$) or $I_{K(DR)}$ ($V_{1/2}$ control –5.3 ± 2.8 mV vs. paxilline –11.8 ± 5.9 mV; $n = 6$, $p > 0.05$, 6.1H and F). Due to the complex nature of recording K_{Na} channel currents, the voltage-dependence of channel activation could not be assessed accurately, therefore data is not included.

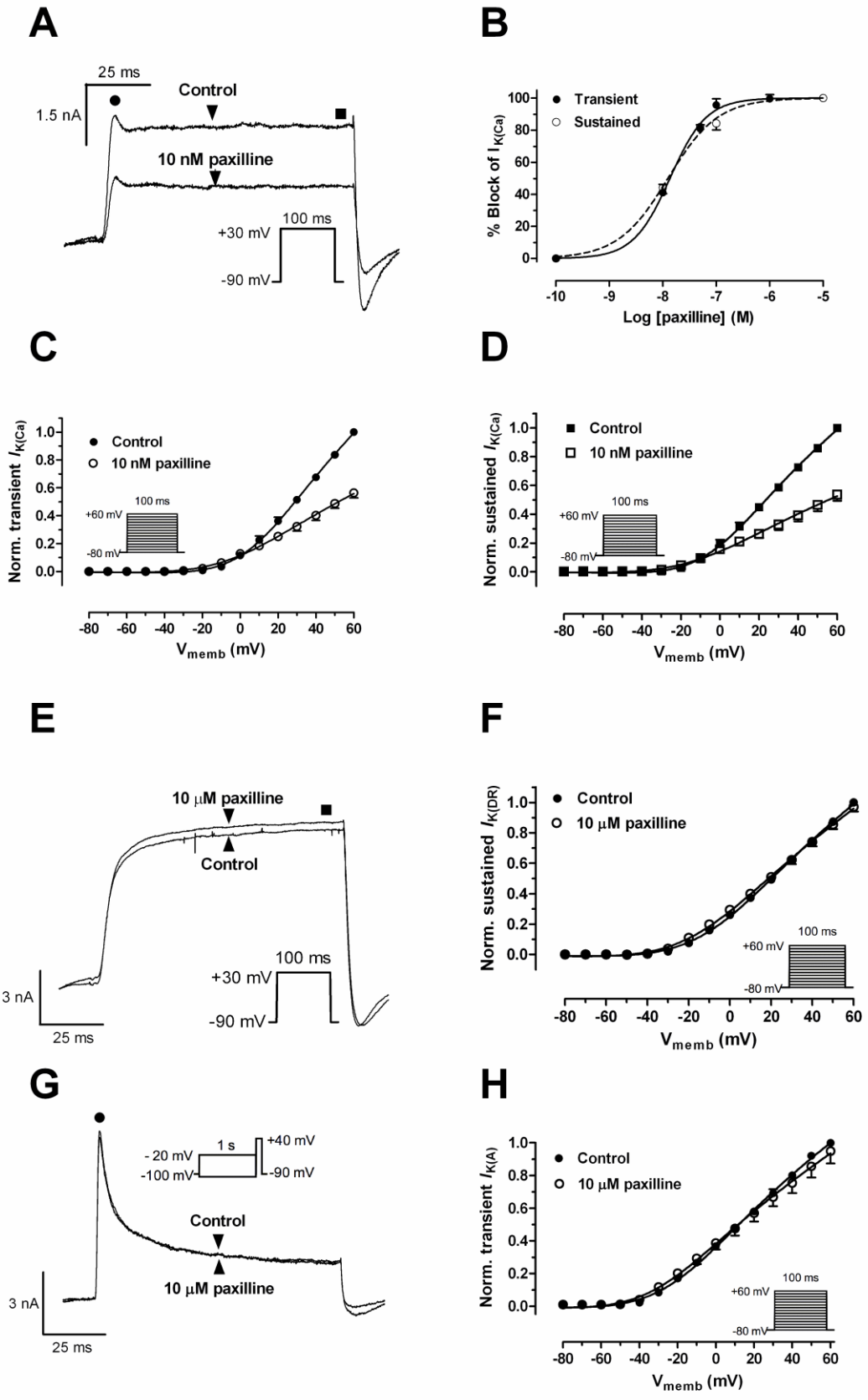

A
25 ms
Control
1.5 nA
10 nM paxilline
100 ms
+30 mV
-90 mV
B
Transient
Sustained
% Block of $I_{K(Ca)}$
Log [paxilline] (M)
C
Control
10 nM paxilline
Norm. transient $I_{K(Ca)}$
100 ms
+60 mV
-80 mV
V_{memb} (mV)
D
Control
10 nM paxilline
Norm. sustained $I_{K(Ca)}$
V_{memb} (mV)
E
10 μM paxilline
Control
100 ms
+30 mV
-90 mV
3 nA
25 ms
F
Control
10 μM paxilline
Norm. sustained $I_{K(DR)}$
V_{memb} (mV)
G
1 s
- 20 mV
-100 mV
+40 mV
-90 mV
Control
10 μM paxilline
3 nA
25 ms
H
Control
10 μM paxilline
Norm. transient $I_{K(A)}$
V_{memb} (mV)

Figure 6.1: Effects of paxilline on DUM neuron $I_{BK(Ca)}$, $I_{K(DR)}$ and $I_{K(A)}$. **(A)** Representative superimposed $I_{BK(Ca)}$ traces showing the typical effect of 10 nM paxilline. **(B)** Concentration-response curve for paxilline block of transient (closed circle) and sustained (closed square) $I_{K(Ca)}$. Data were fitted with Eq. 2 (see chapter 2, section 5) yielding IC_{50} values of 14 ± 4 and 13 ± 6 nM for transient and sustained $I_{K(Ca)}$, respectively. Transient **(C)** and sustained **(D)** $I_{BK(Ca)}$-V curves for control (closed symbols) and after the addition 10 nM paxilline (open symbols) (n = 5). (E and G) Representative traces of the typical lack of effect of 10 μM paxilline on $I_{K(DR)}$ **(E)** and $I_{K(A)}$ **(G)** (n = 6, p > 0.05). Measurement points are indicated by closed circles (transient) and closed squares (sustained). $I_{K(DR)}$-V **(F)** and $I_{K(A)}$-V **(H)** curves for control (closed symbols) and in response to 10 μM paxilline (open symbols) (n = 6). I-V data were fitted with Eq. 1 (see chapter 2, section 5). Currents were recorded in response test pulse protocols shown in the inset of each panel.

6.2.2 Effects of paxilline on I_{Na} and I_{Ca}

Although paxilline activity in vertebrates is selective for BK_{Ca} channels, it was important to confirm that the anti-insectan activity was not due to interactions with other important voltage-gated ion channels. Firstly, the effects of paxilline on the activity of Na_V channels in cockroach DUM neurons were examined. Rapidly-inactivating I_{Na} were evoked by –10 mV test pulses from a V_h of –90 mV. Subsequent exposure to 10 μM paxilline failed to alter Na_V current amplitude or inactivation kinetics (1.3 ± 4.6 % block, n = 5, p > 0.05; Figure 6.2A). The voltage-dependence of channel activation was assessed using depolarising membrane steps from –90 to +60 mV in 10-mV increments. 10 μM paxilline also failed to modify the voltage-dependence of activation as evidenced by no change in the $V_{1/2}$ (control –42.0 ± 4.3 mV, vs. paxilline –41.8 ± 3.5 mV, n = 5) and V_{rev} (control 31.6 ± 6.1 mV, vs. 10 μM paxilline 31.6 ± 8.4 mV; n = 5, p > 0.05, Figure 6.2B).

Paxilline activity was also evaluated on Ca_V channel currents in isolated cockroach DUM neurons. In order to minimise Ca^{2+} induced current rundown, Ba^{2+} was substituted as the charge carrier through Ca_V channels (Wicher and Penzlin, 1997). Dual pulse protocols were used to evoke both mid-to-low-voltage-activated (M-LVA) and high-voltage-activated (HVA) Ca_V channel current subtypes, which have been described

in cockroach DUM neurons (Chong, Hayes et al., 2007). Alternating +30 mV and –30 mV test pulses were used to elicit small amplitude transient HVA and large amplitude slowly inactivating M-LVA currents, respectively. 10 μM paxilline induced a modest block of both HVA and M-LVA Ca_V channel currents (14.0 ± 3.6% and 12.9 ± 3.9%, respectively; $n = 6$, $p < 0.05$, Figure 6.2C and D). Families of depolarising test pulses from –90 mV to +20 mV in 10-mV intervals revealed no significant shift in the presence of 10 μM paxilline in respect to the reversal potential (V_{rev} control 33.7 ± 4.1 mV vs. paxilline 32.1 ± 1.9 mV and $V_{1/2}$ control –49.4 ± 3.1 mV vs. paxilline –48.7 ± 1.4 mV; $n = 3$, $p > 0.05$, Figure 6.2B).

These findings indicate that paxilline inhibits insect BK_{Ca} channels, while failing to affect Ca_V, Na_V and other K_V channel subtypes in insects. Given the insect toxicity of paxilline to a number of insect genera (Belofsky, Gloer et al., 1995) these effects support the BK_{Ca} channel as a potential lethal target in insects. However, as previous studies of paxilline and related indole diterpines only report anti insectan activity via feeding and growth trials we decided it was important to observe the effects of the toxin through acute toxicity assays.

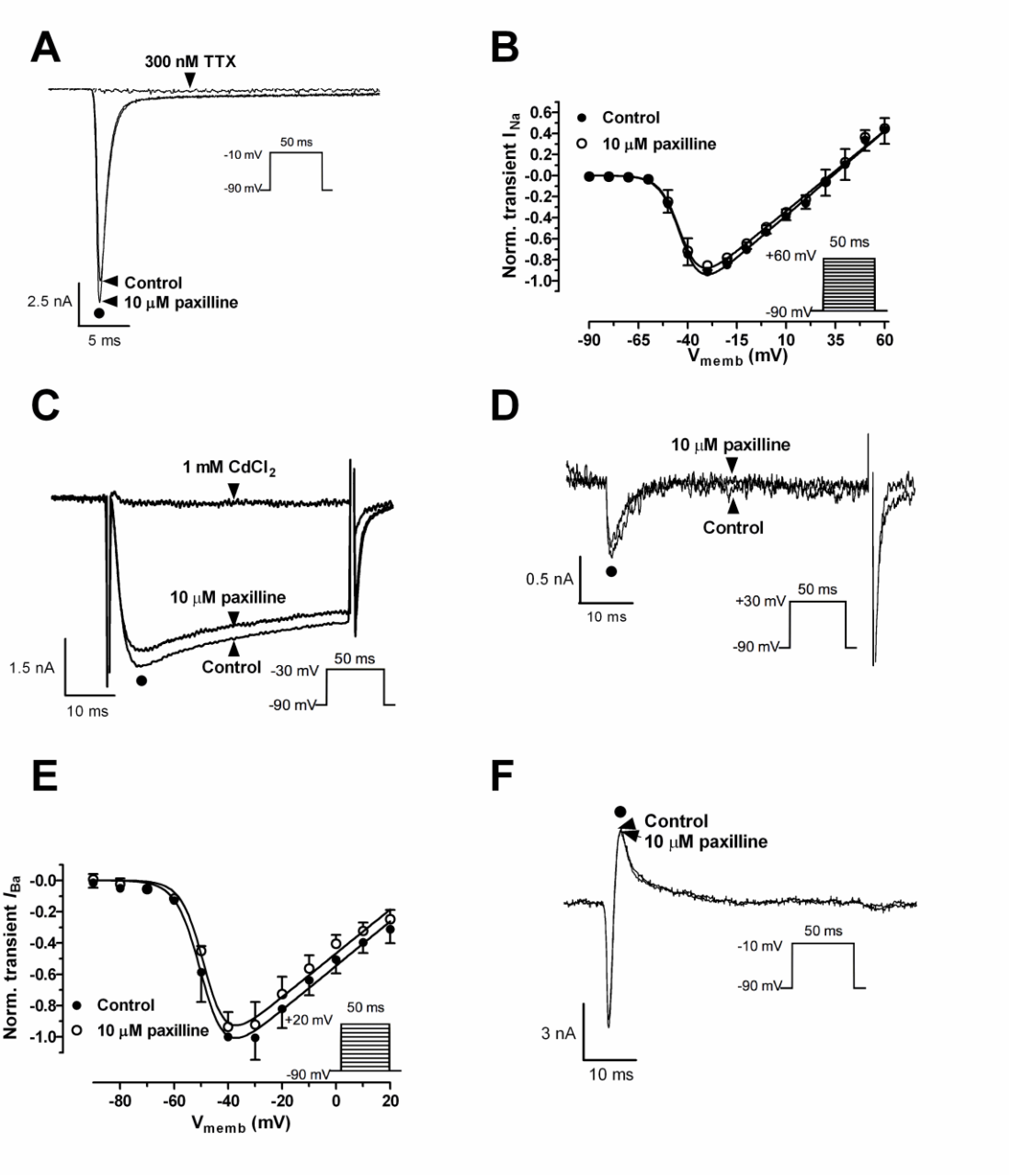


Figure 6.2: Effects of paxilline on DUM neuron I_{Na}, I_{Ba} and $I_{K(Na)}$. (A) Typical superimposed I_{Na} traces illustrating the lack of a significant effect 10 μM paxilline, and complete block following the addition of 300 nM TTX. **(B)** I_{Na}-V curves for control (closed circles) and following the addition of 10 μM paxilline (open circles) (n = 5). (C–D) Representative M-LVA **(C)** and HVA **(D)** I_{Ba} in the presence of 10 μM paxilline and complete block following the addition of 1 mM $CdCl_2$ (C). **(E)** I_{Ba}-V curves for control (closed circles) and in the presence of 10 μM paxilline (open circles) (n = 5). **(F)** Superimposed $I_{K(Na)}$ traces demonstrating the typical effects in the presence of 10 μM

paxilline. Currents were elicited using the test pulse protocols illustrated within the insets of each panel. *I-V* data were fitted with Eq. 1 (see chapter 2, section 5).

6.2.3 THE NEUROTOXIC ACTIVITY OF K_{CA} CHANNEL TOXINS IN ARTHROPODS

Paxilline, as well as archetypal BK_{Ca} channel blockers IbTx and ChTx, were selected to assess the ability of BK_{Ca} channel block to induce neurotoxicity in invertebrates. Given that paxilline is a largely hydrophobic molecule, ethanol (1 part ethanol to 470 parts insect saline) was used to assist the solubility of the high paxilline concentrations used in acute toxicity experiments. Acute toxicity testing was carried out in house crickets (*Acheta domesticus*) by intrathoracic injections. Control experiments revealed that the presence of ethanol did not influence the lethality assays. However, increasingly higher concentrations of paxilline also failed to induce insect lethality. Acute toxicity testing in house crickets of all selective BK_{Ca} channel blockers resulted in minor signs of toxicity including twitching limbs and intermittent abdominal concentrations, at no time however were there any deaths due to toxin injection. At toxin concentrations of >2 nmol/g no signs of toxicity were evident at time points beyond two hours (Figure 6.3). These minor effects are contrary to the lethal neurotoxicity demonstrated by κ-HXTX-Hv1c in house crickets (LD_{50} 167 pmol/g after 48 hrs) (Wang, Connor et al., 2000). These results indicate that selective block of the insect BK_{Ca} channel is not sufficient to induce the potent lethal effects demonstrated by κ-HXTX-Hv1c.

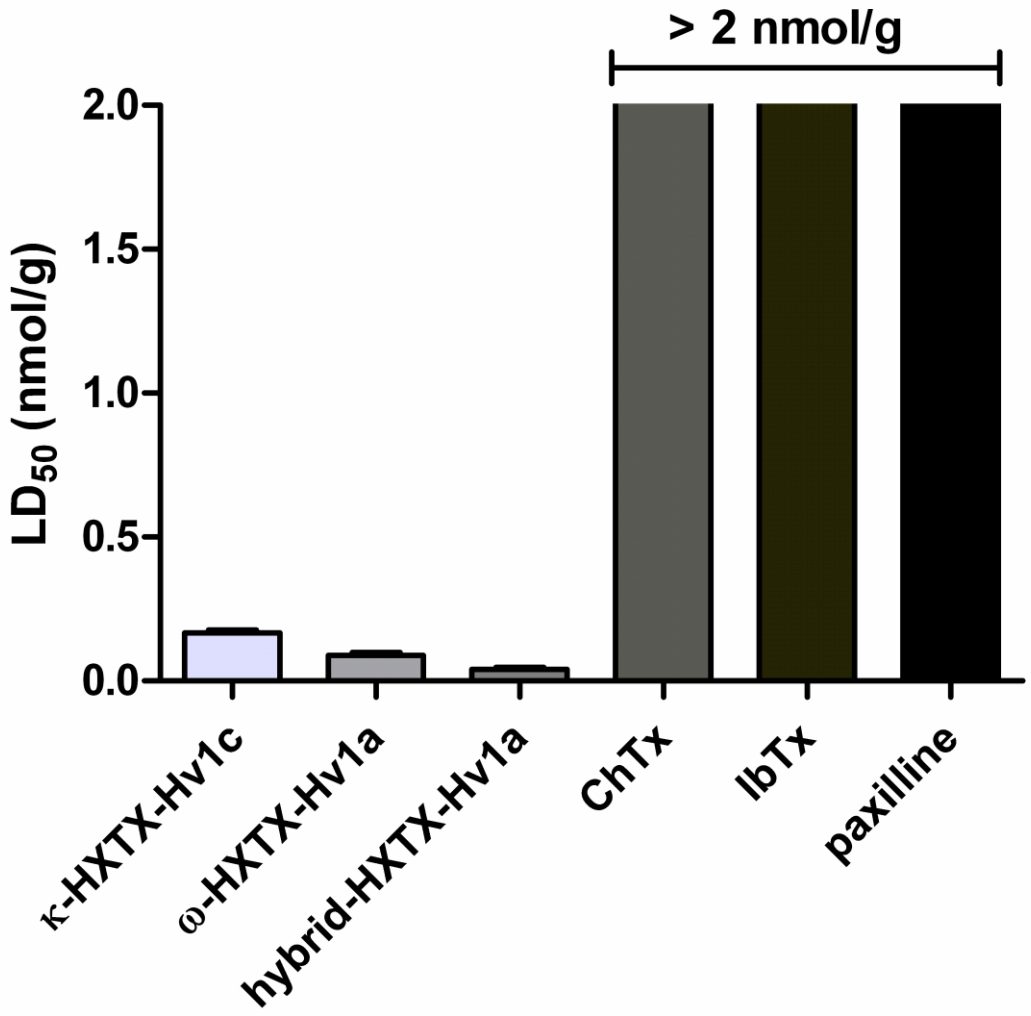


Figure 6.3: Insecticidal activities of insect-selective spider neurotoxins and BK_{Ca} channel blockers in the house cricket, *Acheta domestica*. LD_{50} values at 48 hr post injection in *A. domestica* were 167 ± 10 pmol/g, 89 ± 10 pmol/g and 41 ± 7 pmol/g for κ-HXTX-Hv1c (Wang, Connor et al., 2000; Tedford, Maggio et al., 2007), ω-HXTX-Hv1a (Wang, Smith et al., 1999) and hybrid-HXTX-Hv1a (Suping Wen, Glenn King and Graham Nicholson, unpublished results), respectively. Charybdotoxin (ChTx), iberiotoxin (IbTx) and paxilline displayed no insecticidal activity in house crickets at doses up to 2 nmol/g. All data represents the mean LD_{50} value ± SE recorded at 48 hr post-injection from at least three independent trials.

6.2.4 κ-HXTX-Hv1c on K_{Na} channel currents

Due to the inability of a number of classical BK_{Ca} channel blockers to induce insect lethality, other potential targets needed to be considered. Of the K_V channels known to be present in DUM neurons only the K_{Na} channel remained untested as a potential target of κ-HXTX-Hv1c. Accordingly, K_{Na} channel currents in DUM neurons were elicited in the presence of the inward I_{Na} (Figure 6.4F) and isolated through offline subtraction routines (see chapter 2 for further details). I_K and I_{Na} were evoked by 50-ms voltage-pulses to –10 mV from a holding potential of –90 mV. These currents were recorded in the presence of 1 mM $CdCl_2$, 30 nM IbTx and internal KF to block Ca_V and BK_{Ca} channel currents. Unfortunately 4-AP could not be used to block $I_{K(A)}$ as it also inhibits $I_{K(Na)}$ (Wicher, Walther et al., 2001). The absence of $I_{K(A)}$ block when recording K_{Na} channel currents causes somewhat of a dilemma given the reported modest inhibition of $I_{K(A)}$ by κ-HXTX-Hv1c in DUM neurons. In the presence of 1 μM κ-HXTX-Hv1c K_{Na} currents were reduced by 27.6 ± 9.3% (n = 4, p < 0.05; Figure 6.4E). Due to the lack of $I_{K(A)}$ block, moderate block of K_{Na} in the presence of κ-HXTX-Hv1c does not necessarily confirm or eliminate this channel as a possible target. However, the current inhibition does not appear to be substantially greater than the 24.9 ± 3.6% (n = 4, p < 0.05) from $I_{K(A)}$ inhibition at –10 mV (Figure 6.7H). These results further highlight the need to identify more selective toxins for the isolation of channel currents in native insect neurons.

