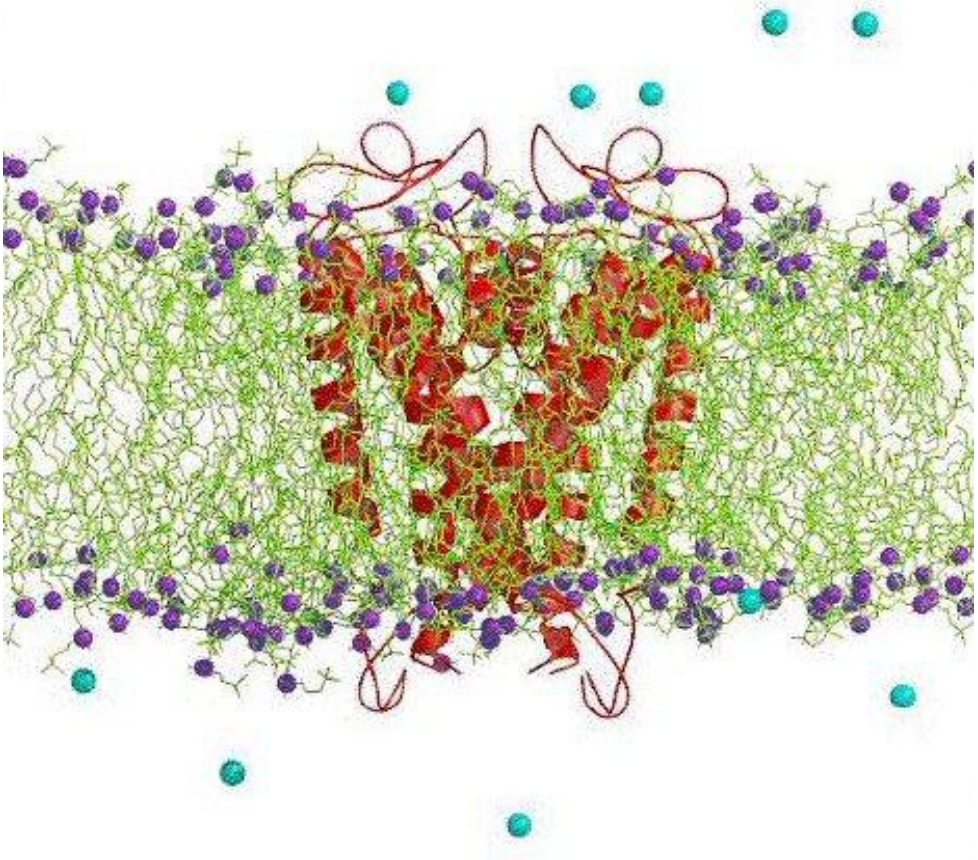
STRUCTURE-FUNCTION STUDIES OF INSECTICIDAL ATRACOTOXINS



PhD Thesis

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ABSTRACT

Part I

The κ-atracotoxins (κ-ACTXs, previously the Janus-faced atracotoxins) are a family of five insect-selective excitatory peptide neurotoxins containing 36-37 residues with four disulfide bonds. Toxins from this family were isolated from the venom of the Blue Mountains funnel-web spider (*Hadronyche versuta*) and Toowooba funnel-web spider (*Hadronyche infensa*). The NMR solution structure and primary sequence of the prototypic member κ-ATCT-Hv1c provided few clues as to the likely molecular target. In order to characterise the site of action and phylogenetic specificity of these toxins, whole-cell patch-clamp electrophysiology was employed using isolated DUM neurons from the American cockroach (*Periplaneta americana*). κ-ACTX-Hv1c had no effect on the gating or kinetics of I_{Na} or I_{Ca} at concentrations up to 1 μM. However, at the same concentration, κ-ATCT-Hv1c reduced K_v channel currents by 56 ± 7% (n = 5). Subsequent experiments in insect DUM neurons indicated that inhibition of the macroscopic I_K was due to a block of calcium-activated K_v (K_{Ca}) channels, with an IC_{50} of 2.3 nM and 2.9 nM for peak and late $I_{K(Ca)}$ respectively (n = 5), and not 'A-type' or delayed-rectifier K_v channels. Insect selectivity was confirmed by a lack of activity on rat dorsal root ganglion (DRG) neuron global I_K as well as $I_{K(Ca)}$ at doses up to 1 μM. κ-ACTX-Hv1c is a selective insect K_{Ca} (BK_{Ca}) channel pore-blocker, not a gating modifier, as inhibition of insect $I_{K(Ca)}$ occurred in the absence of any voltage-dependent actions on channel activation. Specificity for the insect BK_{Ca} channel was validated by κ-ACTX-Hv1c induced inhibition of $I_{K(Ca)}$ from the cloned insect K_{Ca} channel α-subunit (*pSlo*) expressed in HEK293 cells (IC_{50} of 240 nM). The 80-fold reduction in IC_{50}, most likely indicates that κ-ACTX-Hv1c interacts with the auxillary subunits that form part of the wild-type channel, in a manner similar to the BK_{Ca} blocker, charybdotoxin (ChTX), as previously reported. Phyletic selectivity of κ-ACTX-Hv1c was confirmed by the 9776-fold

