CHARACTERISATION OF NOVEL INSECTICIDAL ION CHANNEL TOXINS FROM ARANEOMORPH AND MYGALOMORPH SPIDER VENOMS

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ABSTRACT

The high potency and selectivity of various peptide neurotoxins within spider venoms means these toxins are being considered as leads for the development of new environmentally-benign biopesticides that target pest insects. Currently, the ω-HXTX family of 37-residue arthropod-selective peptide neurotoxins from Australian hexathelid spider venoms are considered a prime candidate for biopesticide development. ω-HXTX-Hv1a, a prototypic member of the ω-HXTX-1 family, was electrophysiologically characterised by voltage-clamp analysis using the whole-cell patch-clamp technique on cockroach dorsal unpaired median (DUM) neurons. ω-HXTX-Hv1a exerted a reversible, concentration-dependent, voltage-independent block of barium currents (I_{Ba}) through mid-low voltage-activated (M-LVA) and high voltage-activated (HVA) voltage-activated calcium (Ca_v) channels without alteration in the activation or inactivation kinetics of the Ca_v channel. To improve the structural stability of this disulfide-rich peptide under biologically reducing conditions, and thereby increase its commercial viability, a synthetic ω-HXTX-Hv1a mutant was produced with the replacement of one disulfide bond with a $Sec^{1,4}$ diselenide bridge. The selenocysteine mutant had comparable oral activity to the native toxin in blowflies and there was no significant difference between the native and diselenide toxin in terms of block of M-LVA and HVA Ca_V channels. This demonstrated that selenocysteine substitution had the potential to improve peptide stability without altering the biological activity of the toxin.

There is a continuous need to identify novel insecticidal peptide toxins for biopesticide development. By screening the venom of mygalomorph Sydney funnel-web (*Atrax robustus*) and Eastern mouse (*Missulena bradleyi*) spiders; two novel insect-selective peptide neurotoxins were isolated: ω-HXTX-Ar1a from *A. robustus* is a homolog of ω-HXTX-Hv1a, and ω-AOTX-Mb1a from *M. bradleyi* has up to 59% homology with the ω-HXTX family. In acute toxicity tests in house crickets, these neurotoxins induced potent neuroexcitatory symptoms followed by paralysis and death. Vertebrate nerve-muscle preparations showed that the toxins lacked overt vertebrate toxicity at

concentrations up to 1 μM. To further characterise the molecular target of ω-HXTX-Ar1a and ω-AOTX-Mb1a on insects, whole-cell patch-clamp experiments were undertaken on cockroach DUM neurons. ω-HXTX-Ar1a induced a reversible, and the ω-AOTX-Mb1a an irreversible, block of both M-LVA and HVA Ca_v channels. The level of block was concentration-dependent and occurred in the absence of alterations in the voltage-dependence of Ca_v channel activation. The block was voltage-independent, suggesting that these toxins are Ca_v channel pore blockers rather than channel gating modifiers. Both ω-HXTX-Ar1a and ω-AOTX-Mb1a are promising biopesticide candidates and their activity on M-LVA and HVA Ca_v channels validates insect Ca_v channels as a novel molecular target for insecticides.

Apart from their insecticidal properties, spider venom can cause serious envenomation and death in vertebrates and invertebrates. Male *M. bradleyi* spiders are clinically important, but the toxin primarily responsible for the envenomation syndrome in humans has not previously been identified. By separating whole male *M. bradleyi* venom and testing for activity, a 42-residue peptide (δ-AOTX-Mb1a) was isolated. In a chick biventer cervicis nerve-muscle preparation, 85 nM concentration of δ-AOTX-Mb1a caused an increase in resting tension, muscle fasciculation and a decrease in indirect twitch tension. These effects were neutralised by *A. robustus* antivenom. The toxic effects were attributed to inhibition of peak tetrodotoxin-sensitive sodium current, a slowing of sodium current inactivation and a hyperpolarising shift in the voltage at half-maximal activation as determined by whole-cell patch-clamp analysis on rat dorsal root ganglion neurons. In acute insect toxicity bioassays, δ-AOTX-Mb1a displayed only moderate insecticidal activity in house crickets (*Acheta domesticus*), with doses up to 2 nmol/g causing reversible neurotoxic symptoms including involuntary spasms and slight loss of coordination within 24 hours. At this dose, lethality was only observed in 60% of crickets after 48 hours. δ-AOTX-Mb1a is highly toxic to vertebrates through its action on sodium channels, but has relatively low biological activity against invertebrates.