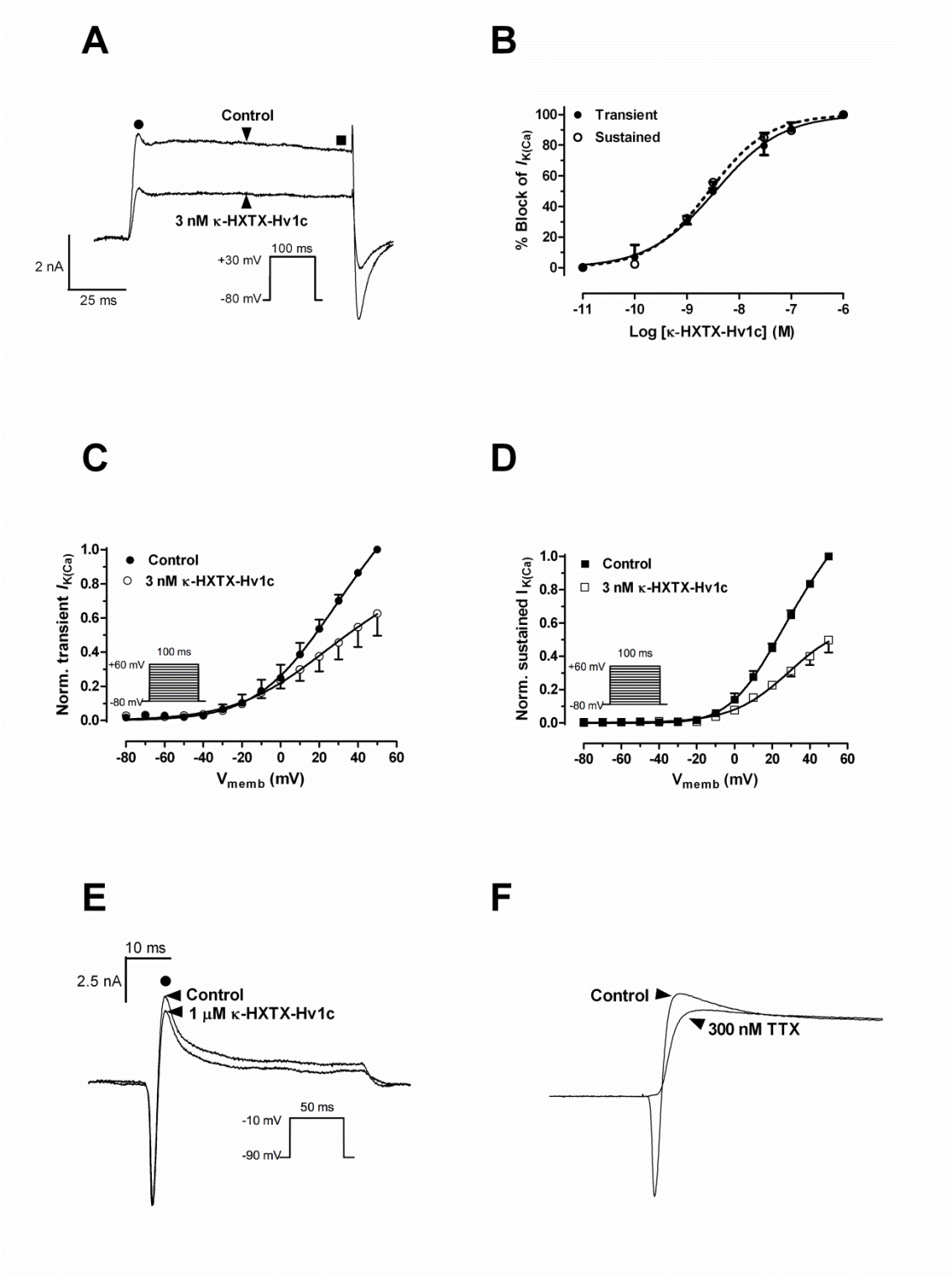


Figure 6.4: Effects of κ-HXTX-Hv1c on DUM neuron $I_{BK(Ca)}$ and $I_{K(Na)}$. **(A)** Superimposed $I_{BK(Ca)}$ traces illustrating the effect 3 nM synthetic κ-HXTX-Hv1c. The transient (circle) and late (square) $I_{BK(Ca)}$ are indicated. **(B)** Concentration-response curves of transient (closed circles), and sustained (open circles), $I_{BK(Ca)}$ in the presence of synthetic κ-HXTX-Hv1c. Data were fitted with Eq. 2 (see chapter 2, section 5), generating IC_{50}

values of 3.5 ± 0.9 and 3.0 ± 0.8 nM for transient and sustained $I_{BK(Ca)}$, respectively (n = 3–4). Representative transient **(C)** and sustained **(D)** $I_{BK(Ca)}$-V for control (closed symbols) and in the presence of 3 nM synthetic κ-HXTX-Hv1c (open symbols) (n = 3–6). I-V data were fitted with Eq.1 (see chapter 2, section 5). **(E)** Superimposed $I_{K(Na)}$ traces demonstrating the typical effects of 3 nM synthetic κ-HXTX-Hv1c. **(F)** A representative trace illustrating the isolation of $I_{K(Na)}$ using 300 nM TTX. The sodium sensitive portion of the I_K is blocked by TTX, the remaining current is digitally subtracted offline in order to isolate $I_{K(Na)}$ (see chapter 2 for further details). All test pulse protocols used to evoke currents are illustrated within the insets of each panel.

6.2.5 INSECTICIDAL ACTIVITY OF 4-AMINOPYRIDINE

Due to the reported moderate effects of κ-HXTX-Hv1c on insect $I_{K(A)}$ (Gunning, Maggio et al., 2008) we decided to investigate the potential of A-type transient K_V channel blockers to induce neurotoxic symptoms in insects, before further exploring the role the A-type transient K_V channels in κ-HXTX-Hv1c activity. 4-AP is often used to isolate $I_{K(A)}$ in DUM neurons (Grolleau and Lapied, 2000, Grolleau, 1995) and was selected to assess the ability of $I_{K(A)}$ block to mimic the lethal, neurotoxic activity of κ-HXTX-Hv1c.

Acute insect toxicity testing in house crickets resulted in overt signs of neurotoxicity within 15 minutes following the injection of 4-AP at concentrations >100 nmol/g. Signs of neurotoxicity were initially characterised by twitching of legs and antennae and intermittent abdominal contractions. Within the first hour symptoms progressed to uncoordinated movement and the absence of righting reflexes, characterised as knockdown. Signs of neurotoxicity including knockdown and death were recorded up to 48 hrs at which point crickets had either recovered or expired. At concentrations >100 nmol/g knockdown occurred within the first hour, while death was recorded at >24 hr post injection. The 4-AP dose-response curve yielded respective LD_{50} and KD_{50} values of 435 ± 10 nmol/g and 448 ± 9 nmol/g, determined 48 hr post injection.

The progressive spastic paralysis, followed by a period of flaccid paralysis and death is reminiscent of the phenotype exhibited by κ-HXTX-Hv1c in a number of invertebrates. These results suggest that block of $I_{K(A)}$ may be sufficient to induce lethal neurotoxicity in insects, and therefore insect A-type transient K_V channels may be a lethal target of κ-

HXTX-Hv1c worthy of further investigation.

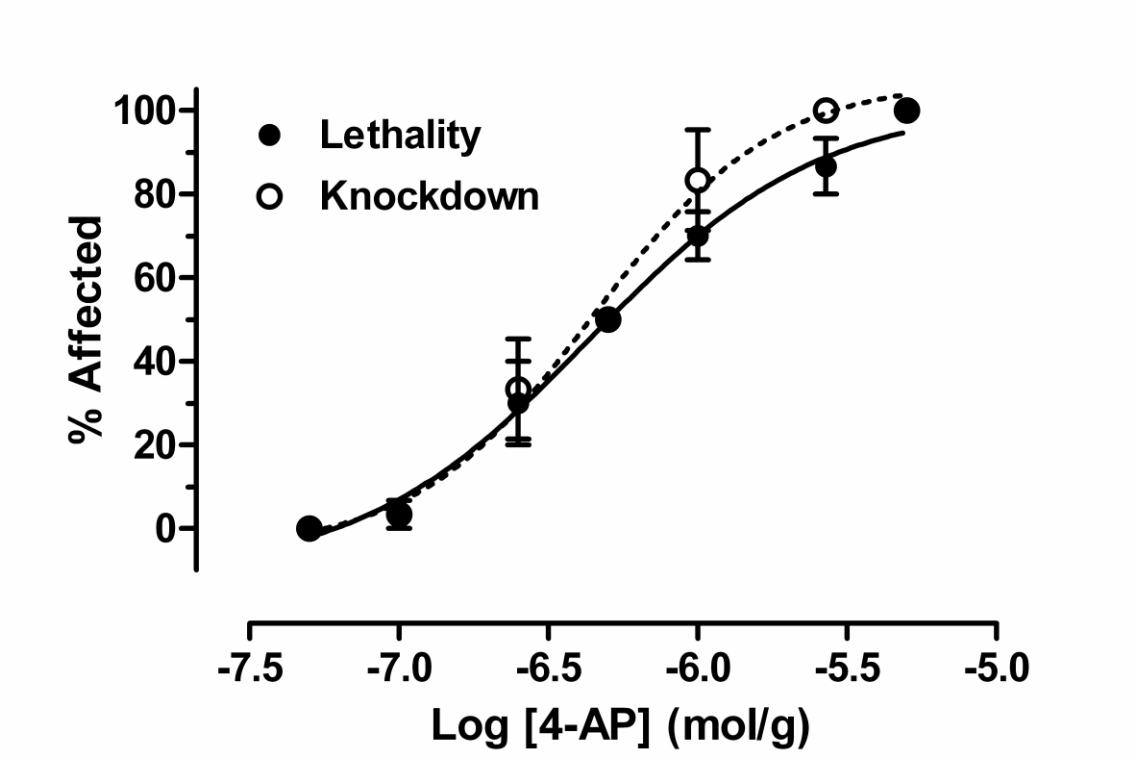


Figure 6.5: The acute toxicity of 4-aminopyridine in house crickets, *Acheta domesticus*. Log concentration-response curve for death (closed circles) and knockdown of A. domestica with 4-aminopyridine at 48 hr post injection. Data was fitted with Eq. 2 (see chapter 2, section 5) generating LD_{50} and KD_{50} values of 435 ± 10 and 448 ± 7 nmol/g, respectively. All data represents the mean LD_{50} and KD_{50} values ± SE recorded at 48 hr post-injection from at least three independent trials.

6.2.6 Effects of κ-HXTX-Hv1c on A-type transient K_V channel subtypes

The moderate inhibition of $I_{K(A)}$ by κ-HXTX-Hv1c, highlights the A-type transient K_V channel as a potential insecticidal target of κ-HXTX-Hv1c. In order to further explore the effects of κ-HXTX-Hv1c on insect A-type transient K_V channels, DUM neurons were exposed to increasing concentrations of κ-HXTX-Hv1c under voltage-clamp conditions in the presence of TTX, Cd^{2+} and iberiotoxin to block Na_V, Ca_V and K_{Ca} channels, respectively. Previous studies have shown that $I_{K(A)}$ are inactivated by depolarised membrane potentials while $I_{K(DR)}$ remain unaffected (Covarrubias, Wei et al., 1991). Accordingly, $I_{K(A)}$ were recorded in isolation from $I_{K(DR)}$ using a depolarising prepulse protocol. Briefly, 1-s prepulses were applied in 10-mV increments from –120

mV to +40 mV, followed by a 100-ms test pulse to +40 mV (Figure 6.6A and B). A leftward shift in the steady-state responses was evident with recordings made up to 15 minutes following the formation of the whole cell configuration (Figure 6.6C). Subsequently, no current recordings were made within the first 15 minutes following the initiation of the whole-cell configuration. Prepulse potentials more depolarised than –20 mV were found to completely inactivate $I_{K(A)}$ as determined by a subsequent test pulse to +40 mV, while a 1-second prepulse at potentials more negative than –90 mV were found to be sufficient for complete A-type transient K_V channel availability (Figure 6.6D; n = 3). Therefore alternating prepulse potentials of –100 mV and –20 mV were used to evoke $I_{K(DR)}$ with and without $I_{K(A)}$, respectively, therefore enabling subsequent offline removal of the contaminating $I_{K(DR)}$ by current subtraction routines (see chapter 2 for further details).

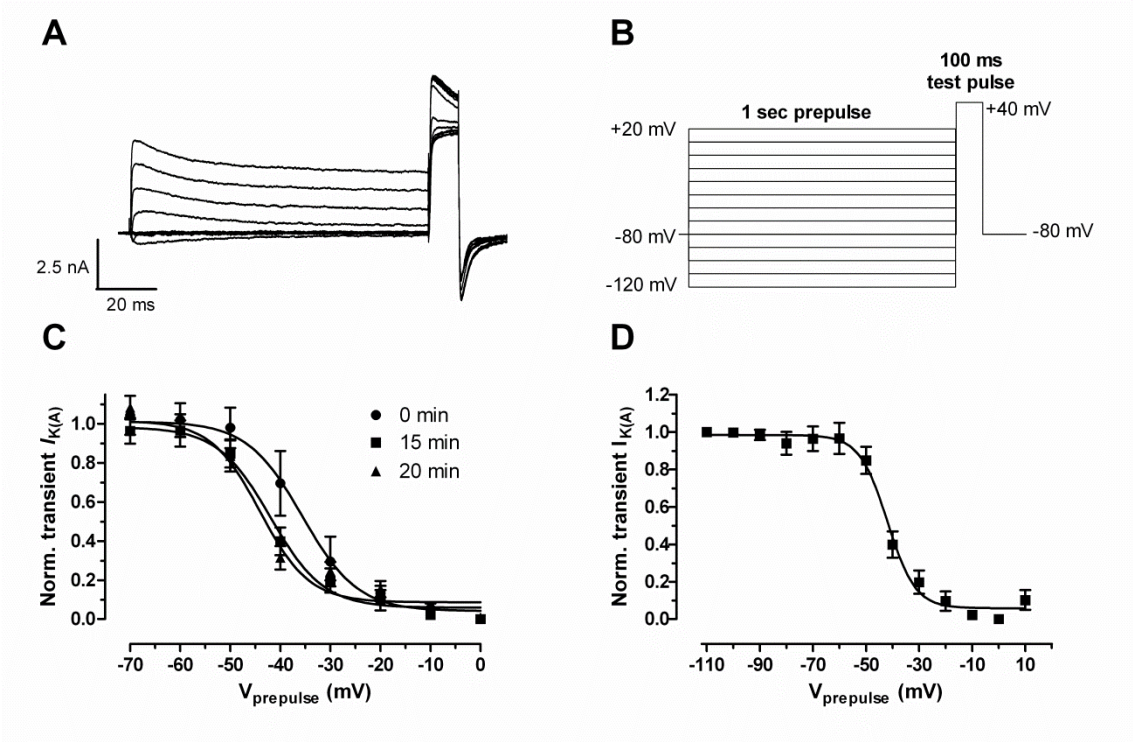


Figure 6.6: Steady-state inactivation of $I_{K(A)}$ and $I_{K(DR)}$. (A) Representative traces of the steady-state inactivation of $I_{K(A)}$ and $I_{K(DR)}$ evoked by voltage pulse protocols illustrated in **(B)**. Neurons were held at –80 mV and 1-ms prepulses from –120 to +10 mV followed by 100-ms pulses to +40 mV were used to evoke $I_{K(A)}$ at 0.3 Hz. **(C)** Steady-state curves for $I_{K(A)}$ measured at 0, 15 and 20 min after achieving the whole-cell configuration. Data was fitted with Eq. 2 (chapter 2, section 5). **(D)** Steady-state curve at 15 min following initiation of the whole-cell configuration.

Interestingly, in the presence κ-HXTX-Hv1c at concentrations of up to 5 μM, no more than 30% of $I_{K(A)}$ was inhibited (n = 5; Figure 6.7G). Similarly to the inhibition of BK_{Ca} channels, reduction of $I_{K(A)}$ occurred without any significant changes in the voltage dependence of activation (control $V_{1/2}$ –24.8 ± 9.5 mV vs. toxin –35.0 ± 14.2 mV, n = 4, p > 0.05, Figure 6.7H). From these results it was determined that κ-HXTX-Hv1c appears to only block a portion of the $I_{K(A)}$ with no apparent effects on the voltage-dependency of channel activation. In order to explain these results we considered the possibility that multiple channel currents may contribute to the $I_{K(A)}$ in DUM neurons.

While it is known that several members of the shaker channel family contribute to the K_V current in insects, the lack of selective blockers has made the identification of these channels difficult in native neurons. Given the high homology shared with the mammalian counterparts of these distinct families of K_V channels we decided to test a number of mammalian K_V channel blockers on insects. According to expression studies on *Drosophila* K_V channels, the *shal* and *shaker* genes appear to encode A-type transient K_V channel currents while *shab* and *shaw* encode for delayed-rectifier channels with somewhat slower activation and inactivation kinetics.

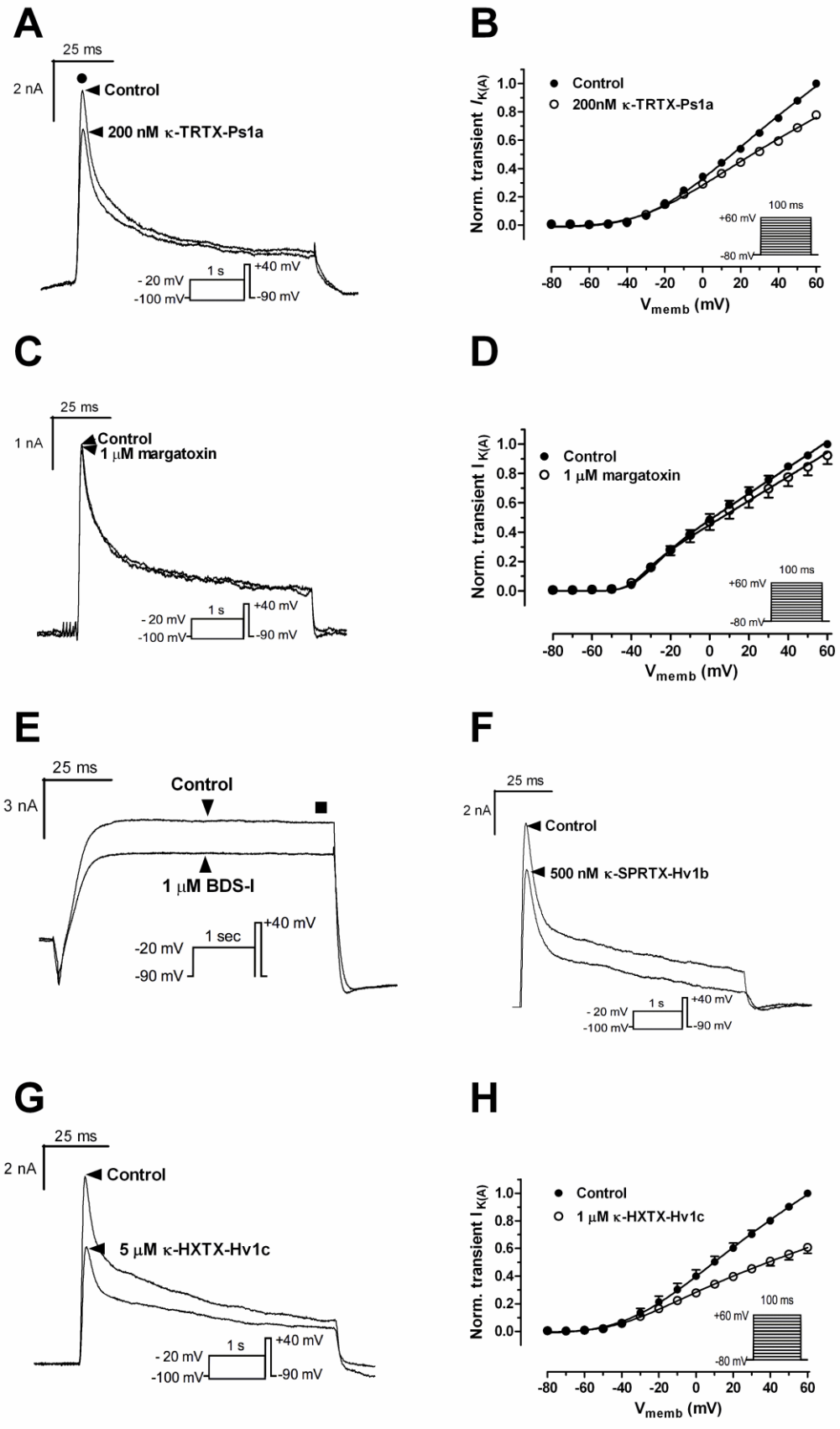
Initially, the selective K_V1 (Shaker-like) channel blocker margatoxin from the venom of the scorpion *Centruroides margaritatus* (Garcia-Calvo, Leonard et al., 1993; Bednarek, Bugianesi et al., 1994) was tested on DUM neuron $I_{K(A)}$. Illustrated in Figure 6.7C, 1 μM margatoxin failed to alter the amplitude or kinetics of the $I_{K(A)}$ (4.0 ± 2.7% inhibition; n = 4, p > 0.05). Furthermore, margatoxin did not alter the threshold of channel activation nor did it modify the $V_{1/2}$ ($V_{1/2}$ control –31.2 ± 1.5 mV vs. margatoxin –35.5 ± 3.2 mV; n = 3, p > 0.05, Figure 6.7D).

As *shal* is believed to encode for the majority of transient K_V channels in embryonic *Drosophila* neurons the effects of K_V4 (Shal-like) channel blockers on $I_{K(A)}$ were examined. PaTx1 (now renamed κ-TRTX-Ps1a) is known to block K_V4 and K_V2 (Shab-like) channels in vertebrates (Diochot, Drici et al., 1999). Given the high homology of κ-TRTX-Ps1a with the insecticidal BK_{Ca} channel toxins isolated from the spider *Eucratoscelus constrictus* (Chapter 3 and (Windley, Escoubas et al., 2011)) this toxin was subsequently tested on DUM neuron $I_{K(A)}$. Using the dual prepulse protocol to evoke $I_{K(A)}$, the application of 200 nM κ-TRTX-Ps1a was found to reduce $I_{K(A)}$ by 20.6 ± 1.2% (n = 4; p < 0.05; Figure 6.7A). Comparatively, vertebrate $K_V4.3$ channels

expressed in COS cells were almost completely abolished at concentrations greater than 200 nM (Diochot, Drici et al., 1999), highlighting either that insects display differing sensitivities or that the $I_{K(A)}$ of DUM neurons comprises of other K_V4-like (Shal) channels relatively insensitive to κ-TRTX-Ps1a.

Although the effects of κ-TRTX-Ps1a on insect $I_{K(A)}$ were significant, they may represent a non-selective block of Kv2 (Shab) channels. Therefore it was necessary to test a toxin known to selectively block mammalian K_V4 (Shal-like) channels and not affect K_V2 (Shab-like) channels. HpTx-2 (now renamed κ-SPRTX-Hv1b) does not modulate the activity of Shaker, Shab or Shaw currents in vertebrates, nor does it inhibit mammalian Ca_V and Na_V channel currents (Sanguinetti, Johnson et al., 1997). Not surprisingly, application of 500 nM κ-SPRTX-Hv1b was found to block 23.9 ± 4.7% (n = 6, $p < 0.05$) of the $I_{K(A)}$ in DUM neurons. This appears to be a saturating concentration as a similar degree of block was also seen in the presence of 100 nM κ-SPRTX-Hv1b (24.4 ± 5.7, n = 6). Interestingly, the portion of K_A current block correlated well with the results observed in the presence of κ-HXTX-Hv1c.