increase in IC_{50} against *mSlo* channels. Interestingly κ-ACTX-Hv1c, like ChTX, failed to potently block the *dSlo* channel with the IC_{50} >10 μM.

Additional experiments on DUM neuron $I_{K(Ca)}$ using alanine mutants confirmed the pharmacophore of bioactive residues κ-ACTX-Hv1c comprises Arg^8, Pro^9, Val^{29} and Tyr^{31}, previously identified by acute toxicity tests in house flies (*Musca domestica*). Interestingly, the functionally critical Arg^8 and Tyr^{31} residues align extremelyç well with the Lys-Phe/Tyr diad conserved amongst structurally dissimilar K_v channel toxins, providing a possible basis for targeting of the toxin to K^+ channels. Using a panel of 8 mutants (R8E, R8Q, R8K, R8H, Y31W, Y31F, Y31L and Y31V) the mechanism of interaction was investigated further. The Arg^8 residue appears to interact with the channel via hydrogen bonding from the δ-guanido group to carbonyl groups on the extracellular surface of the channel, as evidenced by the high potency of the R8H mutant. The imidazole group of His is an adequate substitute for the δ-guanido group of arginine. In contrast the R8E, R8Q and R8K had reduced potency indicating that the positive charge of the amino group of Arg does not directly interact with the target nor is the alkyl group of Arg critical for binding to the target. The critically important Tyr^{31} interacts with the channel via non-specific hydrophobic interactions as substitution for an aromatic ring (Y31F & Y31W) maintains the potency of the toxin. In contrast substitution to small less hydrophobic side chains (Y31V, Y31L and Y31A) reduced potency. It appears therefore that Tyr^{31} in conjunction with Ile^2 and Val^{29}, that lie at either side of the primary pharmacophore, appear to act as ‘gasket’ residues to exclude bulk solvent from disrupting the Arg^8-channel interaction.

This study has identified κ-atracotoxins as potential lead compounds in the development of new biopesticides and validates insect BK_{Ca} channels as potential insecticide targets.

Part II

The second part of this thesis was to determine the target site for the 'hybrid' toxin FW178 from the venom of the Blue Mountains funnel-web spider (*H. versuta*). FW178 is a unique toxin that shares little homology to other known atracotoxins. In order to identify the site of action of this toxin, whole-cell patch-clamp electrophysiology was employed using isolated DUM neurons from the American cockroach (*Periplaneta americana*). FW178 failed to inhibit insect I_{Na} , $I_{K(DR)}$ or $I_{K(A)}$ at doses up to 1 μM. However, further studies demonstrated that η-ACTX-Hv1a blocks voltage-gated calcium (Ca_V) channel currents in DUM neurons as well as $K_{(Ca)}$ channel currents carried by *pSlo* channels with IC_{50} values of 409 nM and 671 nM, respectively. FW178 therefore blocks cockroach Ca_V currents with approximately the same potency as ω-ACTX-Hv1a, a known insect M-LVA and HVA Ca_V channel blocker, while it blocks cockroach *pSlo* channels with about a 4-fold lower potency than κ-ACTX-Hv1c.