For completeness, the K_V3 (Shaw) channel toxin BDS-I from the sea anemone (*Anemonia sulcata*) (Yeung, Thompson et al., 2005) (generously provided by Dr Sylvie Diochot, IPMC, CNRS, Valbonne, France) was tested on the DUM neuron K_A and K_{DR} currents using the dual pulse protocol to evoke channel currents. At concentrations up to 1 μM, BDS-I was found to block the delayed-rectifier portion of the current recorded following a depolarising prepulse potential to –20 mV (Figure 6.7E). However at 1 μM the 8.2 ± 2.3% block of $I_{K(DR)}$ and additional 2.0 ± 4.6% block $I_{K(A)}$ by BDS-I were considered not significant (n = 3, $p > 0.05$; Figure 6.7E). Unfortunately due to limited supplies, higher concentrations could not be tested. Subsequently, it is still possible that a fraction of the K_V current which lacks prepulse inactivation ($I_{K(DR)}$) may be partially represented by a K_V3-like, Shaw channel current. Covarrubias and colleagues have shown that the *Drosophila* Shaw channel expressed in Xenopus oocytes posses similar voltage-independent properties (Covarrubias, Wei et al., 1991). This suggests that the major channel subtype responsible for mediating $I_{K(DR)}$ in cockroach DUM neurons is the K_V3-like Shaw channel, however BDS-I either does not block insect Shaw channels or the concentrations tested were insufficient.


A
25 ms
2 nA
Control
200 nM κ-TRTX-Ps1a
1 s
- 20 mV
-100 mV
+40 mV
-90 mV
B
Control
200nM κ-TRTX-Ps1a
Norm. transient $I_{K(A)}$
1.0
0.8
0.6
0.4
0.2
0.0
100 ms
+60 mV
-80 mV
-80 -60 -40 -20 0 20 40 60
V_{memb} (mV)
C
25 ms
1 nA
Control
1 μM margatoxin
1 s
- 20 mV
-100 mV
+40 mV
-90 mV
D
Control
1 μM margatoxin
Norm. transient $I_{K(A)}$
1.0
0.8
0.6
0.4
0.2
0.0
100 ms
+60 mV
-80 mV
-80 -60 -40 -20 0 20 40 60
V_{memb} (mV)
E
25 ms
3 nA
Control
1 μM BDS-I
1 sec
-20 mV
-90 mV
+40 mV
F
25 ms
2 nA
Control
500 nM κ-SPRTX-Hv1b
1 s
- 20 mV
-100 mV
+40 mV
-90 mV
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25 ms
2 nA
Control
5 μM κ-HXTX-Hv1c
1 s
- 20 mV
-100 mV
+40 mV
-90 mV
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Control
1 μM κ-HXTX-Hv1c
Norm. transient $I_{K(A)}$
1.0
0.8
0.6
0.4
0.2
0.0
100 ms
+60 mV
-80 mV
-80 -60 -40 -20 0 20 40 60
V_{memb} (mV)

Figure 6.7: Effects of K_V channel modulators on DUM neuron $I_{K(A)}$ and $I_{K(DR)}$. Superimposed $I_{K(A)}$ traces illustrating the typical effects of 200 nM κ-TRTX-Ps1a **(A)**, 1 μM margatoxin, 500 nM κ-SPRTX-Hv1b **(C)** and 5 μM synthetic κ-HXTX-Hv1c **(G)**. **(E)** Representative trace of the typical effect of 1 μM BDS-I on DUM neuron $I_{K(DR)}$. $I_{K(A)}$-*V* for control (closed symbols) and in the presence of 200 nM κ-TRTX-Ps1a **(B)**, 1 μM margatoxin **(D)** and 5 μM synthetic κ-HXTX-Hv1c **(H)** (open symbols) (n = 3–6). *I-V* data were fitted with Eq. 1 (see chapter 2, section 5). All test pulse protocols used to evoke currents are illustrated within the insets of each panel.

6.2.7 κ-HXTX-Hv1c and κ-SPRTX-Hv1b share a common target

In order to determine whether both κ-SPRTX-Hv1b and κ-HXTX-Hv1c block the same portion of $I_{K(A)}$, a number of co-application experiments were designed. Similar to previous experiments, $I_{K(A)}$ were evoked using a dual prepulse protocol and isolated following offline subtraction. Initially, DUM neurons were exposed to a saturating concentration of κ-SPRTX-Hv1b until equilibrium was achieved, after which the perfusing solution was replaced with a saturating concentration of κ-HXTX-Hv1c. $I_{K(A)}$ were recorded for a further 5 minutes to evaluate the occurrence of any additional current block. Following inhibition of $I_{K(A)}$ by 500 nM κ-SPRTX-Hv1b (23.9 ± 4.7% inhibition) , subsequent application of 500 nM κ-HXTX-Hv1c failed to further inhibit the current, which remained at 23.0 ± 4.1% inhibition (n = 3, $p < 0.05$; Figure 6.8C). In the complementary experiment, 500nM κ-SPRTX-Hv1b also failed to cause further inhibition of the current following exposure to 500 nM κ-HXTX-Hv1c (n = 3; Figure 6.8D). These findings suggest that κ-HXTX-Hv1c inhibits the same channel as the established selective K_V4 blocker κ-SPRTX-Hv1b.

While κ-HXTX-Hv1c only blocks a portion of the transient K_A current in DUM neurons the effect is dose dependent. The concentration-response curve for block of transient $I_{K(A)}$ by κ-HXTX-Hv1c yielded an IC_{50} of 36.7 ± 0.2 nM (n = 3; Figure 6.8A), a value just over 10-fold less potent than for inhibition of BK_{Ca} channels (Figure 6.1B) . Percentage block of the $I_{K(A)}$ was normalised against the maximum fraction of the current inhibited in order to analyse the concentration dependency (Figure 6.8A). Furthermore, channel block was found to display voltage-independent activity (Figure 6.7H) therefore is likely to act at or near the channel pore.

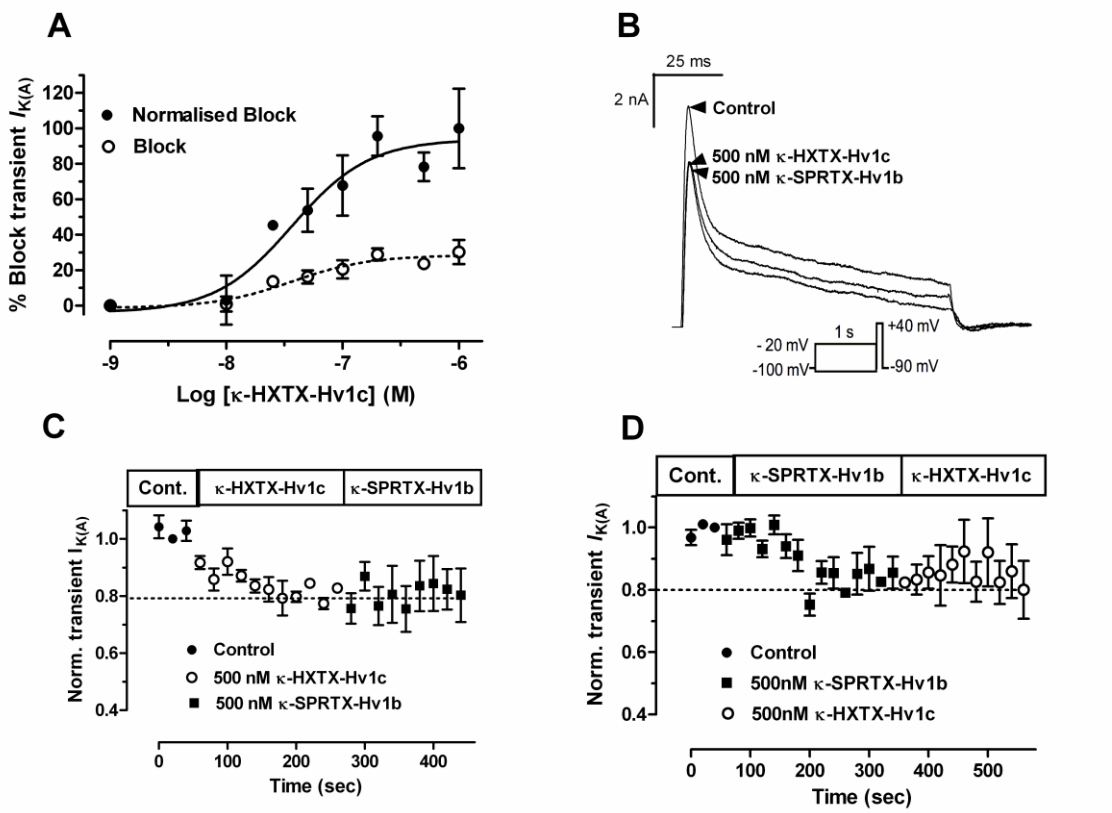

Figure 6.8: κ-HXTX-Hv1c and κ-SPRTX-Hv1b block the same portion of insect $I_{K(A)}$. (A) Concentration-response curve for κ-HXTX-Hv1c block of $I_{K(A)}$ in DUM neurons (n = 3). Data was fitted with Eq. 2 (see chapter 2, section 5) yielding an IC_{50} of 36.7 ± 2.2 nM and a maximal inhibition of only 28.4 ± 4.0% of $I_{K(A)}$ (open circles). Data was also normalised against maximal percentage block (closed circles). (B–D) κ-HXTX-Hv1c and κ-SPRTX-Hv1b share the same target in cockroach DUM neurons. **(B)** Representative trace of DUM neuron $I_{K(A)}$ illustrating the lack of further inhibition by 500 nM κ-SPRTX-Hv1b following prior block by 500 nM κ-HXTX-Hv1c. Complementary experiments to show lack of additional block by κ-SPRTX-Hv1b **(C)** or κ-HXTX-Hv1c **(D)** following block by κ-HXTX-Hv1c or κ-SPRTX-Hv1b respectively (n = 3). All $I_{K(A)}$ were evoked by the test pulse protocol illustrated in the inset of trace (C).

6.2.8 NEUROTOXIC ACTIVITY OF K_A CHANNEL TOXINS IN ARTHROPODS

Acute insect toxicity assays were used to then assess the neurotoxicity of Shal channel blockers in house crickets. No neurotoxic symptoms were observed following the injection of up to 5 nmol/g of κ-SPRTX-Hv1b at a 48 hr endpoint. In order to eliminate the possibility of an obligate relationship where both BK_{Ca} (Slo) and K_V4 (Shal) channel block are required for insecticidal activity, insects were also simultaneously injected with κ-SPRTX-Hv1b and the selective BK_{Ca} (Slo) channel blocker, IbTx (1:1). Again, following toxin injection of doses up to 2 nmol/g the crickets failed to exhibit any overt signs of neurotoxicity. These results indicate that concurrent block of Slo and Shal channels is insufficient to result in the insecticidal activity induced by κ-HXTX-Hv1c.

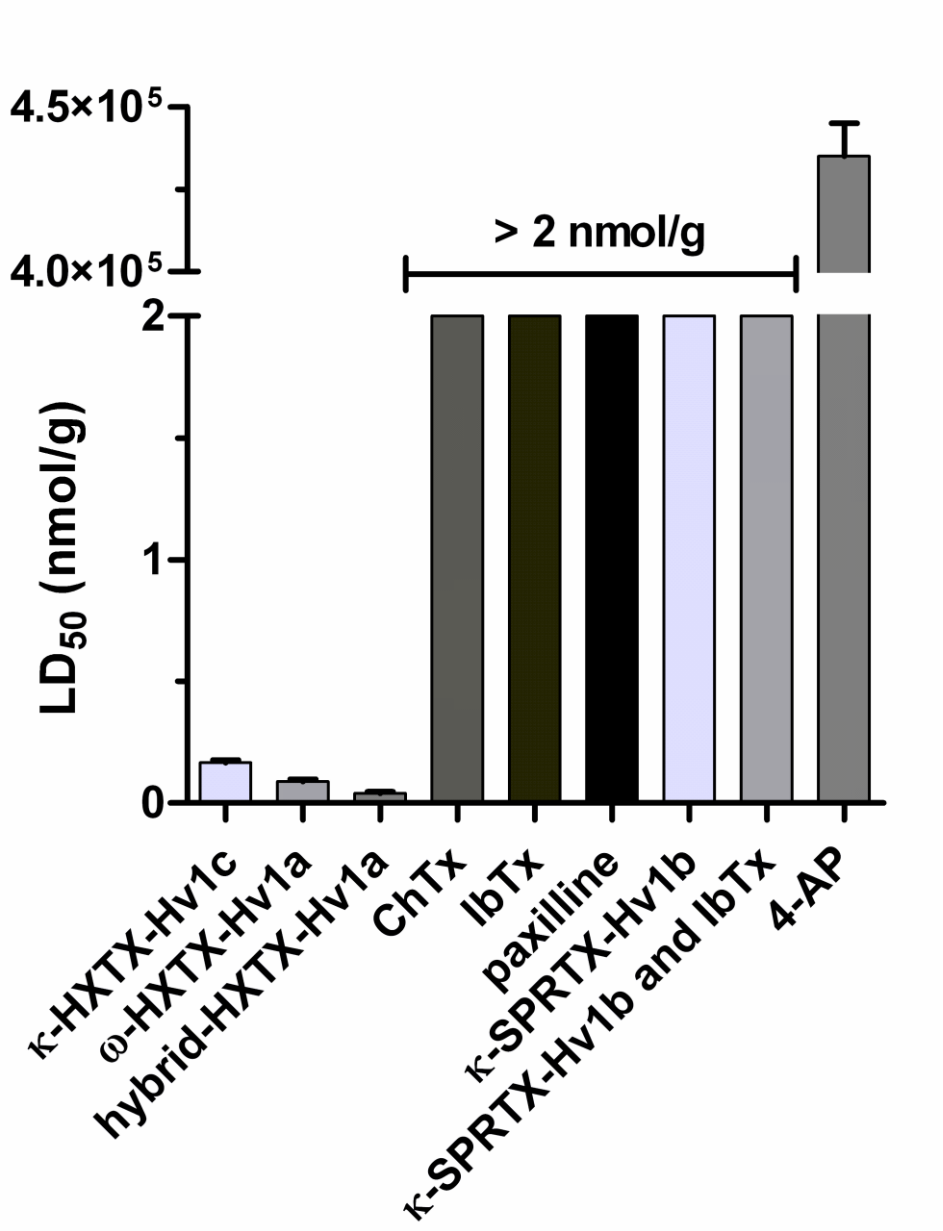

Figure 6.9: The insecticidal activities of BK_{Ca} and K_V4 (Shal) channel blockers in the house cricket, *Acheta domestica*. LD_{50} values at 48 hr post injection in *A. domestica* were 167 ± 10 pmol/g, 89 ± 10 pmol/g and 41 ± 7 pmol/g for κ-HXTX-Hv1c (Wang, Connor et al., 2000), ω-HXTX-Hv1a (Wang, Smith et al., 1999) and hybrid-HXTX-Hv1a (Suping Wen, Glenn King and Graham Nicholson, unpublished results), respectively. Charybdotoxin (ChTx), iberiotoxin (IbTx) and paxilline showed no insecticidal activity in house crickets at doses up to 2 nmol/g. The K_V4 (Shal) channel blocker κ-SPRTX-Hv1b also failed to exhibit any insecticidal activity at doses up to 5 nmol/g nor did a combination of κ-SPRTX-Hv1b and IbTx (1:1) injected simultaneously at doses up to 2

nmol/g. 4-aminopyridine, blocker of DUM neuron $I_{K(A)}$ recorded an LD_{50} of 435 ± 10 μmol/g at 48 hrs post injection (see Figure 6.5). All data represents the mean LD_{50} value ± SE recorded at 48 hr post-injection from at least three independent trials.

6.3 DISCUSSION

The aim of this study was to confirm that the lethal target of κ-HXTX-Hv1c was the insect BK_{Ca} channel. This initially involved acute toxicity studies using a range of classical peptidic and non-peptidic BK_{Ca} channel blockers. Firstly it was necessary to confirm that each toxin blocks BK_{Ca} channels in insects. This had previously confirmed for the peptide scorpion toxins iberiotoxin (selective BK_{Ca} blocker; (Windley, Escoubas et al., 2011)) and charybdotoxin (BK_{Ca} and $K_V1.3$ channel blocker; (Gunning, Maggio et al., 2008)). For the non-peptidic indole diterpine, paxilline, this study reports for the first time a highly selective block of cockroach BK_{Ca} channels.

6.3.1 PAXILLINE IS A SELECTIVE BLOCKER OF K_{CA} CHANNELS

While the anti-insectan paxilline is known to selectively block mammalian BK_{Ca}, little work has been done on its effects in insects. In this study paxilline was found to potently block DUM neuron $I_{BK(Ca)}$ with a IC_{50} of 14 nM; this value was comparable to the effects exhibited on mammalian $I_{K(Ca)}$ (DeFarias, Carvalho et al., 1996; Sanchez and McManus, 1996; Strobaek, Christophersen et al., 1996; Li and Cheung, 1999; Longland, Dyer et al., 2000; Molinari, Sullivan et al., 2000). Due to the role of BK_{Ca} channels in the regulation of action potential duration and subsequently neurotransmitter release, the effects of paxilline have been extensively analysed on a number of vertebrate channels (DeFarias, Carvalho et al., 1996; Sanchez and McManus, 1996; Strobaek, Christophersen et al., 1996; Smith, McLeay et al., 1997; Li and Cheung, 1999; Longland, Dyer et al., 2000; Molinari, Sullivan et al., 2000). Toxin application failed to modify a variety of other mammalian channels, including; hSK1 (human small conductance K_{Ca}), mouse delayed-rectifier K_V (minor effect), L-type Ca_V and Na_V channels (Knaus, McManus et al., 1994). Similarly, we also found that paxilline inhibits insect BK_{Ca}, while failing to affect Ca_V, Na_V and other K_V channel subtypes in insects. Furthermore, paxilline is considered the most potent non-peptidic blocker of vertebrate (Knaus, McManus et al., 1994) and, following this study, insect BK_{Ca} channels.

Given the insect toxicity of paxilline to a number of insect genera (Belofsky, Gloer et al., 1995) these effects support the BK_{Ca} channel as a potential lethal target in insects. However, as previous studies of paxilline and related indole diterpines only report anti insectan activity via feeding and growth trials we decided it was important to observe the effects of the toxin through acute toxicity assays.

Importantly, paxilline failed to cause overt toxicity in crickets even at high concentrations. This result was somewhat unexpected given the previously reported insecticidal activities of paxilline in feeding assays (Belofsky, Gloer et al., 1995). Potentially, these results indicate that while paxilline block of the insect BK_{Ca} channel does not cause lethal neurotoxicity, it may be useful in at least reducing crop destruction by phytophagous insect pests. Nevertheless, these results further confirm that selective inhibition of the BK_{Ca} channel is not sufficient to result in the potent, excitatory phenotype exhibited by κ-HXTX-Hv1c in multiple insect orders (Wang, Connor et al., 2000; Maggio and King, 2002a; Maggio and King, 2002b; Tedford, Maggio et al., 2007).

Due to the lack of insecticidal activity presented by a number of classical BK_{Ca} channel blockers that block DUM neuron BK_{Ca} channels, the possibility that the BK_{Ca} channel was not the insecticidal target of κ-HXTX-Hv1c and may only contribute to the overall toxicity of the toxin was considered. Subsequently, further characterisation of the effects of κ-HXTX-Hv1c on insect K_V channels were undertaken in an attempt to identify the lethal insecticidal target. In order achieve this aim; the effects of κ-HXTX-Hv1c on a number of other known K_V channel subtypes in DUM neurons were examined.

6.3.2 Channel subtypes underlying delayed-rectifier and A-type transient K_V channels

In contrast to the block of BK_{Ca} channels, inhibition of A-type transient K_V channels is potentially linked to insecticidal activity. In support, the effects of the A-type transient K_V channel blocker 4-aminopyridine (4-AP) (Grolleau and Lapied, 1995b) were found to be lethal to house crickets with a LD_{50} of 435 nmol/g at a 48 hr endpoint. Although this value was high in comparison to the LD_{50} of κ-HXTX-Hv1c, these results were not unexpected as micromolar concentrations of 4-AP are also required to block A-type

transient K_V channels (Grolleau and Lapied, 1995b). As a result, the moderate effects of 1 μM κ-HXTX-Hv1c on A-type transient K_V channels (Gunning, Maggio et al., 2008) were assessed in more detail. By way of background, in *Drosophila*, four genes namely *shaker, shab, shaw* and *shal* encode four different K_V channel subfamilies (Kamb, Iverson et al., 1987; Papazian, Schwarz et al., 1987; Pongs, Kecskemethy et al., 1988; Schwarz, Tempel et al., 1988; Timpe, Schwarz et al., 1988; Butler, Wei et al., 1989; Gasque, Labarca et al., 2005). Although many more K_V genes have been isolated from vertebrates they all fall into the same four families; K_V1–K_V4, respectively (Coetzee, Amarillo et al., 1999). When expressed individually, *shaker* and *shal* genes encode A-type transient K_V channel currents, while *shaw* and *shab* genes encode for delayed-rectifier K_V channels (Kamb, Iverson et al., 1987; Iverson, Tanouye et al., 1988; Timpe, Schwarz et al., 1988; Wei, Covarrubias et al., 1990; Salkoff, Baker et al., 1992; Tsunoda and Salkoff, 1995). These genes appear to be expressed individually and the formation of heteromultimeric channels across the subfamilies has not been identified in coexpression experiments (Covarrubias, Wei et al., 1991; Salkoff, Baker et al., 1992). Therefore, it is likely that this molecular barrier exists between native currents, at the very least between shaker-like channels. However, the lack of selective blockers for these currents has made it impossible to dissect the individual channel currents in native neurons. For example, it was not until the discovery of κ-TRTX-Ps1a that it was known that the K_V4 channel was responsible for the I_{to1} in the rat ventricular myocyte (Diochot, Drici et al., 1999). The discoveries of selective toxins are also vital to the identification and characterisation of channels contributing to insect K_V currents in native neurons.

6.3.2.1 κ-SPRTX-Hv1b and κ-HXTX-Hv1c block the same K_V channel

In the present study, the effects of κ-HXTX-Hv1c on $I_{K(A)}$ were found to be somewhat more complex than initially perceived. κ-HXTX-Hv1c was found to block only a portion of DUM neuron $I_{K(A)}$, with an IC_{50} value 10-fold less than on BK_{Ca} channels. Furthermore, κ-HXTX-Hv1c was also established to block the same portion of DUM neuron $I_{K(A)}$ as the mammalian K_V4 blocker κ-SPRTX-Hv1b. Previously, κ-SPRTX-Hv1b has been characterised to block mammalian $K_V4.2$ and 4.3 channels and prolong action potential duration in isolated rat ventricular myocytes (Sanguinetti, Johnson et al., 1997), similarly to κ-HXTX-Hv1c (see chapter 5). Importantly, κ-SPRTX-Hv1b was also found not to modulate *shaker*-like ($K_V1.4$) (Sanguinetti, Johnson et al., 1997),

K_V3.4 (*shaw*-like) or K_V2.1 (*shab*-like) channels (Zarayskiy, Balasubramanian et al., 2005). Furthermore, following inhibition of insect $I_{K(A)}$ with κ-SPRTX-Hv1b, the subsequent application of κ-HXTX-Hv1c failed to produce any additional block. This result was also replicated in the reverse experiment. These findings provide further evidence that these peptides act on the same molecular target in insect DUM neurons, namely a K_V4-like Shal channel.

Given the Shal K_V channel subfamily demonstrates the highest degree of functional and structural conservation between insects and vertebrates, it is likely that vertebrate-active K_V4 toxins will affect Shal channels in cockroaches similarly. While we only saw a partial 23% block of the prepulse sensitive component of the $I_{K(A)}$ in the presence of high concentrations of κ-SPRTX-Hv1b, the effect mimicked that of κ-HXTX-Hv1c at saturating concentrations. This data implies that both toxins potentially target the same K_V channel; however confirmation of block using an expression system is necessary.

κ-SPRTX-Hv1b has previously been shown to exhibit voltage-dependent block on mammalian K_V4 channels (Sanguinetti, Johnson et al., 1997; Zarayskiy, Balasubramanian et al., 2005) and studies suggest that the toxin binds to the S3-S4 linker of the K_V4 channel (Zarayskiy, Balasubramanian et al., 2005). In this study we found that κ-HXTX-Hv1c does not alter the voltage kinetics of insect K_A channel activation, subsequently it is unlikely that κ-HXTX-Hv1c targets the same site on the channel as κ-SPRTX-Hv1b.

Finally, although A-type transient K_V channel blocker 4-AP was found to be lethal, insect toxicity testing established that κ-SPRTX-Hv1b was not insecticidal. Furthermore, when both IbTx and κ-SPRTX-Hv1b were injected into house crickets simultaneously there was no indication of any overt toxicity implying that the combined block of BK_{Ca} and K_V4-like (Shal) channels is not lethal.

6.3.3 CONCLUSIONS AND FUTURE DIRECTIONS

Although these results bring us no closer to identifying the lethal target of κ-HXTX-Hv1c, they do draw attention to κ-HXTX-Hv1c as a tool for exploring the complexity

of DUM neuron $I_{K(A)}$. This study is the first to indicate that multiple channel subtypes may contribute to DUM neuron $I_{K(A)}$. Moreover, κ-HXTX-Hv1c has been established as an important tool for exploring insect BK_{Ca} and K_V4-like Shal channels and how they relate to each other in terms of structure and function.

This study highlights the need to carry out more thorough investigations into toxin activities before drawing conclusions about lethal targets. It has often been assumed that the insecticidal targets of spider toxins are those with which the toxin acts on with the highest potency and that these toxins interact with only one target. As a consequence, only a discrete number of channel targets are assessed and other potential activities remain unknown. The presence of target promiscuity may in fact be essential to toxin activity or potency and in that case this knowledge would be particularly important for the development of non-peptide mimetic structures with comparable target affinities.

Furthermore, this study identifies the need to characterise the potential of various insect voltage-gated ion channels involved in insecticidal activity. As a consequence, this research suggests that block of K_V4-like Shal and BK_{Ca} are unlikely to be lethal to insects and subsequently the lethal target of κ-HXTX-Hv1c remains elusive. As a result of the rapid neurotoxicity, the next logical potential target of κ-HXTX-Hv1c is a neurotransmitter-gated ion channel. γ-Aminobutyric acid (GABA)-, glutamate- and acetylcholine-gated ion channels play a vital role in the modulation of insect nervous system and, notably, they are already acknowledged targets of existing agrochemical insecticides. Subsequently, the next chapter investigates these receptors as potential targets of κ-HXTX-Hv1c.

Chapter Seven

Modulation of the insect nicotinic acetylcholine receptor by κ-HXTX-Hv1c

Monique J. Windley

[2012]

7.1 INTRODUCTION

Although κ-HXTX-Hv1c is believed to target the insect voltage-gated calcium-activated potassium channel (Gunning, Maggio et al., 2008) this channel is unlikely to be the insecticidal target (see chapter 6). Therefore, this study aimed to assess the effects of the insecticidal κ-HXTX-Hv1c on nicotinic acetylcholine (nACh), γ-aminobutyric acid (GABA) and L-glutamate receptors due to their vital role in the modulation of fast synaptic transmission in the insect nervous system.

Nicotinic receptors (nAChR) play an important role in the regulation of fast synaptic transmission in the insect central nervous system (see ref. (Thany, 2010) for a review). nAChR belong to a family of allosteric ligand gated channels which are targeted by a range of commercially important insecticides including: neonicotinoids (Tomizawa and Casida, 2003; Matsuda, Shimomura et al., 2005; Ihara, Brown et al., 2006), spinosyns (Salgado, 1998; Salgado, Sheets et al., 1998; Salgado and Saar, 2004; Watson, Chouinard et al., 2010), and nereistoxin analogs (Lee, Tomizawa et al., 2003; Raymond Delpech, Ihara et al., 2003). Many of the insecticides targeting insect nAChR act as competitive and non-competitive antagonists, while those that activate receptors act as full or partial agonists (Millar and Denholm, 2007).

Evidence suggests that the insect gene family encoding nAChR is even more diverse than in mammals. Presently, 10 nAChR subunit encoding genes have been described in *Drosophila melanogaster* (Sattelle, Jones et al., 2005), 10 in *Anopheles gambiae* (Jones, Grauso et al., 2005), 11 in *Apis mellifera* (Jones, Raymond-Delpech et al., 2006) and 12 in *Tribolium castaneum* (Jones and Sattelle, 2007). Many of these genes undergo alternate splicing and post-translational modifications of the subunit mRNA which may contribute to further diversity (Sattelle, Jones et al., 2005). Although the identification and characterisation of insect nAChR has been limited, at least four subtypes have been identified in cockroach neurons. Firstly, two groups of cockroach nAChR have been identified based on sensitivity to the snake neurotoxin α-bungarotoxin (α-BgTx) (Tomizawa and Yamamoto, 1992; Tornøe, Bai et al., 1995). Moreover, varying sensitivities of α-BgTx susceptible nAChR to various nicotinic agonists reveal two additional subtypes correlated with a desensitizing (nAChD) or non-desensitizing (nAChN) time course of channel inactivation (Bai and Sattelle, 1993; Buckingham, Lummis et al., 1993; Su and O'Dowd, 2003; Salgado and Saar, 2004). α-BgTx

insensitive nAChR are similarly divided into two groups based on agonist susceptibility, namely nAChR1 and nAChR2 (Courjaret and Lapied, 2001; Courjaret, Grolleau et al., 2003; Thany, Courjaret et al., 2008). Unlike vertebrates, nAChR are predominantly found in the insect CNS (see ref. (Thany, Tricoire-Leignel et al., 2010) for a review). Furthermore, vertebrate classifications cannot be applied directly to insects as their pharmacologies and gating kinetics are quite different (Lapied, Corronc et al., 1990).

In vertebrates, the neuronal actions of L-glutamate are mediated by two distinct glutamate neurotransmitter receptor classes in the CNS: ionotropic (Stawski, Janovjak et al., 2010) and metabotropic (Nicoletti, Bockaert et al., 2011). All known vertebrate ionotropic L-glutamate receptors comprise of cation channels and are classified into three subtypes: α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA), kainate (KA) and *N*-methyl-D-aspartate (NMDA) receptors. In contrast, much less is known about the properties L-glutamate receptors (GluR) in insects. Although the genome of *Drosophila* contains at least 30 glutamate receptors (Littleton and Ganetzky, 2000), far fewer have presently been identified in native neurons. In insects, at least two ionotropic L-glutamate-gated cation channels have been found to mediate excitatory neurotransmission at the neuromuscular junction, rather than in the CNS (Usherwood, 1994; DiAntonio, 2006; Fedorova, Magazanik et al., 2009). In addition, two insect ionotropic L-glutamate receptors that gate chloride channels (Glu-Cls) have been discovered to play an inhibitory role in insect CNS neurons (Raymond, Sattelle et al., 2000). In particular, these anion-gated Glu-Cls are important sites of action of insecticides such as ivermectin (Kane, Hirschberg et al., 2000), and in some cases fipronil (Raymond, Sattelle et al., 2000). Moreover, only Glu-Cls are found in CNS neurons (not cation-gated GluR) and as they are already known insecticidal targets this study will only examine the effects of κ-HXTX-Hv1c on Glu-Cls.

Ionotrophic GABA receptors also mediate inhibitory neurotransmission in the insect nervous system. Similarly to insect GluR, GABA receptors are permeable to chloride ions (Sattelle, Lummis et al., 1991). Vertebrate GABA receptors are divided into three subtypes based on structure and pharmacology, namely $GABA_{A,}$ $GABA_B$ and $GABA_C$. $GABA_A$, $GABA_C$ receptors are coupled to chloride channels while $GABA_B$ receptors modulate potassium or calcium channels through a G-protein linked second messenger system (Barnard, Skolnick et al., 1998). However, as for vertebrate GluR and nAChR,

GABA receptor classification cannot be applied directly to insects as their receptors possess different pharmacologies. To date, three GABA receptor (GABAR) subunit classes called RDL, LCCH3 and GRD have been cloned from *Drosophila* (Ffrench-Constant, Mortlock et al., 1991; Henderson, Soderlund et al., 1993; Harvey, Schmitt et al., 1994). These classes are encoded by three genes which appear to undergo alternate splicing and multiple post-transcriptional modifications to create molecular diversity. However, only two GABA-CLR subtypes have been clearly identified in insects as picrotoxin-sensitive and picrotoxin-insensitive GABA-gated chloride (GABA-Cl) channels (Hue, 1998; Le Corronc, Alix et al., 2002). Importantly, insect GABA-Cl receptors are the targets of commercial insecticides such as dieldrin (Tanaka, Scott et al., 1984) and fipronil (Hosie, Baylis et al., 1995).

Insect nACh, GABA-Cl and Glu-Cl receptors all belong to the Cys loop superfamily of ionotropic neurotransmitter receptors. They are comprised of four distinct subunits and an extracellular neurotransmitter binding domain which interact to form a heterologous pentamer around a central pore (Karlin, 2002). These three neurotransmitter-gated receptors play vital roles in the insect nervous system and as such are known insecticidal targets. Subsequently, the following chapter details the characterisation of the insecticidal spider toxin κ-HXTX-Hv1c on these receptor targets.

7.2 RESULTS

7.2.1 CHARACTERISTICS OF NACHR CHANNEL CURRENTS IN DUM NEURONS

In order to characterise the effects of κ-HXTX-Hv1c on nAChRs, 10 μM nicotine was pressure applied onto the surface of isolated cockroach DUM neurons to evoke nicotinic acetylcholine receptor channel currents (I_{nAChR}). Whole-cell patch-clamp recordings were made from DUM neurons voltage-clamped at a holding potential (V_h) of –50 mV.

Protocols were designed to evoke channel currents with increasing duration pulses of nicotine through a small diameter glass pipette (<0.5 MΩ when filled with agonist). This procedure involved using a single concentration of agonist loaded into a pressure ejection system, was adapted from that used previously. (McCaman, McKenna et al., 1977; Lapied, Corronc et al., 1990; Courjaret, Grolleau et al., 2003). In order to reduce desensitisation in response to the agonist, a pneumatic pressure ejection (Picospritzer) system was used for millisecond control of agonist delivery, with pulses delivered at 1

min intervals. According to testing under oil emersion, the logarithmic concentration of agonist at any point of the cell body would be approximately proportional to the agonist pulse duration under a constant pressure (McCaman, McKenna et al., 1977; Lapied, Corronc et al., 1990; Courjaret, Grolleau et al., 2003, see Chapter 2 for more details). Consequently, varying pulse durations of nicotine were applied to DUM neurons to assess the concentration dependence of I_{nAChR} in response to nicotine. Channel currents were evoked with nicotine pulses greater than 3 ms and maximal activation was evident at pulses greater than 12 ms (Figure 7.1). In DUM neurons, nicotine application was shown to elicit fast activating I_{nAChR}, which decayed over a period of 5–10 seconds depending on the nicotine concentration (altered via pulse duration, see Figure 7.1A).

7.2.2 Modulation of insect nAChR by α-bungarotoxin

nAChR channel currents in DUM neurons are partially sensitive to the snake α-neurotoxin α-bungarotoxin (Lapied, Corronc et al., 1990; Tornøe, Bai et al., 1995). Accordingly, DUM neuron I_{nAChR} were evoked in the presence of 500 nM α-bungarotoxin. nAChR currents were elicited in response to 10 ms nicotine pulses. Following the application of 500 nM α-bungarotoxin, nAChR currents were reduced to 82.3 ± 0.4 % of control (n = 3; Figure 7.1D). These results indicate that a small portion of the global I_{nAChR} is α-bungarotoxin-sensitive, in agreement with previous studies on DUM neurons (Lapied, Corronc et al., 1990).

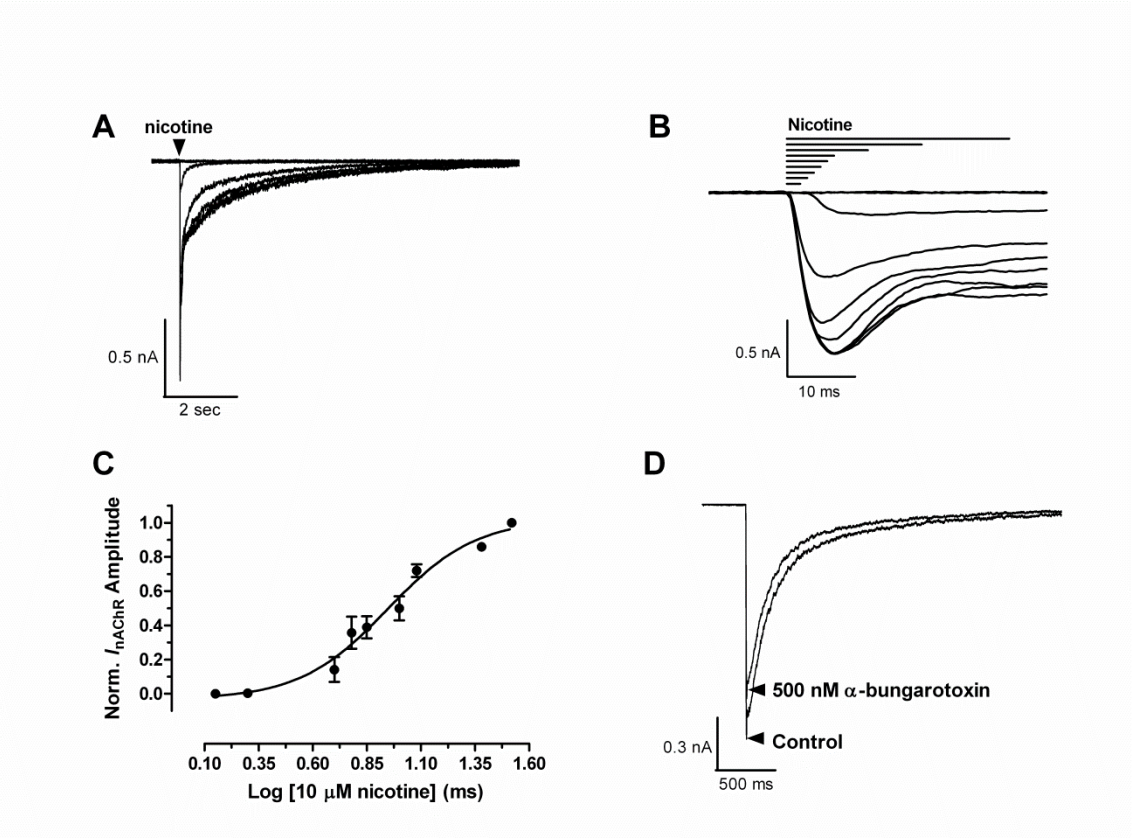

Figure 7.1: Isolation and characterisation of nAChR channel currents in DUM neurons. (A–C) I_{nAChR} were evoked in response to application of 10 μM nicotine via a pressurised ejection system. **(A)** Superimposed traces representing typical I_{nAChR} evoked by varying the pulse duration of nicotine from 4 to 33 ms. **(B)** Magnification of the initial 50 ms of trace (A) highlighting the peak I_{nAChR} amplitude. The timecourses for consecutive nicotine applications are represented above the traces. **(C)** Concentration-response curve for I_{nAChR} amplitude in response to nicotine application, yielding an EC_{50} of 8.0 ± 0.3 ms (n = 3). **(D)** Superimposed I_{nAChR} evoked by 10-ms pulses of 10 μM nicotine before and 10 min after perfusion with 500 nM α-bungarotoxin.

7.2.3 PHARMACOLOGICAL EFFECTS OF κ-HXTX- HV1C ON I_{NACHR}

In order to examine the effects of κ-HXTX-Hv1c on I_{nAChR}, channel currents were evoked by 10-ms pulses of nicotine. In the first 15 minutes following patching onto a neuron, a gradual shift in nicotine sensitivity was noted; subsequently recordings were only made 15 min after establishing the whole-cell configuration—whereby the responses to nicotine remained consistent. In the presence of concentrations from 200 nM to 1 μM κ-HXTX-Hv1c I_{nAChR} decay rates were significantly prolonged within 5

min of toxin application ($p < 0.05$; Figure 7.2A and C), while current amplitude remained unaltered ($p > 0.05$; Figure 7.2E and F). As seen in Figure 7.2A, the effects on maximal I_{nAChR} amplitude were minimal with only a 15.8 ± 4.6% inhibition of peak current ($n = 5$, $p > 0.05$), however an apparent slower secondary component following partial decay of the current was more prominent post-toxin. The secondary component was more apparent at higher toxin concentrations (> 200 nM) as the effects on current decay were enhanced. In the presence of 500 nM κ-HXTX-Hv1c, a 98.9 ± 9.7% ($n = 5$, $p < 0.05$) increase in the secondary component was evident. Measurements were taken at the maximum amplitude (Figure 7.2B, closed circle) in response to toxin and compared with control amplitudes at the same time point. It is possible that the secondary component represents an enhancement of the activity of one of the channel subtypes contributing to the I_{nAChR}; however the nAChR subtypes in cockroach DUM neurons still remain relatively uncharacterised. Subsequently, the ability of κ-HXTX-Hv1c to enhance I_{nAChR} amplitude remains unclear.

In order to compare the data at varying concentrations of κ-HXTX-Hv1c, the current duration was measured at 20% of peak I_{nAChR} amplitude ($duration_{20}$). Control currents decayed rapidly with a $duration_{20}$ of 2.2 ± 0.3 seconds ($n = 27$), while $duration_{20}$ values in the presence of 500 nM and 1 μM κ-HXTX-Hv1c were increased to 9.8 ± 3.6 sec ($n = 6$) and 8.2 ± 1.8 sec ($n = 4$), respectively. Data was normalised to control currents evoked in response to 10 ms pulses of 10 μM nicotine. The current was altered in a concentration-dependent manner with an EC_{50} 182.5 ± 1.0 nM and a maximum enhancement of 552 ± 39% in the presence of 500 nM κ-HXTX-Hv1c ($n = 4–6$; Figure 7.2D). Notably, κ-HXTX-Hv1c did not directly activate I_{nAChR} at concentrations up to 1 μM (Figure 7.2E). This indicates that κ-HXTX-Hv1c does not act as an agonist of DUM neuron nAChRs.

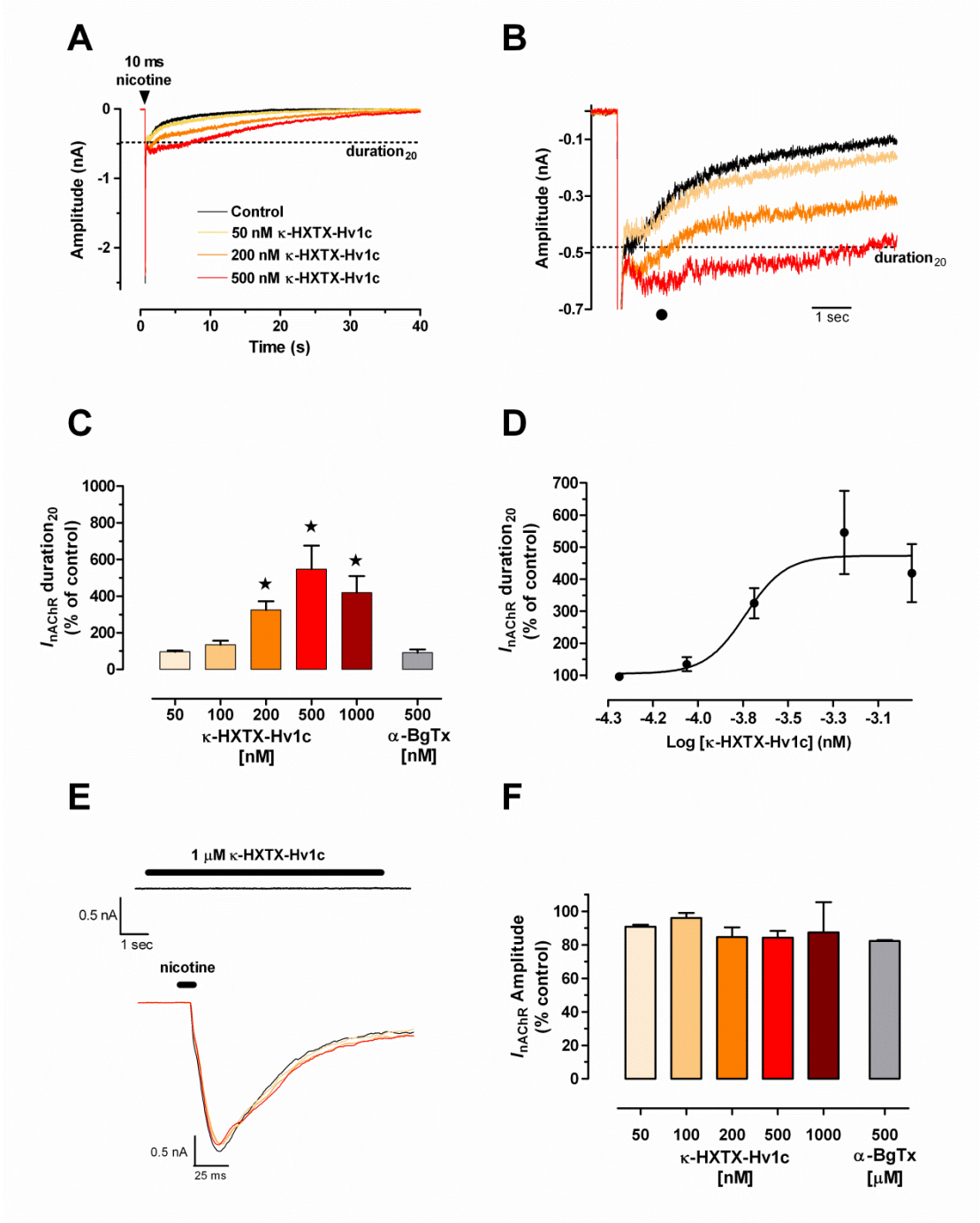

Figure 7.2: Concentration dependent effects of κ-HXTX-Hv1c on nAChR mediated currents in DUM neurons. **(A)** Superimposed traces representing the effect of 50, 200 and 500 nM κ-HXTX-Hv1c on DUM neuron I_{nAChR}. Inward currents were evoked in response to 10-ms pulse applications of 10 μM nicotine. duration$_{20}$: duration of I_{nAChR} at 20% of current amplitude. **(B)** Magnification of the initial 7 seconds of trace (A) highlighting the effect of κ-HXTX-Hv1c on the I_{nAChR} rate of decay and the enhanced secondary component (closed circle). **(C)** Comparison of I_{nAChR} duration$_{20}$ values in the presence of various concentrations of κ-HXTX-Hv1c and 500 nM α-bungarotoxin (α-

BgTx) as a percentage of control currents. **(D)** Concentration-response curve for I_{nAChR} $duration_{20}$ in the presence of κ-HXTX-Hv1c. Data were fitted with Eq. 2 (chapter 2, section 5) yielding an EC_{50} value of 182.5 ± 1.0 nM (n = 3–8) **(E)** Below is a representative trace illustrating the typical effects of 50–500 nM κ-HXTX-Hv1c on the initial transient portion of I_{nAChR} and above–the typical effect of 1 μM κ-HXTX-Hv1c in the absence of nicotine is shown. **(F)** Comparison of I_{nAChR} amplitude in response to various concentrations of κ-HXTX-Hv1c and 500 nM α-BgTx, expressed as a percentage of control. All I_{nAChR} were evoked by 10-ms nicotine pulses at 1-min intervals. Stars represent results significantly different from control values (n = 3–8, p < 0.05, one-way ANOVA).