Interestingly FW178 has an LD_{50} of 38 ± 3 pmol/g when injected into *M. domestica* as compared to the LD_{50} values for ω-ACTX-Hv1a (86.5 ± 1.3 pmol/g) and κ-ACTX-Hv1c (91 ± 5 pmol/g). This makes FW178 at least two-fold more potent than any other atracotoxin isolated from Australian funnel-web spiders. Despite this, FW178 only blocks cockroach Ca_V channels with a similar potency to ω-ACTX-Hv1a, and blocks cockroach $BK_{(Ca)}$ with 4-fold less potency than κ-ACTX-Hv1c. Therefore the striking potency of FW178 may result from a synergistic action to block insect Ca_V and $BK_{(Ca)}$ channels. Not surprising the pharmacophore of FW178 (refer section **4**.3) contains elements of the pharmacophore of both ω-ACTX-Hv1a and κ-ACTX-Hv1c. Thus FW178 directly block insect $K_{(Ca)}$ channels, but the toxin also enhances this action by indirectly reducing current through these channels by block of the transient inward flow of calcium through Ca_V channels. Therefore FW178 represents the first known dual-target, self-synergizing toxin and is an excellent lead compound for the development of a novel insecticide.

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LIST OF ABBREVIATIONS

ACh	Acetylcholine
ACTX	Atracotoxin
AP	Action Potential
ATP	Adenosine tri-phosphate
α-BGT	α-Bungarotoxin
α-LTx	α-Latrotoxin
α-ScTX	α-Scorpion toxin
4-AP	4-amino pyridine
BAPTA	1,2-bis-(2-aminophenoxy)ethane-*N*,*N*,*N*,*N*-tetra acetate
BK_{Ca}	Large-conductance Ca^{2+}-activated K^+ channels
BTX	Batrachotoxin
β-Sctx	β-Scorpion toxin

CAMs	Cell adhesion molecules
CF-NIS	Ca^{2+}-free normal insect saline
CICR	Ca^{2+}-induced Ca^{2+}-release
ChTX	Charybdotoxin
CMF-PBS	Ca^{2+}-and Mg^{2+}-free phosphate buffered saline
DHP	Dihydropyridine
DRG	Dorsal Root Ganglion
DUM	Dorsal Unpaired Median
ET_{50}	Time to 50% paralysis/death
GABA	γ-aminobutyric acid
GSH	Glutathione
GST	Glutathione-S-transferase
HaTX-1	Hanatoxin-1
HEK293	Human embryonic kidney 293 cells
HpTX	Heteropodatoxin
HoTx	Hololenatoxin
IBX	Iberiotoxin
ICK	Inhibitory cystine knot
IFM	Hydrophobic triad of isoleucine, phenylalanine and methionine
Ig	Immunoglobulin
i.p	Intraperitoneal
$I_{Ca(tLVA)}$	Transient low voltage-activated calcium current
$I_{Ca(mLVA)}$	Maintained low voltage-activated calcium current
$I_{Ca(HVA)}$	High voltage-activated calcium current
$I_{Ca(r)}$	Calcium resting current
$I_{Cl(Ca)}$	Calcium-activated chloride current
$I_{K(A)}$	‘A-like’ potassium current
$I_{K(tCa)}$	Transient calcium-activated potassium current
$I_{K(mCa)}$	Maintained (or Late) calcium-activated potassium current
$I_{K(DR)}$	Delayed rectifier potassium current
$I_{K(IR)}$	Inward rectifier potassium current

$I_{K(r)}$	Potassium resting current
$I_{K(t)}$	Total (macroscopic) potassium current
$I_{K(Na)}$	Sodium-activated potassium current
I_{Na}	Sodium current
$I_{(mNa)}$	Maintained sodium current
$I_{(rNa)}$	Sodium resting (or background) current
LA	Local Anesthetics
MSG	Mushroom shaped accessory gland
NIS	Normal insect saline
NMR	Nuclear magnetic resonance
NPPB	5-nitro-2-(3-phenylpropylamino) benzoic acid
NSW	New South Wales
NVPs	Nucleopolyhedroviruses
PLTX	*Plectreurys* spider toxin
rp-HPLC	Reversed phase high performance liquid chromatography
SITS	4-acetamido-4'-isothiocyanostilbene-2,2'-disulfonic acid
SK_{Ca}	Small-conductance Ca^{2+}-activated K^+ channels
dSlo	*Drosophila* Slo-poke potassium channel
mSlo	Murine Slo-poke potassium channel
pSlo	*Periplaneta* Slo-poke potassium channel
STX	Saxitoxin
TAG	Terminal abdominal ganglion
TEA-Cl	Tetra ethyl ammonium chloride
TTX	Tetrodotoxin
VGCC	Voltage-gated calcium channels
VGKC	Voltage-gated potassium channels
VGSC	Voltage-gated sodium channels
VUM	Ventral Unpaired Median

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