7.2.3.1 Modulation of the agonist concentration-response by κ-HXTX-Hv1c

In order to identify any modulation of nAChR agonist sensitivity we examined the ability of 200 nM κ-HXTX-Hv1c to prolong current decay in response to varying concentrations of nicotine. Duration measurements at 20% of maximal current amplitude ($duration_{20}$) at a given nicotine pulse duration were compared in the absence and presence of κ-HXTX-Hv1c. The data was fitted by Eq. 2 (see chapter 2, section 5). A minor but significant shift in the nicotine concentration-response curve was observed following κ-HXTX-Hv1c application (EC_{50} 6.0 ± 0.2 ms in comparison to 8.9 ± 0.4 ms for control, n = 3, p < 0.05; Figure 7.3C and D). These results seem to indicate that 200 nM κ-HXTX-Hv1c moderately increases nAChR sensitivity to nicotine. Potentially the shift would be greater in the presence of higher concentrations of κ-HXTX-Hv1c; however for the sake of consistency an EC_{50} concentration was used.

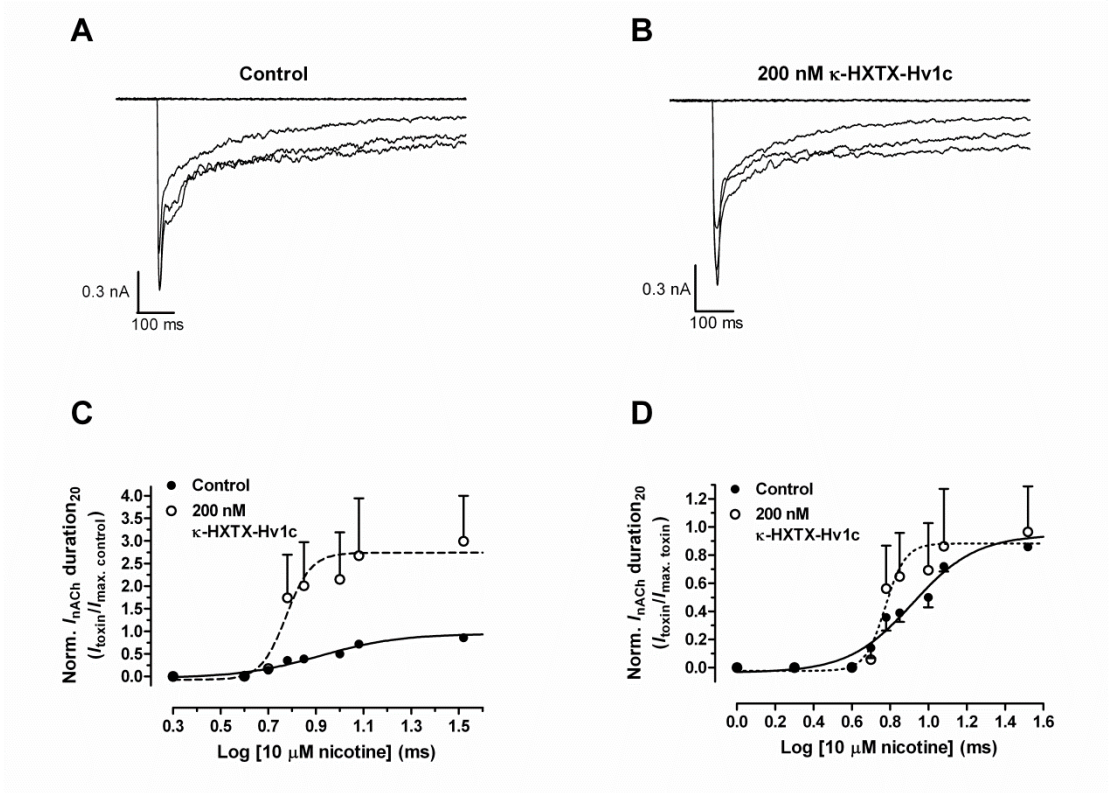

Figure 7.3: Effects of κ-HXTX-Hv1c on the nicotine sensitivity of nAChR. (A) Representative control inward I_{nAChR} evoked in response to applications of 10 μM nicotine at durations of 2, 4, 7, 20 and 56 ms, at 1-min intervals. **(B)** Representative I_{nAChR} evoked by 2, 4, 7, 20 and 56 ms pulses of nicotine in the presence of 200 nM κ-HXTX-Hv1c. **(C)** Concentration-response curve for control (closed circles) and 200 nM κ-HXTX-Hv1c (open circles) I_{nAChR} duration$_{20}$ in response to nicotine application (n = 3), yielding EC_{50} values of 8.9 ± 0.4 ms and 6.0 ± 0.2 ms, respectively. Data normalised to maximal control response. **(D)** Concentration-response curve for control (closed circles) and 200 nM κ-HXTX-Hv1c (open circles) in response to applications of 10 μM nicotine (n = 3). Data is normalised to the maximal response in each data set, the response to nicotine is significantly different in the presence of 200 nM κ-HXTX-Hv1c ($p < 0.05$, one-way ANOVA). Concentration-response data were fitted by Eq. 2 (see chapter 2, section 5).

7.2.3.2 MECHANISMS OF NACHR MODULATION BY Κ-HXTX-HV1C

To further characterise the nature of the toxin induced slowing of I_{nAChR} decay; current-voltage experiments were carried out. The membrane potential was stepped from a holding potential of –50 mV to test potentials from –90 to +30 mV in the presence and absence of κ-HXTX-Hv1c. To limit the influence of transient K_V and Ca_V channel currents membrane potentials were held at the test potential for 3.8 seconds prior to nicotine application. 300 nM TTX was included in solutions to block Na_V channel currents. Normalised current amplitude was plotted against membrane potential values as shown in Figure 7.4. The initial portion of the plot between –90 and –50 follows a single linear function however there is an inflection at –50 mV with currents reaching zero amplitude between –10 and +10 mV. This data is analogous with other studies on DUM neuron nAChR voltage-dependency (Courjaret and Lapied, 2001) No shift in voltage-dependence was observed following toxin application (Figure 7.4B), suggesting that κ-HXTX-Hv1c does not alter ionic selectivity of the channel.

Although it appears that channel currents were blocked by ~50% at hyperpolarised potentials, it is believed that this was a side effect of holding the membrane for prolonged periods of time under stressful conditions. Furthermore, under test conditions closer to the native conditions of the cell (V_h –50 mV) changes in current amplitude were minimal (see Figure 7.2F).

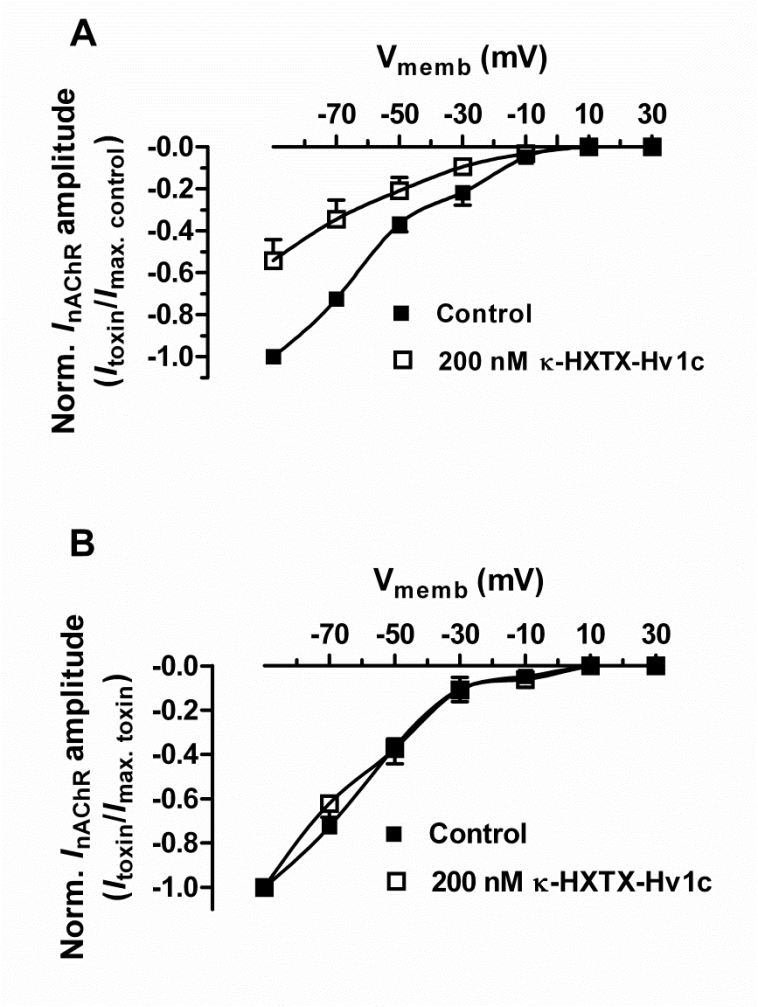

Figure 7.4: Current-voltage relationships for nAChR induced currents in the presence of κ-HXTX-Hv1c. I_{nAChR} were evoked by 10-ms pulses of 10 µM nicotine at 1-min intervals. Currents were recorded in the presence of depolarising voltage-pulse protocols from –90 mV to + 30 mV applied at 20-mV intervals from a holding potential of –50 mV. I_{nAChR} were recorded in the absence (closed squares) and presence (open squares) of 200 nM κ-HXTX-Hv1c. **(A)** Data were normalised to maximal control I_{nAChR} amplitude (n = 3). **(B)** Data were normalised to maximal I_{nAChR} amplitude of each data set (n =3).

7.2.3.3 κ-HXTX-Hv1c reverses nAChR channel desensitisation in response to nicotine

A particularly unique feature of κ-HXTX-Hv1c is its ability to seemingly reactivate nACh receptor currents in the presence of nicotine while they are in a fully or partially desensitised state. The toxin alone does not induce channel activation and thus cannot act as a receptor agonist. Subsequently, due to the ability of κ-HXTX-Hv1c to slow

I_{nAChR} decay rate we decided to test whether the toxin could reverse slow desensitisation of the nACh receptor. In order to induce slow desensitisation, 10 μM nicotine was continuously perfused for a period of 3.5–5 min (210–300 sec). After 100 seconds of uninterrupted nicotine perfusion I_{nAChR} decay (desensitisation) was found to reach 80.5 ± 5% of control amplitude (n = 5). Notably, some differences where seen in the initial decay timecourse in the absence of toxin, this is most likely due to the mixed population of nAChR subtypes present (Lapied et al., 1990; Bai and Sattelle, 1993; Salgado and Saar, 2004; Courjaret and Lapied, 2001) Subsequent, simultaneous application of nicotine and 500 nM κ-HXTX-Hv1c was found to cause a 33.7 ± 15.7% (n = 3) recovery of current amplitude, thus partially reversing desensitisation. Moreover, following the cessation of nicotine perfusion, I_{nAChR} were completely desensitised (Figure 7.5) this further indicates that the presence of both agonist and toxin are required to reverse desensitisation. Similar results were also seen in the presence of EC_{50} concentrations of κ-HXTX-Hv1c (n = 2). Application of 200 nM κ-HXTX-Hv1c following partial (~80%) I_{nAChR} desensitisation recovered 32.6 ± 2.9 of current amplitude (Figure 7.5B).

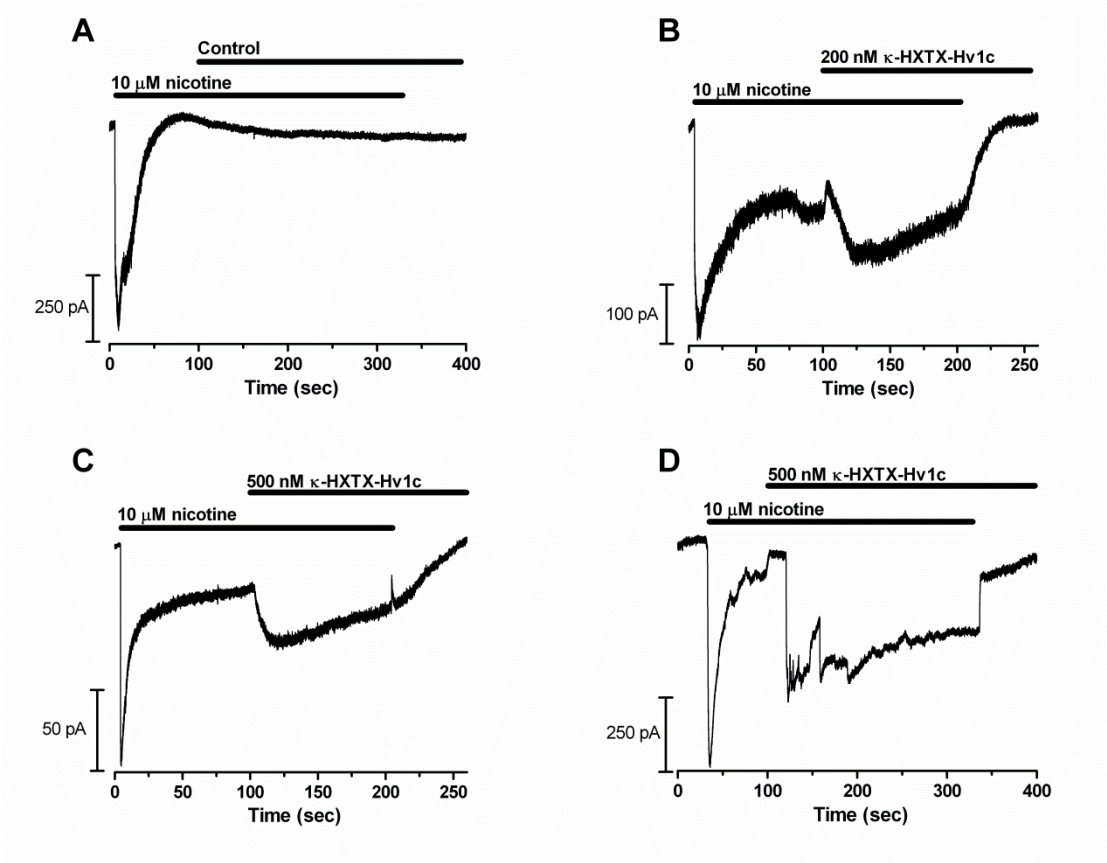

Figure 7.5: Effects of κ-HXTX-Hv1c on partially and fully desensitised nAChR channels. (B–D) I_{nAChR} desensitisation in response to extended application of nicotine is reversed in the presence of κ-HXTX-Hv1c. I_{nAChR} were recorded in response to 3.5–5 min continuous applications 10 μM nicotine via a pressure ejection system. Concentrations of 0 nM **(A)**, 200 nM **(B)** and 500 nM **(C–D)** κ-HXTX-Hv1c were applied at 100 seconds via a fast perfusion system, following full or partial inactivation of I_{nAChR}, represented by trace currents nearing 0 nA amplitude. I_{nAChR} were recorded in the presence of both κ-HXTX-Hv1c and nicotine for up to 250 seconds. I_{nAChR} activation was not maintained in the absence of nicotine. Note: traces (A) and (D) were recorded over a period of 400 sec, while (B) and (C) were recorded over 250 ms.

7.2.4 PROPERTIES OF GLUTAMATE INDUCED INWARD CURRENTS IN DUM NEURONS

Glutamate has an inhibitory effect on the excitatory activity of DUM neurons (Washio, 1994; Washio, 2002). The inhibitory effects are mediated by the conduction of chloride ions through glutamate-activated chloride channels (Sattelle, 1992; Washio, 1994; Zhao, Salgado et al., 2004) which appear to only be found in insects (Sattelle, 1992; Cleland, 1996; Cully, Paress et al., 1996).

The actions of glutamate on cockroach DUM neurons were examined under voltage-clamp conditions. Pressure application of 100 μM glutamate for periods of >2.5 ms induced an inward transient current. Picrotoxin, a potent blocker of invertebrate glutamate receptor (Glu-Cl) currents (Sattelle, 1992; Washio, 1994; Hamon, Le Corronc et al., 1998) was shown to block 35.1 ± 2.6% (n = 3) of current amplitude at a concentration of 100 μM (Figure 7.6D). The presence of a picrotoxin insensitive component of Glu-Cl currents ($I_{Glu\text{-}Cl}$) has previously described in DUM neurons (Raymond, Sattelle et al., 2000) which may explain the moderate inhibition at a what is believed to be a saturating concentration.

Steady-state protocols were used to obtain recordings at membrane potentials between –70 and +30 mV, with a V_h of –50 mV. Families of $I_{Glu\text{-}Cl}$ were evoked by 40-ms pulses of 100 μM glutamate (Figure 7.6E). The normalised maximum amplitude of the transient inward current was plotted against membrane potential. The $I_{Glu\text{-}Cl}$-V plot shows an inward current at negative potentials which decreases in amplitude as the membrane potential approaches 0 mV. At membrane potentials of approx. 0 mV no significant outward current was evident; this is in agreement with the calculated equilibrium potential for chloride ions (E_{Cl}) under symmetrical chloride conditions. This supports the assertion that the channels activated by glutamate conduct chloride ions.

In order to measure the glutamate sensitivity of the channel, various duration pulses of 100 μM glutamate were applied via pressure ejection. The mean values of peak $I_{Glu\text{-}Cl}$ amplitude were then plotted against the logarithm of glutamate pulses (Figure 7.6C). The data was fitted with a logistic function (Eq. 2, chapter 2, section 5) yielding an EC_{50} value of 16.5 ± 0.5 ms (n = 5). The maximal response to glutamate was evident at pulse durations greater than 320 ms.

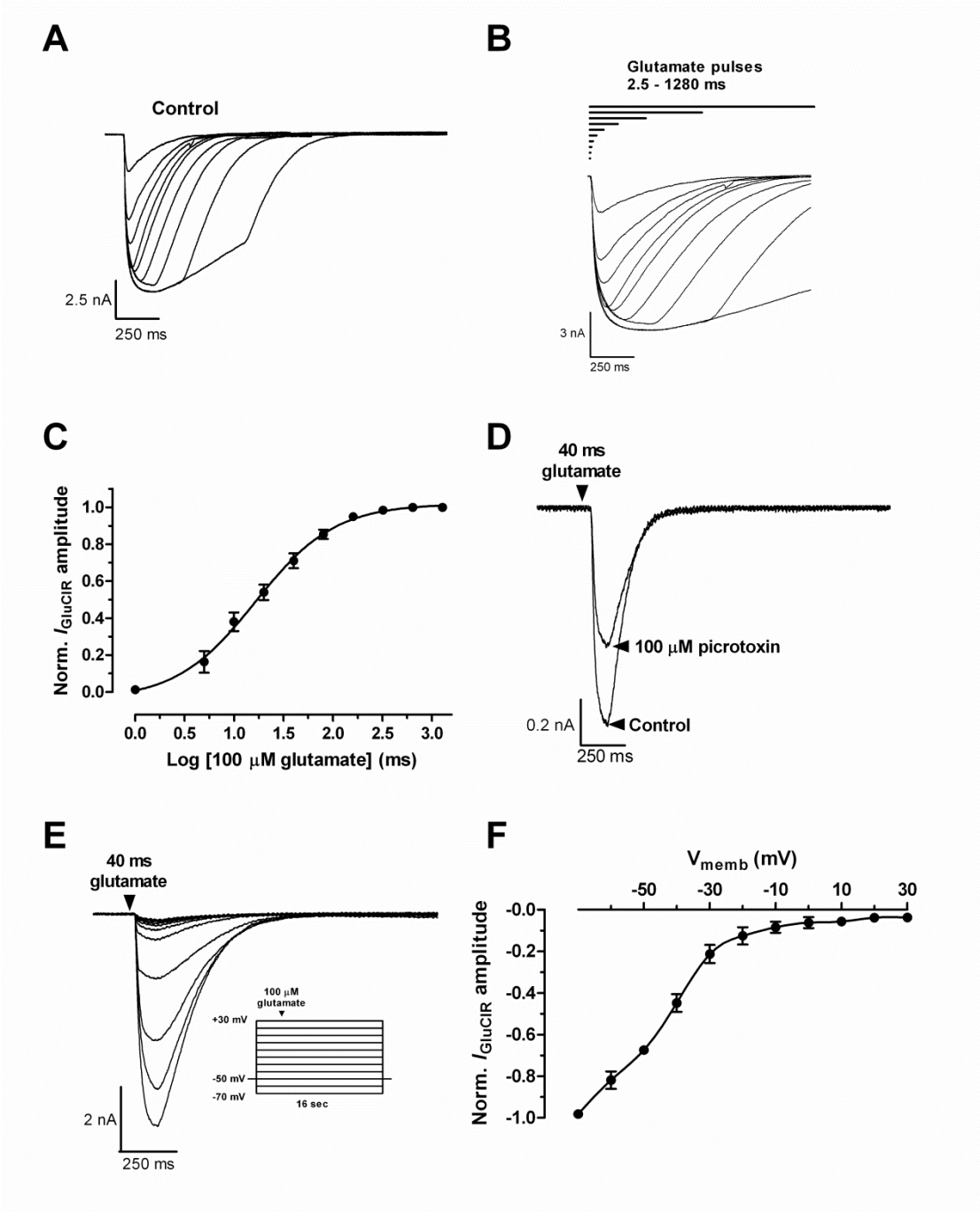

Figure 7.6: Properties of glutamate-mediated currents in DUM neurons. $I_{Glu\text{-}Cl}$ were evoked in response to 40-ms pulses of 100 µM glutamate at 1-min intervals. **(A)** Representative control inward $I_{Glu\text{-}Cl}$ evoked in response to applications of 100 µM glutamate. Currents were induced by glutamate pulse durations of 2.5, 5, 10, 20, 40, 80, 160, 320, 640 and 1280 ms, at 1-min intervals. **(B)** Magnification of the initial 1280 ms of trace (A) highlighting the glutamate pulse durations employed to evoke $I_{Glu\text{-}Cl}$. Note: with longer pulse durations a significant notch in the current trace is evident. This directly corresponds to the end of the agonist pulse and inactivation of the channel. **(C)**

Concentration-response curve for $I_{Glu\text{-}Cl}$ in response to glutamate application (n = 3), yielding an EC_{50} value of 16.5 ± 0.5 ms. Data normalised to maximal response and fitted with Eq. 2 (see chapter 2, section 5). **(D)** Superimposed traces representing the typical effect of 100 µM picrotoxin on $I_{Glu\text{-}Cl}$. **(E)** Representative $I_{Glu\text{-}Cl}$ traces evoked in the presence of test pulse potentials ranging from –70 mV to +30 mV at 10-mV intervals. The test pulse protocol used to evoke families of $I_{Glu\text{-}Cl}$ is shown in the inset. **(F)** Normalised $I_{Glu\text{-}Cl}$ amplitude recorded in the presence of changing membrane potentials: reversal potential ~0 mV (n = 3).

7.2.5 Pharmacological effects of κ-HXTX-Hv1c on glutamate mediated currents

In order to assess the effects of κ-HXTX-Hv1c on Glu-Cl channels, $I_{Glu\text{-}Cl}$ were evoked by 40-ms pulses of 100 µM glutamate. According to the glutamate concentration-response data (Figure 7.6C) a 40-ms pulse will evoke $I_{Glu\text{-}Cl}$ at 70% of maximum amplitude. $I_{Glu\text{-}Cl}$ evoked at 1-min intervals were recorded in the presence and absence of 1 µM κ-HXTX-Hv1c, at a V_h of –50 mV. In the presence of 1 µM κ-HXTX-Hv1c $I_{Glu\text{-}Cl}$ peak amplitude was increased by 21.0 ± 5.2% (n = 4, $p < 0.05$) while at 50 nM currents were only increased by 8.0 ± 2.7% (n = 3, $p > 0.05$; Figure 7.7). Increases in amplitude were evident within 2 min of toxin application. Alternately, $I_{Glu\text{-}Cl}$ duration at 20% of maximal peak amplitude ($duration_{20}$) was not significantly altered at any concentration of κ-HXTX-Hv1c tested (n = 3–4, $p > 0.05$; Figure 7.7D).

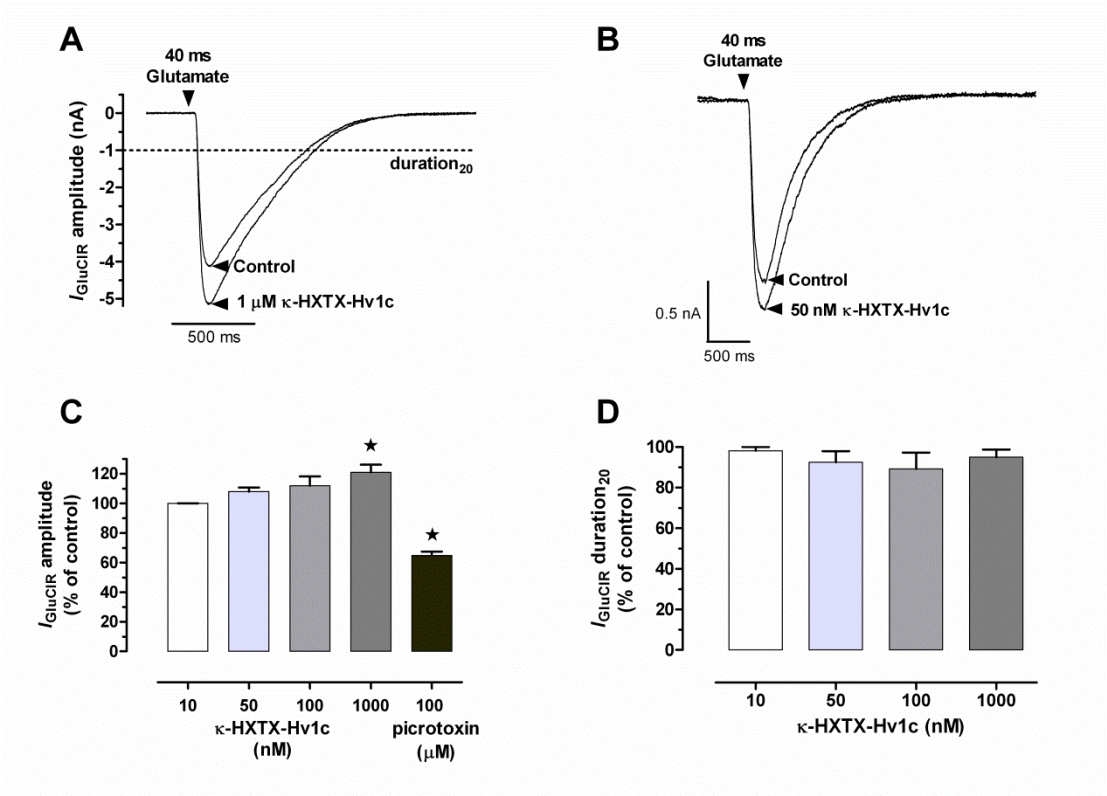

Figure 7.7: Effects of κ-HXTX-Hv1c on I_{Glu-Cl} in DUM neurons. (A–D) I_{Glu-Cl} were evoked in response to 40-ms pulses of 100 µM nicotine at 1-min intervals. Superimposed traces represent the typical effects of 1 µM **(A)** and 50 nM **(B)** κ-HXTX-Hv1c on I_{Glu-Cl}. (C–D) Comparison of I_{Glu-Cl} duration$_{20}$ **(C)** and amplitude **(D)** in response to 10, 50, 100 and 1000 nM κ-HXTX-Hv1c. Results were expressed as a percentage of control values. duration$_{20}$: I_{Glu-Cl} duration at 20% of current amplitude (see A). Only I_{Glu-Cl} amplitude in response to 1000 nM was considered significantly different from control currents (★, $p < 0.05$) after statistical analysis with one-way ANOVA.

Furthermore, due to the effects of κ-HXTX-Hv1c on I_{Glu-Cl} amplitude, the sensitivity of Glu-Cl channels to glutamate was assessed. Peak I_{Glu-Cl} amplitude was recorded in response to varying pulse durations of 100 µM glutamate in the absence and presence of toxin. Data was fitted with Eq. 2 (see chapter 2, section 5). Prior to toxin perfusion the data yielded an EC_{50} of 16.5 ± 0.5 ms while the EC_{50} value was reduced to 10.5 ± 2.2 ms in the presence of 1 µM κ-HXTX-Hv1c ($n = 3$, $p < 0.05$; Figure 7.8). Figure 7.8C indicates that peak I_{Glu-Cl} amplitude is increased at all pulse durations of glutamate tested in the presence of toxin. Subsequently, amplitude of I_{Glu-Cl} is even increased

beyond the maximum amplitude achieved under control conditions (i.e. at pulse durations >320 ms).

Although increases in current amplitude are evident at all glutamate pulse durations tested, it was not completely clear whether this was due to increased sensitivity to glutamate. Subsequently, data recorded in the presence of κ-HXTX-Hv1c was normalised to the maximum amplitude recorded under the same conditions. Figure 7.8D illustrates that while there is an increase in the maximum current amplitude there is no change in receptor sensitivity as evidenced by the lack of a leftward shift in the concentration-response curve. Therefore, it is likely that the increases in $I_{Glu\text{-}Cl}$ seen in the presence of κ-HXTX-Hv1c are a result of a mechanism other than an increase in channel sensitivity to glutamate.

However, despite these significant but modest actions, it is unlikely that an enhancement of an inhibitory response is responsible for the potent *excitatory* neurotoxicity caused by κ-HXTX-Hv1c. Furthermore, a significant enhancement of $I_{Glu\text{-}Cl}$ amplitude ($p < 0.05$) was only evident at a concentration of 1 μM, which seems unlikely to be potent enough to correlate well with the results seen in insect bioassays (Wang, Connor et al., 2000; Maggio and King, 2002b; Tedford, Maggio et al., 2007; Gunning, Maggio et al., 2008).

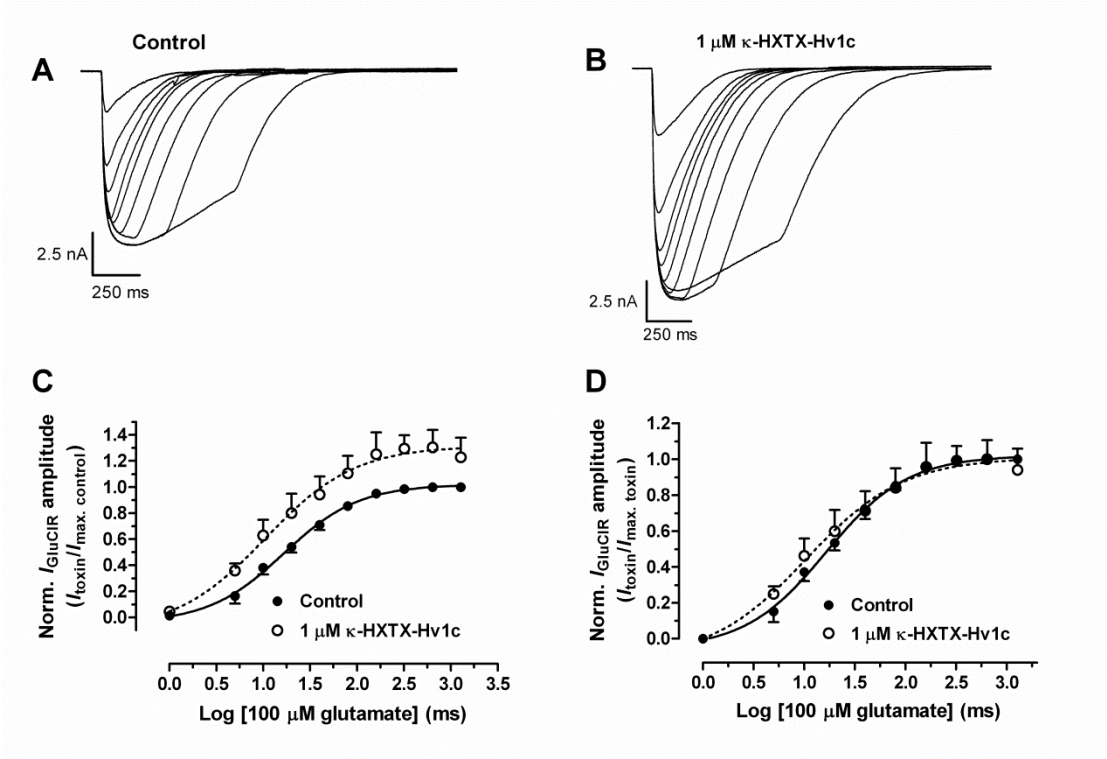

Figure 7.8: Effects of κ-HXTX-Hv1c on Glu-Cl sensitivity to glutamate. (A) Representative control inward $I_{Glu\text{-}Cl}$ evoked in response to applications of 100 μM glutamate. Currents were induced by glutamate pulse durations of 2.5, 5, 10, 20, 40, 80, 160, 320, 640 and 1280 ms, at 1-min intervals. **(B)** Representative $I_{Glu\text{-}Cl}$ traces evoked by 2.5, 5, 10, 20, 40, 80, 160, 320, 640 and 1280 ms pulses of glutamate in the presence of 1 μM κ-HXTX-Hv1c. Note: with longer pulse durations a significant notch in the current trace is evident. This directly corresponds to the end of the agonist pulse and inactivation of the channel. **(C)** Concentration-response curve for control (closed circles) and 1 μM κ-HXTX-Hv1c (open circles) $I_{Glu\text{-}Cl}$ $duration_{20}$ in response to glutamate application (n = 3), yielding EC_{50} values of 16.5 ± 0.5 ms and 10.7 ± 2 ms, respectively. Data normalised to maximal control response. The response to glutamate is significantly enhanced in the presence of 1 μM κ-HXTX-Hv1c ($p < 0.05$, one-way ANOVA). Concentration-response data were fitted by Eq. 2 (see chapter 2, section 5). Typical family of $I_{Glu\text{-}Cl}$ evoked by the protocol illustrated in (A). **(D)** Concentration-response curve for control (closed circles) and 1 μM κ-HXTX-Hv1c (open circles) $I_{Glu\text{-}Cl}$ $duration_{20}$ in response to glutamate application. Data were normalised to the maximal peak amplitude in each data set.

7.2.6 CHARACTERISTICS OF GABA MEDIATED CHLORIDE CURRENTS IN DUM NEURONS

The inhibitory neurotransmitter γ-aminobutyric acid (GABA) is widely distributed in the CNS of insects and its effects mediated by a $GABA_A$ receptor (Bicker, Schafer et al., 1988). To assess the effects of κ-HXTX-Hv1c on the activity of GABA evoked chloride channel currents ($I_{GABA\text{-}Cl}$), DUM neuron $I_{GABA\text{-}Cl}$ were evoked by short pulses of 100 µM GABA delivered via a pneumatic pressure ejection system. At ejection periods less than 10-ms no discernible currents were evident. In the presence of symmetrical chloride solutions inward $I_{GABA\text{-}Cl}$ were evoked by GABA pulses while cells were held at –50 mV.

Receptor sensitivity to 100 µM GABA was evaluated by analysing the agonist concentration-response relationship. Increasing duration GABA pulses between 10 and 600 ms evoked currents of varying amplitudes. Maximal amplitudes were elicited in response to pulse durations greater than 300-ms (Figure 7.9C and D). Normalised current amplitudes were plotted against the logarithm of pulse durations. The data was fitted by a Logistic function (Eq. 2, chapter 2, section 5) yielding an EC_{50} value of 74.8 ± 0.4 ms (n = 7; Figure 7.9D).

To determine whether GABA currents were carried by chloride ions the response induced by GABA at varying membrane potentials was assessed. The same voltage step protocol used for glutamate currents was employed for GABA related experiments. In response to membrane potentials between –70 to +30 mV inward currents were evoked by 100 ms-pulses of GABA (Figure 7.9A). However, at membrane potentials around 0 mV no currents were evident. Normalised current amplitude was plot against membrane potential values as shown in Figure 9B (n = 5). The initial portion of the plot between –70 and –40 mV follows a single linear function however there is an inflection at –30 mV with currents reaching zero amplitude between –10 and +10 mV which is in agreement with the calculated equilibrium potential for chloride ions (E_{Cl} = 0 mV) under symmetrical Cl^-.

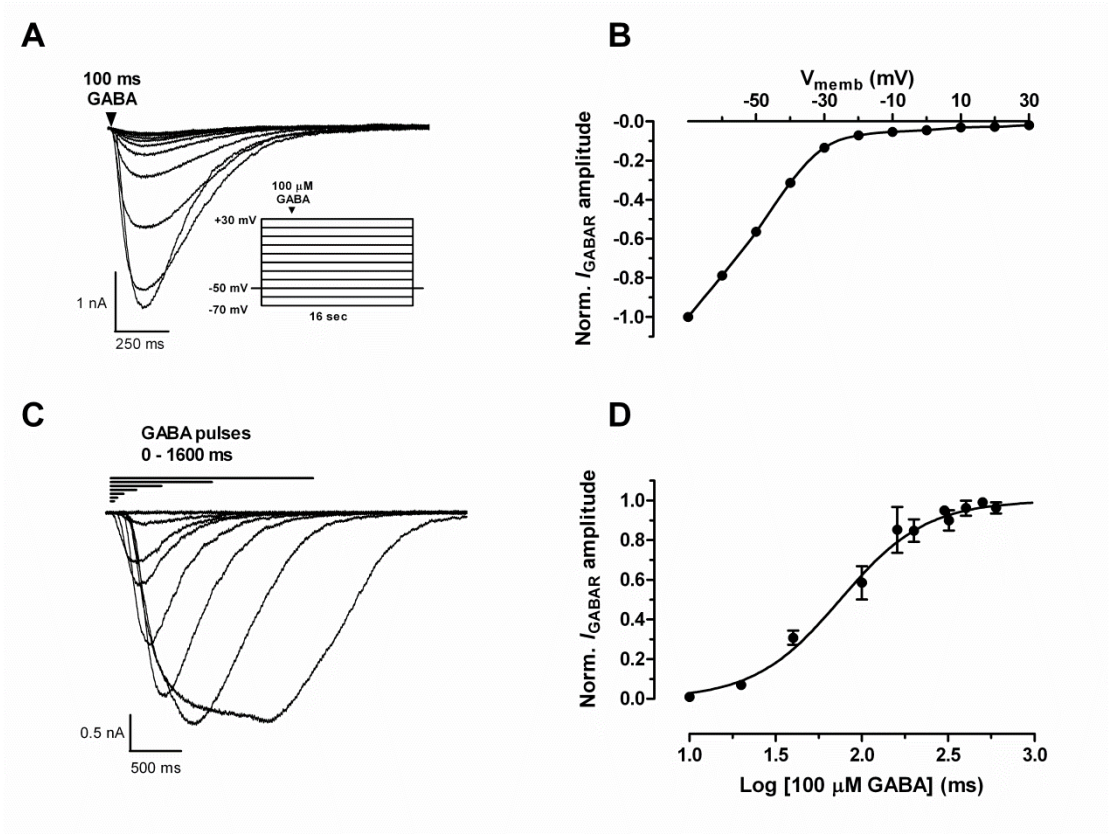

Figure 7.9: Isolation and characterisation of $I_{GABA\text{-}Cl}$ in DUM neurons. $I_{GABA\text{-}Cl}$ were evoked in response to 100-ms pulses of 100 µM GABA at 1-min intervals. **(A)** Representative $I_{GABA\text{-}Cl}$ traces were evoked in the presence of test pulse potentials ranging from –70 mV to +30 mV at 10-mV intervals. **(B)** Normalised $I_{Glu\text{-}Cl}$ amplitude recorded in the presence of changing membrane potentials: reversal potential ~0 mV (n = 5). **(C)** Representative inward $I_{GABA\text{-}Cl}$ evoked in response to applications of 100 µM GABA. Currents were induced by GABA pulse durations of 25, 50, 100, 200, 400, 800 and 1600 ms, at 1-min intervals. A representation of GABA pulses is indicated above the trace. **(D)** Concentration-response curve of $I_{GABA\text{-}Cl}$ in response to varying durations of 100 µM GABA application, yielding an EC_{50} value of 74.8 ± 0.4 ms (n = 7). Data was fitted by Eq. 2 (see chapter 2, section 5).

7.2.7 PHARMACOLOGICAL EFFECTS OF κ-HXTX-HV1C ON INSECT GABA MEDIATED CURRENTS

To further confirm that GABA mediates I_{Cl} in DUM neurons, currents were evoked in the presence of 100 μM picrotoxin. Following the application of picrotoxin, GABA-evoked currents were reduced to 18.8 ± 2.4% of control currents ($p < 0.05$, $n = 4$). These results again reflect that GABA-mediated currents are carried by chloride ions (Le Corronc, Alix et al., 2002). Current block occurred within 5 min and partial washout was evident. Due to the activity of κ-HXTX-Hv1c on other transmitter-mediated currents, the effects of 1 μM κ-HXTX-Hv1c were assessed on GABA-Cl currents evoked by 100-ms pulses of GABA. In the presence of toxin, $I_{GABA\text{-}Cl}$ remained unaltered (Figure 7.10B). GABA currents were recorded in the presence of toxin (100 nM and 1 μM) for up to 10 min however no significant change in current duration or current amplitude was observed ($n = 4–5$, $p > 0.05$; Figure 7.10C and D). Therefore the effects of κ-HXTX-Hv1c on chloride currents appear to be confined to those mediated by glutamate.

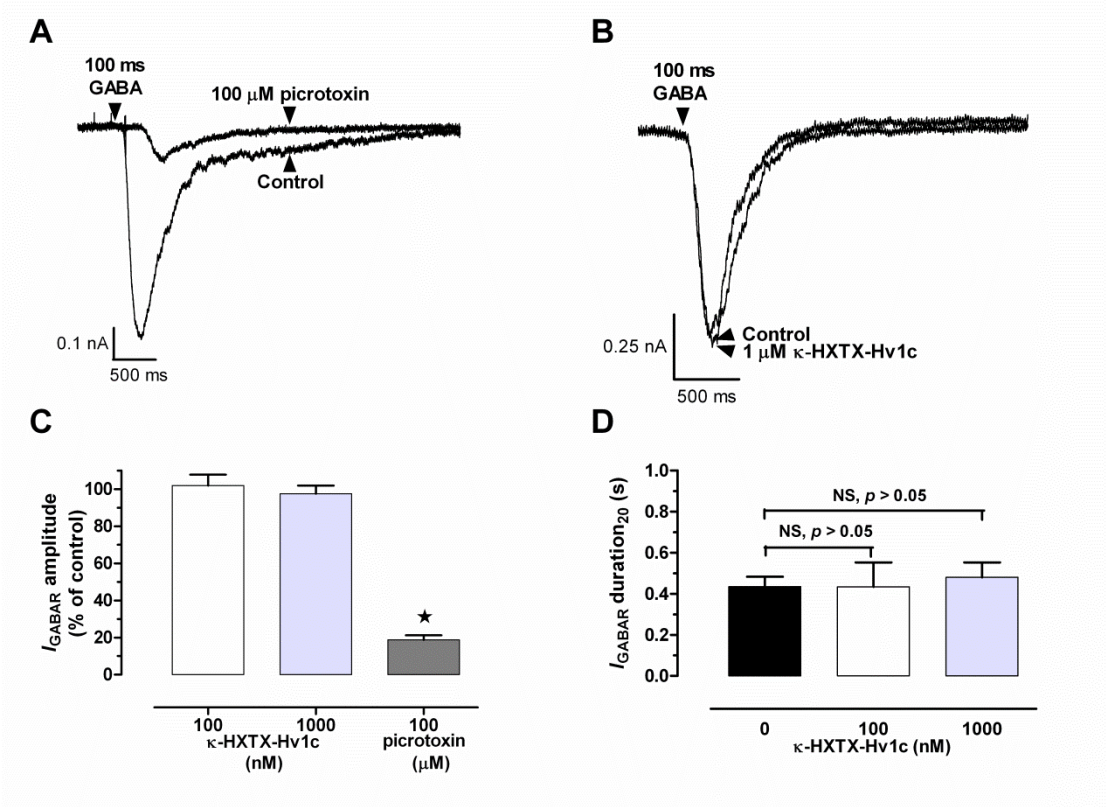

Figure 7.10: The effects of κ-HXTX-Hv1c and picrotoxin on $I_{GABA-Cl}$ in DUM neurons. $I_{GABA-Cl}$ were evoked by 100-ms pulse applications of 100 μM GABA at 1-min intervals. The membrane potential was held at –50 mV. Superimposed traces representing the typical effect of 100 μM picrotoxin **(A)** and 1 μM κ-HXTX-Hv1c **(B)** on $I_{GABA-CL}$. (C–D) Comparison of $I_{GABA-Cl}$ amplitude **(C)** and duration$_{20}$ **(D)** in response to κ-HXTX-Hv1c and picrotoxin. Amplitude was measured as a percentage of $I_{GABA-Cl}$ under control conditions while duration is represented as a duration$_{20}$ value (duration at 20% of amplitude). No significant changes were found in the presence of κ-HXTX-Hv1c ($p > 0.05$, one-way ANOVA). 100 μM picrotoxin (star) significantly reduced I_{GABA} amplitude to 18.8 ± 2.4% of control ($n = 4$, $p < 0.05$).

7.3 DISCUSSION

The aim of this study was to assess the action of κ-HXTX-Hv1c on neurotransmitter-activated receptors given the importance of these receptors in the modulation of the insect nervous system. In this study the effects of the insecticidal peptide neurotoxin κ-HXTX-Hv1c were tested on GABA-, glutamate- and nicotine-mediated channel currents in cockroach DUM neurons. Importantly, unlike the calcium-activated K_V channel, previously believed to be the insecticidal target of κ-HXTX-Hv1c, these neurotransmitter-activated channel targets have already been validated as lethal targets for a range of agrochemical insecticides including fipronil, (GABA receptors), avermectins (glutamate receptors) and neonicotinoids such as imidacloprid (nicotinic acetylcholine receptor) (Gant, Eldefrawi et al., 1987; Narahashi, 1996; Matsuda, Buckingham et al., 2001; Buckingham, Biggin et al., 2005; Raymond-Delpech, Matsuda et al., 2005; Millar and Denholm, 2007).

While the effects of κ-HXTX-Hv1c on the glutamate receptor were small but significant, increasing the activity of an inhibitory neurotransmitter receptor (Cleland, 1996; Narahashi, Zhao et al., 2010) is unlikely to be responsible for the excitatory phenotype seen in insect assays (Wang, Connor et al., 2000; Tedford, Maggio et al., 2007; Gunning, Maggio et al., 2008). In addition, no significant effects were seen on $I_{GABA\text{-}Cl}$ at concentrations up to 1 μM. Therefore, the major finding of this study was that κ-HXTX-Hv1c significantly delays I_{nAChR} decay at concentrations as low as 200 nM. Applications of nanomolar concentrations of κ-HXTX-Hv1c were found to substantially prolong the apparent inactivation kinetics of nAChR channel currents while failing to alter the amplitude or kinetics of the initial transient portion of the current. Furthermore, κ-HXTX-Hv1c did not evoke channel currents in the absence of nicotine but it did moderately alter receptor sensitivity to nicotine. In addition, the activity of κ-HXTX-Hv1c did not appear to alter the ion selectivity of the channel as evidenced by no shift in the reversal potential in the *I-V* data.

A number of allosteric modulators of the mammalian nAChR also demonstrate a similar ability to slow I_{nAChR} decay (Hurst, Hajos et al., 2005; Bertrand and Gopalakrishnan, 2007; Gronlien, Hakerud et al., 2007). Furthermore, some of these toxins also have the ability to reverse receptor desensitisation to nicotine (Barron, McLaughlin et al., 2009).

Accordingly, we tested the ability of κ-HXTX-Hv1c to reactivate partially or fully desensitised nAChRs. In order to achieve this goal we applied long pulses of nicotine and found that concurrent application of nicotine and κ-HXTX-Hv1c resulted in channel reactivation, resulting in an apparent reversal of receptor desensitisation.

7.3.1 κ-HXTX-Hv1c is an allosteric modulator of nAChRs

There are several hypotheses that could explain the activity of κ-HXTX-Hv1c on I_{nAChR} in DUM neurons. Firstly, we can exclude the possibility that κ-HXTX-Hv1c activates a completely new channel current as we have shown that the toxin is incapable of producing a response in the absence of nicotine, further supporting the conclusion that κ-HXTX-Hv1c targets the nAChR in cockroach DUM neurons. Secondly, the ability of κ-HXTX-Hv1c to induce a prolongation of the kinetics of I_{nAChR} decay may purely be a result of channel being stabilised in the open conformation, subsequently increasing current amplitude as more channels become open for longer periods of time.

This mechanism of positive allosteric modulation has been previously described for a number of compounds targeting the mammalian α7 nAChR. Positive allosteric modulators (PAMs) that target mammalian nAChRs are differentiated into two groups – those that do not alter desensitisation properties (type I) and those that prolong desensitisation (type II) (Bertrand and Gopalakrishnan, 2007). PAMs are predicted to bind away from the agonist binding site but have been shown to enhance channel gating in the presence of an agonist (Bertrand and Gopalakrishnan, 2007). Three different binding sites have been previously identified for PAMs targeting mammalian nAChR. Glantamine targets the subunit interface domain (Iorga, Herlem et al., 2006), PNU-1205996 targets a site in the middle of the transmembrane domain (Young, Zwart et al., 2008) and NS-1738 targets a site at the extracellular-transmembrane junction (Bertrand, Bertrand et al., 2008).

Somewhat akin to the effects of κ-HXTX-Hv1c, type II PAMs significantly delay nAChR current decay. Furthermore, PAMs of mammalian nAChRs enhance current amplitudes and increase receptor agonist sensitivity. As stated previously PNU-1205996 binds to the transmembrane domain (Young, Zwart et al., 2008) and stabilises the channel in the open conformation, subsequently delaying channel desensitisation (Barron, McLaughlin et al., 2009). Studies indicate that PNU increases channel open

time, has no effect on ion selectivity and little effect on unitary conductance (Hurst, Hajos et al., 2005). Although the changes in receptor kinetics are not sufficient to activate receptors in the absence of an agonist, evidence does suggest that PNU is able to lower energy barriers for agonist-induced activation therefore preparing the receptor to undergo gating transitions (Barron, McLaughlin et al., 2009).

Additionally, PNU-1205996 is one of a growing number of allosteric modulators with the ability to reopen nAChR channels from a desensitised state (Hurst, Hajos et al., 2005; Gronlien, Hakerud et al., 2007). So far only type II PAMs seem to possess the ability to evoke nAChR reactivation (Hurst, Hajos et al., 2005; Gronlien, Hakerud et al., 2007). The similar characteristics of the reactivated current including a slow rate of decay and a slow onset would suggest that the same mechanism is responsible for the effects seen on the current decay in the presence of κ-HXTX-Hv1c.

The results of the present study indicate that the reactivation of nAChR currents following desensitisation is only applicable in the presence of an agonist. Following the removal of nicotine, currents evoked in the presence of κ-HXTX-Hv1c decayed rapidly. This data suggests that while κ-HXTX-Hv1c is capable of reactivating desensitised nAChRs, the toxin is only able to do so in the presence of the agonist. Presumably the toxin acts by activating receptors that are already bound by agonist but are desensitised, therefore inducing a conducting state.

7.3.2 Insect nAChR subtypes

The presence of a number of distinct nAChR may explain the lack of increase in current amplitude in response to the positive allosteric modulation by κ-HXTX-Hv1c. The results indicate that the transient, rapidly decaying portion of the current is unaffected by κ-HXTX-Hv1c while the secondary sustained component undergoes significant slowing of inactivation. Moreover, a clear increase in the initial portion of the secondary component, particularly in the presence of high concentrations of κ-HXTX-Hv1c, might suggest an enhancement of a component which is obscured by the initial transient current.

Investigations on cockroach neurons have identified two distinct nAChR channel subtypes which demonstrate α-BgTx sensitive and resistant activities. α-BgTx sensitive

receptors include channel subtypes with mixed nicotinic/muscarinic activity (Lapied, Corronc et al., 1990), those with rapid desensitisation (nAChD) and another with non-desensitising properties (Salgado and Saar, 2004). nAChD receptors have been described in cockroach thoracic neurons with rapid current decay and selective sensitivity to 100 nM imidacloprid, an insecticidal compound of the neonicotinoid family (Courjaret and Lapied, 2001). Furthermore, imidacloprid (IMI) and other related compounds which have been found to bind to the nAChD receptor in the insect CNS, displace binding of α-BgTx (Tomizawa and Yamamoto, 1992; Lind, Clough et al., 1999). In contrast, nAChN receptors are activated by neonicotinoids and are allosterically targeted by spinosyn A (Salgado and Saar, 2004), one of the active components of the insecticide spinosad (Salgado, 1998; Salgado, Sheets et al., 1998). nAChN are also selectively blocked by methyllycaconitine (MLA) and more potently targeted by α-BgTx (Salgado and Saar, 2004). However, it is important to note that nAChD and nAChN have been identified in thoracic cockroach neurons and do not appear to be apparent in abdominal DUM neurons as used in the present study. On the other hand, we have shown in this study that while κ-HXTX-Hv1c fails to affect the initial fast component of I_{nAChR} it dramatically prolongs the secondary component. Moreover, at higher concentrations a clear enhancement at the beginning of the secondary component is evident. This enhancement may potentially represent an increase in a nAChN-like component previously obscured by the initial transient component.

α-bungarotoxin sensitive insect nAChRs with mixed nicotinic/muscarinic activities have been shown to induce depolarising responses in cockroach DUM neurons (Lapied, Corronc et al., 1990). Moreover, the slow component of the nicotine-evoked response is selectively inhibited by d-tubocurarine, pirenzepine and gallamine while the fast component was insensitive to these antagonists (Lapied, Corronc et al., 1990). It has been suggested that these receptors may play roles the regulation of neurotransmitter release, driving burst activity and the modulation of rhythmic depolarisations in motorneurons (Thany, 2010). However, no further characterisation of these mixed nAChR has been pursued as they are believed to only contribute to a small portion of the global nAChR current (Thany, 2010).

Alternately, α-bungarotoxin resistant nAChR subtypes are characterised as nAChR1 and nAChR2 based on differing ionic permeation and pharmacologies (Courjaret and Lapied, 2001). The inward currents mediated by nAChR1 and nAChR2 differ particularly in their sensitivities to various antagonists. Studies have found that nAChR1 is selectively blocked by d-tubocurarine, while nAChR2 is preferentially blocked by mecamylamine and α-conotoxin ImI (Courjaret and Lapied, 2001).

Unfortunately, while the successful cloning of various nAChR subunits has been reported in several insect species, few have been successfully expressed (Eastham, Lind et al., 1998; Hermsen, Stetzer et al., 1998; Gao, Deacutis et al., 2007; Millar and Lansdell, 2010). Success has only been achieved with the inclusion of vertebrate non α subunits and as such it is widely believed that expression difficulties may relate to the lack of a presently unidentified insect non α subunit (Millar, 1999). However, given the full characterisation of several insect genomes this theory does seem less likely (Millar and Lansdell, 2010). Another potential answer may be the requirement of specific accessory proteins to produce functional channels (Millar and Lansdell, 2010). Nonetheless, the inability to express functional nAChR subtypes makes it even more important to identify and characterise these subtypes in native neurons.

To summarise, classification of nAChR subtypes is more complex than α-BgTx sensitive and insensitive. As the α-BgTx sensitive component of nAChR in DUM neurons is not well understood, extensive characterisation would be required prior to identifying the channel subtype targeted by κ-HXTX-Hv1c. Consequently, further characterisation is the subject of another investigation and is therefore beyond the scope of this project.

7.3.3 FUTURE DIRECTIONS

Importantly, as we have indicated in this study, PAMs do not directly activate or desensitise nAChR. PAMs enhance receptor activity by causing conformational changes to the ligand binding site (Barron, McLaughlin et al., 2009). The enhancement of receptor activity can manifest as an increase in receptor sensitivity to agonists and/or an augmentation of receptor efficacy during activation. While κ-HXTX-Hv1c causes a modest shift in receptor sensitivity to nicotine at 200 nM it is possible that this activity may be increased at higher concentrations. Moreover, the most significant effects of κ-

HXTX-Hv1c on receptor activity appear to be the ability of the toxin to slow nAChR current inactivation and to reactivate desensitised channels.

Following the identification of the nAChR subtype targeted by κ-HXTX-H-v1c, the next step would be to gain further insight into the mechanism of activity. The implementation of single channel recordings would enable the analysis of open channel times and changes in channel conductance. For instance, single channel recordings would allow us to identify if the reduction in channel current decay is due to a channels remaining in the open configuration for longer periods of time, more channels are becoming activated and furthermore whether toxin binding results in any changes in channel conductance.

7.3.4 Is nAChR a valid insecticidal target?

In conclusion, nicotine receptors are the most abundant excitatory receptors in insects and play important roles in the regulation of fast synaptic neurotransmission. As such, nAChR are important targets of several commercially available insecticides including the neonicotinoids (Tomizawa and Casida, 2003; Matsuda, Shimomura et al., 2005; Ihara, Brown et al., 2006), spinosyns (Salgado, 1998; Salgado, Sheets et al., 1998; Salgado and Saar, 2004; Watson, Chouinard et al., 2010), and nereistoxin analogs (Lee, Tomizawa et al., 2003; Raymond Delpech, Ihara et al., 2003). Of significant interest in regards to this study however, is the activity of spinosyn A. Spinosyn A is the main active component of the commercially available insecticide, spinosad (Salgado, 1998; Salgado, Sheets et al., 1998). Importantly, spinosad can directly excite the insect CNS evoking involuntary muscle contractions and tremors (Salgado, 1998; Salgado, Sheets et al., 1998). Interestingly the prolonged hyperexcitation, followed by paralysis due to neuromuscular fatigue is reminiscent of the phenotype induced by κ-HXTX-Hv1c (Wang, Connor et al., 2000; Tedford, Maggio et al., 2007; Gunning, Maggio et al., 2008). Furthermore, spinosyn A has been shown to activate nAChN receptors in cockroaches and is believed to do so via an allosteric mechanism (Salgado and Saar, 2004). Additionally, spinosyn A appears to evoke a continued increase in current in the presence of acetylcholine (Salgado and Saar, 2004) which appears to be similar to the effects of κ-HXTX-Hv1c on channel current decay. These results would therefore seem to indicate that positive allosteric modulation of nAChR is a valid insecticidal mechanism exploited by spinosyn A and κ-HXTX-Hv1c.

Chapter Eight

Discussion and Concluding Remarks

Monique J. Windley

[2012]

8.1 PROJECT AIMS REVISITED

The overall aim of this project was to characterise the activity of two distinct families of insect-selective spider venom peptide neurotoxins κ-TRTX-Ec2 toxins and κ-HXTX-Hv1c on the insect nervous system in order to promote these toxins as promising leads in the development of bioinsecticides. To achieve these goals, these spider peptide toxins were assayed against a number of important ion channels within the insect nervous system including; voltage-gated potassium (Kv), calcium (Ca_V) and sodium (Na_V), as well as nicotinic-acetylcholine- (nACh), glutamate- and γ-aminobutyric acid-activated (GABA). Voltage-clamp and current-clamp recordings were made from cockroach dorsal unpaired neurons utilising the whole-cell patch-clamp technique. The effects of these toxins to modulate the gating and kinetics of both voltage- and neurotransmitter-gated ion channels were assessed. Insect bioassays were also utilised to validate the insecticidal activities of various toxins that target K_V channel subtypes in house crickets.

8.2 CHARACTERISATION OF κ-TRTX-EC2A, κ-TRTX-EC2B AND κ-TRTX-EC2C

Chapter 3 details the isolation and characterisation of three insecticidal peptides from the venom of the African tarantula, *Eucratoscelus constrictus.* κ-TRTX-Ec2a, -Ec2b and -Ec2c were found to be high affinity blockers of insect BK_{Ca} channels with IC_{50} values of 3.7, 25.3 and 24.6 nM, respectively. Inhibition of insect BK_{Ca} channels by κ-TRTX-Ec2 toxins was voltage-independent. Furthermore, channel block did not demonstrate alleviation at increasingly depolarised potentials, thus it was proposed that the κ-TRTX-Ec2a toxins interact with the turret and/or loop region of the BK_{Ca} channel vestibule and do not project deeply into the pore. Channel inhibition is believed to be a result of a steric rather than a physical occlusion of the channel pore. In addition, substantially higher concentrations of κ-TRTX-Ec2a caused a minor, but significant voltage-independent inhibition of insect delayed-rectifier K_V (K_{DR}). κ-TRTX-Ec2 did not significantly modify insect Ca_V, Na_V channels or other K_V channel subtypes including A-type, transient (K_A).

The κ-TRTX-Ec2 toxins share high homology with a number of mammalian-active peptide toxins isolated from other theraphosid spiders (Figure 8.1). These homologous peptides have been described with a range of activities against Ca_V1, Ca_V3, Na_V, K_V4

and K_V2 channels. In comparison with other homologous theraphotoxins it seems likely that the affinity of κ-TRTX-Ec2 toxins for the BK_{Ca} channel is due to substitutions in the highly conserved Lys4-Trp5-Met6 of the theraphotoxins. In particular the Lys and Phe at positions 4 and 5 of the κ-TRTX-Ec2 toxins are different from other homologous theraphotoxins (See Figure 8.1).

All three κ-TRTX-Ec2 toxins were found to be insecticidal to crickets while only κ-TRTX-Ec2c was found to induce neurotoxic symptoms in mice. Due to the highly homologous nature of these toxins it was proposed that the residues located at the C-terminal region of κ-TRTX-Ec2a and -Ec2b were most likely to be responsible for the phyla selectivity. In particular, it was proposed that the presence of a glutamic residue at position 28 or 29 is likely to confer insect-selectivity as all the remaining mammalian-active theraphotoxin have basic or hydrophobic residues at one or both of these locations (Figure 8.1).

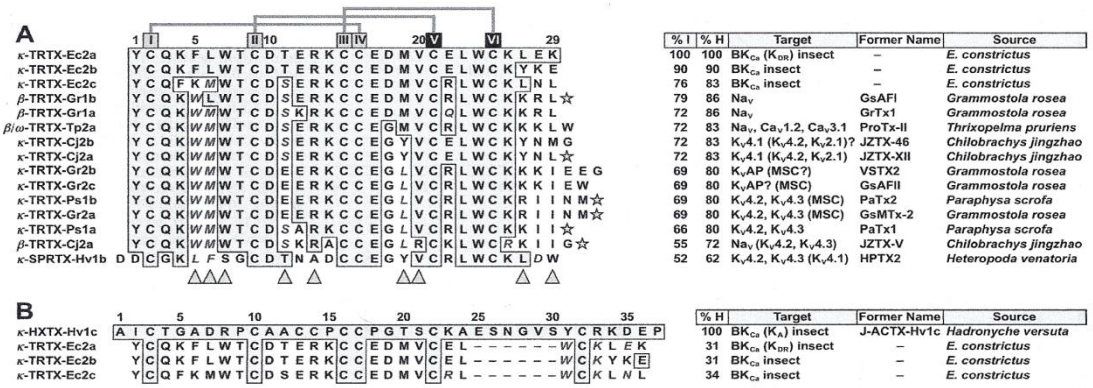

Figure 8.1: Homology of κ-TRTX-Ec2 toxins with short-loop ICK spider toxins. (A) Blast search through ArachnoServer 2.0 Database highlighting peptides with highly homologous sequences. All sequences belong to the short-loop ICK group of tarantula toxins defined by three residues in the loop between C_V and C_{VI} cysteine residues (Escoubas and Rash, 2004). Homologies are shown relative to κ-TRTX-Ec2a: identities are boxed in grey, whereas conservative substitutions are in grey italic text. Stars represent C-terminal amidation. Percentage identity (%I) is relative to κ-TRTX-Ec2a, whereas percentage similarity (%H) includes conservatively substituted residues. The disulfide bonding pattern for the strictly conserved cysteine residues determined for κ-TRTX-Gr2a (Oswald, Suchyna et al., 2002) and κ-TRTX-Ps2a (Diochot, Drici et al., 1999) is indicated above the sequences; it is assumed that κ-TRTX-Ec2 toxins share the

same bonding pattern. The known mammalian targets (unless otherwise indicated) of these toxins are identified on the right, with channels identified in brackets indicating only weak affinity. Those targets annotated with a question mark indicate a likely target based on considerable homology with a toxin of known pharmacology (e.g., κ-TRTX-Gr2a and κ-TRTX-Gr2c). It is noteworthy that κ-TRTX-Ps2b and κ-TRTX-Gr2a possess identical sequences, although they originate from unrelated species. Sequence, posttranslational modifications, disulfide connectivity, and pharmacological data were obtained from the ArachnoServer 2.0 Database. **(B)** Despite sharing a similar phyla and target selectivity, κ-TRTX-Ec2 toxins show only limited homology to κ-HXTX-Hv1c (Gunning, Maggio et al., 2008). Dashes have been inserted to maximise alignment.

8.2.1 LIMITATIONS AND FUTURE DIRECTIONS

The κ-TRTX-Ec2 toxins are important tools in the characterisation of the insect BK_{Ca} channel and in particular they offer the opportunity to hypothesise as to the molecular determinants for insecticidal and target selectivity. As a result, the mutagenesis of selected residues should be used to determine the pharmacophore and particularly the residues important for both the insecticidal and BK_{Ca} channel affinity of these toxins. Importantly, the κ-TRTX-Ec2 toxins represent valuable lead compounds in the development of bioinsecticides and furthermore in the development of phyla selective peptidomimetic insecticidal compounds.

Unfortunately, further characterisation of these toxins has been limited due to the inability to produce sufficient amounts of toxin via synthetic means. In order to obtain sufficient amounts of material for structural data and pursue peptide mutagenesis studies, synthetic production of κ-TRTX-Ec2 is necessary. Regrettably, while reduced linear forms of κ-TRTX-Ec2 toxins were produced via solid phase peptide-synthesis the peptides failed to fold correctly under a variety of different conditions (P. Escoubas and G. Nicholson, pers. comm.). Consequently, further characterisation of the κ-TRTX-Ec2 toxins awaits identification of the appropriate refolding conditions.

8.3 CHARACTERISATION OF κ-HXTX-HV1C

Chapters 4 to 7 detail the characterisation of the excitatory neurotoxin κ-HXTX-Hv1c isolated from the venom of the Blue Mountains funnel-web spider, *Hadronyche versuta*.

κ-HXTX-Hv1c induces excitatory and lethal neurotoxicity in a range of agronomically and medically relevant insects but displays no toxicity towards vertebrates (Wang, Connor et al., 2000; Maggio and King, 2002a; Maggio and King, 2002b; Tedford, Maggio et al., 2007). Studies have shown that κ-HXTX-Hv1c blocks native and expressed cockroach large conductance potassium (BK_{Ca}) channels while having no effect on sodium (Na_V) and calcium voltage-gated (Ca_V) channels (Gunning, Maggio et al., 2008).

In Chapter 5 we examined the effects of the BK_{Ca} channel blocker κ-HXTX-Hv1c on spike activity and action potential generation within the insect nervous system. 100 nM κ-HXTX-Hv1c was found to moderately slow repolarisation by 34% (as measured by an increase in $duration_{20}$) and reduce afterhyperpolarisation (AHP) of evoked action potentials (AP) in cockroach DUM neurons. At 3 nM κ-HXTX-Hv1c the effects on the AHP were substantially reduced while the effects on AP repolarisation were only moderately attenuated. Furthermore, 3 and 100 nM κ-HXTX-Hv1c induced a modest increase in the spontaneous generation of APs in cockroach DUM neurons. At saturating concentrations of the archetypal BK_{Ca} channel blocker iberiotoxin a minor slowing AP repolarisation, a reduction in the AHP and a 10% increase in spontaneous firing frequency were evident. These minor effects were not consistent with the effects seen in the presence of saturating concentrations of κ-HXTX-Hv1c; subsequently another channel target was likely to contribute to neurotoxicity κ-HXTX-Hv1c in cockroach DUM neurons.

Additionally, we characterised the effects of hybrid-HXTX-Hv1c which shares homology with both κ-HXTX-Hv1c and ω-HXTX-Hv1c; an insecticidal toxin from the same venom targeting Ca_V channels (Wang, Smith et al., 1999; Chong, Hayes et al., 2007). Furthermore, hybrid-HXTX-Hv1c contains elements of the pharmacophores of both toxins and targets both Ca_V (Suping Wen and Graham Nicholson, unpublished data) and BK_{Ca} (Simon Gunning and Graham Nicholson, unpublished data) channels in insects. At concentrations of hybrid-HXTX-Hv1c at which K_{Ca} channel block was evident we found that the effects on AP generation were closely mimicked by κ-HXTX-Hv1c. The changes included a moderate decrease in AHP amplitude, a substantial slowing of AP repolarisation and a 58% increase in spontaneous firing frequency. At concentrations of toxin at which Ca_V channel block was evident not only were the

effects on the AHP and repolarisation of the AP maintained but additional effects on membrane potential and AP amplitude observed. In the presence of combined Ca_V and K_{Ca} channel block by hybrid-HXTX-Hv1c, the resting membrane potential was depolarised by up to 20 mV and the AP amplitude was significantly reduced. Furthermore, the spontaneous AP firing frequency was substantially reduced which seems correlate with the paralytic effects of Ca_V channel block in insects (Wang, Smith et al., 1999; Chong, Hayes et al., 2007). Evidently the synergistic effects of BK_{Ca} block (Shao, Halvorsrud et al., 1999; Zhang, Gopalakrishnan et al., 2003; Gunning, Maggio et al., 2008) and Ca_V block (Chong, Hayes et al., 2007) on AP generation correlate well with the initial excitatory and consequent depressant phenotypic seen in insect bioassay experiments (Suping Wen and Graham Nicholson, unpublished data).

As high concentrations of κ-HXTX-Hv1c have a moderate effect on K_A channel currents (Gunning, Maggio et al., 2008), the effects of the classical $I_{K(A)}$ blocker 4-aminopyridine (4-AP) on DUM neuron APs were investigated. A saturating concentration of 4-AP was found to slow AP repolarisation and decrease the AHP to a similar extent as κ-HXTX-Hv1c. On the other hand 4-AP caused a substantial 200% increase in spontaneous firing frequency. These results would tend to suggest that the moderate block of the $I_{K(A)}$ may contribute to the neurotoxic effects of κ-HXTX-Hv1c, as block of the BK_{Ca} channel alone does not appear to be sufficient. Furthermore, the effects of $I_{K(A)}$ block on AP repolarisation and AHP seem to be comparable with the effects of κ-HXTX-Hv1c.

In Chapter 6 the neurotoxic effects of K_{Ca} channel block was assessed in acute toxicity assays. Acute toxicity testing in crickets revealed that several toxins which selectively target insect BK_{Ca} channels were not lethal. Paxilline, iberiotoxin and charybdotoxin all failed to induce the neurotoxic symptoms that were evident following exposure to κ-HXTX-Hv1c. These results, in addition to the effects on APs, led to the conclusion that while the BK_{Ca} channel is targeted with high affinity by κ-HXTX-Hv1c, it is not the *lethal* target. As a consequence it was necessary to consider additional molecular targets that may be important for toxin lethality.

Due to the similar effects of $I_{K(A)}$ blockers and κ-HXTX-Hv1c on AP repolarisation and AHPs it was decided to further explore the moderate block of K_A channel currents by κ-

HXTX-Hv1c (Gunning, Maggio et al., 2008). Subsequent testing of cockroach K_V channels mediating A-type fast transient currents revealed that κ-HXTX-Hv1c was responsible for a partial block of the channel current with an IC_{50} of 36 nM, but that it failed to inhibit delayed rectifier currents. κ-HXTX-Hv1c was found to induce a maximal block of only 29% of the global A-type fast transient current and revealed that more than one channel subtype contributes to such currents in cockroaches. Furthermore, co-application experiments revealed that the mammalian K_V4 channel blocker κ-sparatoxin-Hv1b (formerly heteropodatoxin-2) (Sanguinetti, Johnson et al., 1997; Zarayskiy, Balasubramanian et al., 2005) does not cause further block of cockroach $I_{K(A)}$ following κ-HXTX-Hv1c block and vice versa. These results suggest that κ-HXTX-Hv1c blocks the same portion of the cockroach global K_A current as κ-sparatoxin-Hv1b. We therefore propose that κ-HXTX-Hv1c blocks a K_V4-like channel in insects.

In support of the hypothesis that the lethal target of κ-HXTX-Hv1c is an A-type fast transient K_V4-like channel in insects, the non-selective A-type fast transient current (K_V1 and Kv4) blocker 4-aminopyridine (Stocker, Stuhmer et al., 1990; Wei, Covarrubias et al., 1990; Covarrubias, Wei et al., 1991; Gasque, Labarca et al., 2005; Peng and Wu, 2007) was found to be lethal to insects. However, we found that κ-sparatoxin-Hv1b was not lethal when injected into house crickets (*Acheta domestica*). Consequently, it is likely that the non-selective activity of 4-AP on the A-type channel current and potentially other targets, including BK_{Ca} and K_{Na} channels (Stocker, Stuhmer et al., 1990; Wei, Covarrubias et al., 1990; Covarrubias, Wei et al., 1991; Gasque, Labarca et al., 2005; Peng and Wu, 2007) may be responsible for the substantial increase in spontaneous firing frequency and insecticidal effects of 4-AP. Furthermore, a combination of κ-sparatoxin-Hv1b and iberiotoxin also was not lethal in acute toxicity assays.

Given the lack of any overt lethal toxicity mediated by classical voltage-gated ion channels, this thesis assessed the effects of κ-HXTX-Hv1c on several neurotransmitter-gated ion channels of particular importance within the insect nervous system. Accordingly, the effects of κ-HXTX-Hv1c were investigated on chloride-gated $GABA_A$ (GABA-Cl) and glutamate (Glu-Cl) channel currents and nAChR channel currents from cockroach DUM neurons (Raymond-Delpech, Matsuda et al., 2005). To our knowledge

this is the first comprehensive investigation of any spider peptide toxin on such channels. Concentrations of up to 1μM κ-HXTX-Hv1c failed to modify $GABA_A$ channel currents while causing only a moderate 21% increase in Glu-Cl channel currents. However, an increase in the activity of an inhibitory neurotransmitter-gated receptor such as glutamate would not be responsible for the excitatory neurotoxic effects observed in insects (Cleland, 1996; de Figueiredo, de Lima et al., 2001).

In contrast to the modest effects on glutamate receptors we found that κ-HXTX-Hv1c produced a concentration-dependent slowing of nicotine-evoked nACh receptor current decay with an EC_{50} of 185 nM prolonging current decay 6-fold at a concentration of 500 nM. In addition, κ-HXTX-Hv1c was found to not only cause a slight enhancement in receptor sensitivity to nicotine but was also capable of reversing channel desensitisation by ~34% at 500 nM. These findings are consistent with the action of a positive allosteric modulator of nAChR activity (Gronlien, Hakerud et al., 2007; Bertrand, Bertrand et al., 2008; Barron, McLaughlin et al., 2009; Arias, Gu et al., 2011).

It is highly likely that the pharmacophore of κ-HXTX-Hv1c on BK_{Ca} channels is similar or even identical to the pharmacophore for the modulation of the insect nAChR. As identified by Gunning and colleagues (Gunning, Maggio et al., 2008), the pharmacophore for insect BK_{Ca} inhibition and the insecticidal activity of κ-HXTX-Hv1c are very closely correlated. The functionally critical residues for toxin binding were previously identified by alanine-scanning mutagenesis. The residues critical for insecticidal activity of the toxin against house flies (*Musca domestica*) were identified as Arg8, Pro9 and Tyr31 and the VDR (Cys13-Cys14) (Maggio and King, 2002a; Maggio and King, 2002b). Residues Ile2 and Val29 were also proposed to be important, acting as gasket residues to exclude solvent from the putative toxin binding site (Maggio and King, 2002a; Maggio and King, 2002b). Importantly, these residues were found to be similarly important to the inhibition of the insect BK_{Ca} channel (Gunning, Maggio et al., 2008). Furthermore, as the insecticidal target of κ-HXTX-Hv1c is believed to be the nAChR it follows that the pharmacophore is likely to be the same as for toxin activity against the insect BK_{Ca} channel.

Importantly, the insect nAChR is already recognised as an important insecticidal target (Millar and Denholm, 2007). A number of commercially available insecticides target the insect nAChR, including those that enhance (imidacloprid) (Bai, Lummis et al.,

1991), inhibit (cartap) (Lee, Tomizawa et al., 2003) and allosterically modulate (spinosad) (Salgado and Saar, 2004) receptor function. In support of the positive allosteric modulation of nAChR and its ability to induce lethal actions in insects, κ-HXTX-Hv1c appears to have a mode of action similar to the excitatory insecticide spinosyn A (Salgado, 1998; Salgado, Sheets et al., 1998; Salgado and Saar, 2004; Watson, Chouinard et al., 2010). Spinosyn A is the major active component of the commercial insecticide spinosad which has been characterised to allosterically modify nAChR function (Salgado and Saar, 2004). Moreover, studies have indicated that spinosyn A interacts with a site on insect nAChR which is distinct from other insecticides (Salgado and Saar, 2004). While it should also be taken into consideration that spinosad additionally enhances GABA receptor activity in some insect species, the effect on nAChRs alone is considered to be sufficient to induce excitatory insecticidal activity (Salgado and Sparks, 2005; Orr, Shaffner et al., 2009). Subsequently, we believe that positive allosteric modulation of the nAChR has the ability to induce the excitatory insecticidal actions provoked by κ-HXTX-Hv1c exposure.

Due to significant effect of κ-HXTX-Hv1c on the action potential and firing frequency in the cockroach neurons we believe that while block of BK_{Ca} and K_V4-like channels are not lethal, they may play a supporting role in the development of neurotoxicity. The increase in firing frequency and changes in AP generation induced by BK_{Ca} and K_V4-like channel block may well play a synergistic role with the positive allosteric modulation of nAChR by increasing neurotransmitter release to further enhance the excitotoxicity in the insect nervous system. However, as the effects of BK_{Ca} and K_V4-like channel block are not lethal it is unlikely that the effects on these channels directly result in the allosteric modulation of nAChRs. Furthermore, toxins such as the tremorgenic indole diterpine Aflatrem have also been shown to have dual, unrelated activities against both BK_{Ca} channels and transmitter-gated channels (Knaus, 1994; Yao, 1989).

8.3.1 IMPROVING STRUCTURAL STABILITY

The susceptibility of peptides to enzymatic degradation and ensuing poor stability is a challenge that has somewhat limited the development of peptides for use in insecticide and pharmaceutical drug design. Subsequently, Chapter 4 investigated two methods aimed at improving the stability of peptide spider toxins.

Due to the oral toxicity demonstrated by ω-HXTX-Hv1a against insects (Fletcher, Smith et al., 1997; Mukherjee, Sollod et al., 2006), the ability of peptide cyclisation as a means of improving stability was investigated. It was found that the cyclisation of ω-HXTX-Hv1a did not alter toxin affinity as evidenced by the comparable effects of native and cyclic ω-HXTX-Hv1a on insect Ca_V channels. Improved neurotoxic activity was found when injected into blowflies, however cyclisation reduced oral activity significantly (V. Herzig, *et al.*, unpublished data). The results of a midgut permeation assay using cyclic ω-HXTX-Hv1a revealed a significantly reduced permeation rate as compared to the native toxin (V. Herzig, *et al.*, unpublished data). Subsequently, the slowed permeation through the gut membrane may explain the reduced oral activity as the peptide would be exposed to enzymes within the insect mid-gut for longer periods of time and a reduced amount would reach the target in the insect CNS. Importantly, the maintained affinity for the Ca_V channel and improved neurotoxicity via injection suggests that cyclisation is a valid means of improving the stability of peptide toxins; however an alternative means to improve insect gut permeability seems necessary.

The second aim of Chapter 4 was to look at diselenide replacement of the disulfide bond as a method to improve toxin stability, particularly the Cys13-Cys14 vicinal disulfide bond found in κ-HXTX-Hv1c. The vicinal disulfide ring (VDR) structure is critical to toxin function (Wang, Connor et al., 2000) and subsequently it was hypothesised to act as a redox-activated switch or a special binding recognition site (Wang, Connor et al., 2000). The native κ-HXTX-Hv1c VDR (S-S) was replaced by the more stable diselenide linkage (Se-Se), a modification designed to minimise possible redox reactions while maintaining the ring scaffold of the structure. In order to test the hypothesis, the activity of κ-HXTX-Hv1c against insect BK_{Ca} channels was determined. It was determined that the diselenide replacement did not significantly alter the effect of κ-HXTX-Hv1c on insect BK_{Ca} channels. Furthermore, it was revealed that the replacement of the native VDR (S-S) with a non-reducible dicarba linkage (C-C) did not alter the activity of κ-HXTX-Hv1c against insect BK_{Ca} channels (A. Dantas de Araujo, V. Herzig, M. J. Windley, M. Dziemborowicz S., Mobli, G.M. Nicholson, P. F. Alewood and G. F. King, unpublished data). These results indicate that even when the VDR is modified to incorporate highly stable linkages the activity of κ-HXTX-Hv1c remains unchanged. Subsequently, the critical VDR within κ-HXTX-Hv1c is unlikely to act as a redox activated switch and is more likely to function as a unique binding

structure. The VDR of κ-HXTX-Hv1c is a strong and specific binding site and highlights this toxin as a particularly appealing lead in the design of non-peptide mimetic, especially since the VDR is believed to be implicated in the most important target:toxin interaction (Wang, Connor et al., 2000; Gunning, Maggio et al., 2008). Importantly, this study also validates diselenide replacement as a valid means of improving toxin stability without impeding biological activity.

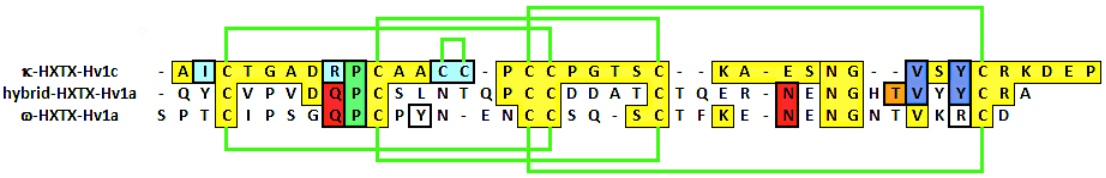

Figure 8.2: Comparison of mature toxin sequences of κ-HXTX-Hv1c, ω-HXTX-Hv1c and hybrid-HXTX-Hv1a. Homologies are shown relative to hybrid-HXTX-Hv1a; identities are boxed in yellow. The experimentally determined disulfide bonding pattern for κ-HXTX-Hv1c (Wang, Connor et al., 2000) and ω-HXTX-Hv1c (Wang, Smith et al., 1999) are represented in green above and below the alignment, respectively. Red shading shows the pharmacophore residues of ω-HXTX-Hv1c (Tedford, Gilles et al., 2004) that are also found in hybrid-HXTX-Hv1a, while the pink shading indicates residues that are unique to the ω-HXTX-Hv1c pharmacophore. Dark blue shading highlights the key functional residues that are common to κ-HXTX-Hv1c (Maggio and King, 2002b) and hybrid-HXTX-Hv1a. Additional key residues that are part of the κ-HXTX-Hv1c pharmacophore are indicated by pale blue shading. Residues highlighted in green are part of the pharmacophore for all three toxins. Residues highlighted in orange are part of the pharmacophore of hybrid-HXTX-Hv1a only. Dashes have been inserted to maximise alignment.

8.3.2 Limitations and future directions

It is recognised that κ-HXTX-Hv1c is not orally or contact active to insects (Mukherjee, Sollod et al., 2006) and therefore cannot be directly delivered as a foliar spray or expressed in transgenically modified foliage. Subsequently, the development of κ-HXTX-Hv1c as a bioinsecticide should focus on recombinant baculovirus expression (Prikhod'ko, Robson et al., 1996; Hughes, Wood et al., 1997; Thiem, 1997; Prikhod'ko, Popham et al., 1998; Elazar, Levi et al., 2001; Kamita, Kang et al., 2005; Wang and St

Leger, 2007) or the design of non-peptide mimetics (Menzler, Bikker et al., 2000; Baell, Duggan et al., 2006). While the development of non-peptide structures has seen only limited success (Zlotkin, Fishman et al., 2000), the tightly constrained pharmacophore (Maggio and King, 2002b; Gunning, Maggio et al., 2008) of κ-HXTX-Hv1c and functional importance of the unique VDR in κ-HXTX-Hv1c recommends them as particularly strong candidates.

Subsequently, future directions in the study of κ-HXTX-Hv1c should involve the identification of the nAChR channel subtype targeted by κ-HXTX-Hv1c. This will help to determine whether κ-HXTX-Hv1c targets a unique nAChR to other insecticides. Following the identification of the subtype targeted by κ-HXTX-Hv1c the site of action on the channel should be determined. The identification of the site of action would be achieved by assessing toxin displacement by other ligands targeting nAChRs, particularly spinosyn A, in the form of radioligand binding assays (Orr, Shaffner et al., 2009) or co-application assays using patch clamp analysis.

Future projects should also address areas such as improving peptide toxin stability and screening for other potential insecticidal compounds in spider venom. Firstly, cyclisation and diselenide replacement appear to be valid methods of improving toxin stability, further studies should be pursued to improve and implement these techniques in the promotion of spider peptides as viable bioinsecticides. Secondly, the identification of the insect nAChR as a viable and lethal target of κ-HXTX-Hv1c means that this channel target could be used as a high throughput screening target for the identification of other insecticidal toxins within spider venom. With this goal in mind it is important to identify the nAChR channel subtype targeted and achieve functional channel expression. Unfortunately, while successful cloning of channel subunits has been achieved very few have been successfully expressed, making this approach very difficult at present (Eastham, Lind et al., 1998; Hermsen, Stetzer et al., 1998; Millar, 1999; Gao, Deacutis et al., 2007; Millar and Lansdell, 2010), although potentially *Drosophila* insect cell lines offer a potential avenue of investigation.

In conclusion, very few spider peptide toxins have undergone such rigorous characterisation as κ-HXTX-Hv1c. In fact no other spider toxins have been tested on such a wide range of both voltage- and neurotransmitter-gated ion channels in insects. The extensive characterisation of κ-HXTX-Hv1c highlights the hazard of identifying a

high affinity target as the lethal insecticidal site of action without comprehensive characterisation, particularly when trying to identify novel insecticidal targets.

Additionally, κ-HXTX-Hv1c is the first spider peptide toxin characterised to target the insect nAChR (Herzig, Wood et al., 2011). Although insect nAChRs are not new targets for insecticides (Millar and Denholm, 2007) κ-HXTX-Hv1c exhibits a novel mode of action as a positive allosteric modulator and therefore is likely to interact with a unique site on the channel receptor. Moreover, given the novel mode of action, κ-HXTX-Hv1c may be important for reducing insect resistance to other nAChR agrochemicals that partially activate nAChRs such as imidacloprid (Buckingham, Lapied et al., 1997). For instance, studies have shown that pyrethroid-resistant strains were more susceptible to insecticides that bound to the channel at different locations than pyrethroids (McCutchen, Hoover et al., 1997). This suggests that if κ-HXTX-Hv1c targets the same nAChR as commercially available insecticides it may be a useful tool in the control of insecticide resistance.

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