Spider peptide toxins that display non-selective toxicity toward both vertebrates and invertebrates can be used as molecular tools to probe the function of, and phylogenetic differences in, receptors and ion channels. Accordingly, ω-CNTX-Cs1a, a 74-residue peptide toxin from the venom of the Central American hunting spider (*Cupiennius salei*) that displays high toxicity in mammalian and insect bioassays, was investigated. Whole-cell patch-clamp experiments showed that ω-CNTX-Cs1a caused a voltage-independent block of mammalian L-type HVA Ca_v channels in rat neurons and neuroendocrine GH3 and GH4 cells, but had no significant effect on other types of HVA, or LVA Ca_v channels. In contrast, ω-CNTX-Cs1a induced a slow voltage-independent, concentration-dependent block of both M-LVA and HVA Ca_v channels in whole-cell patch-clamp experiments performed on cockroach DUM neurons. ω-CNTX-Cs1a shows high selectivity for a subset of mammalian Ca_v channels, but indiscriminate activity on invertebrate Ca_v channels, which makes this toxin useful as a molecular tool for further investigation of mammalian and insect Ca_v channels.

The incredible diversity in the phylogenetic selectivity and ion channel specificity of spider neurotoxins means there is great potential for these toxins as molecular tools, and many may become the defining pharmacology for receptor or ion channel subtypes. As a result of such investigations we now also understand the underlying basis for clinical envenomation syndromes that develop following envenoming, and many are being investigated as environmentally-friendly biopesticides and therapeutic drugs.

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$\tau_{on/off}$: time constants for onset of (on) and recovery from (off) current block
3D: three-dimensional
4-AP: 4-aminopyridine
4VP: 4-vinylpyridine
ACh: acetylcholine
AChE: acetylcholinesterase
ACN: acetonitrile
AOTX: actinopoditoxin
APAF: Australian Proteome Analysis Facility
Arg: arginine
Asn: asparagine
Asp: aspartic acid
ATP: adenosine triphosphate
BCA: bicinchoninic acid
BSA: bovine serum albumin
Bt: *Bacillus thuringiensis*
Ca_V: voltage-activated calcium
cDNA: complementary deoxyribonucleic acid
CMF-PBS: Calcium- and Magnesium-Free Phosphate-Buffered Saline
CNTX: ctenitoxin
Cys: cysteine
DDT: dichlorodiphenyltrichloroethane
DMEM: Dulbecco's Modified Eagle Medium
DNA: deoxyribonucleic acid
DRG: dorsal root ganglion
DTT: dithiothreitol
DUM: dorsal unpaired median
E. coli: *Escherichia coli*
EDTA: ethylenediaminetetraacetic acid
EGTA: ethylene glycol tetraacetic acid
ESI-MS: electrospray ionisation mass spectrometry
FPLC: fast-perfusion liquid chromatography
GABA: gamma-amino butyric acid
Gln: glutamine

Glu: glutamic acid

G_{max}: maximum conductance

GNA: *Galanthus nivalis* agglutinin

GSSG: glutathione disulfide

GST: glutathione S-transferase

HEK: human embryonic kidney

HEPES: 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid sodium salt

HF: hydrogen fluoride

HVA: high voltage-activated

HXTX : hexatoxin

I: current

I_{Ba}: barium currents

IC_{50}: half maximal inhibitory concentration

ICK: inhibitory cystine knot

KD_{50}: median knockdown dose

K_v: voltage-activated potassium

LD_{50}: median lethal dose

LJP: liquid junction potential

Lys: lysine

m/z: mass/charge

MALDI-TOF MS: matrix-assisted laser desorption/ionisation-time of flight mass spectrometry

MeOH: methanol

mLVA: maintained low voltage-activated

M-LVA: mid-low voltage-activated

Na_v: voltage-activated sodium

NIS: normal insect saline

POPs: persistent organic pollutants

Pro: proline

RNSH: Royal North Shore Hospital

Rp-HPLC: reverse phase high pressure liquid chromatography

S: slope factor

SAR: structure-activity relationship

Sec: selenocysteine

Ser: serine

TAG: terminal abdominal ganglion/ganglia

TEA-Br: tetraethylammonium bromide

TEA-Cl: tetraethylammonium chloride

TEA-OH: tetraethylammonium hydroxide

TFA: trifluoroacetic acid

Thr: threonine

tLVA: transient low voltage-activated

Tris: tris(hydroxymethyl)aminomethane

TTX: tetrodotoxin

Tyr: tyrosine

UNSW: University of New South Wales

USEPA: United States Environmental Protection Agency

UTS: University of Technology, Sydney

UV: ultraviolet

UV-VIS: ultraviolet-visible

V: voltage

$V_{1/2}$: voltage of half-maximal activation

V_h: membrane holding potential

V_{rev}: reversal voltage

WHO: World Health Organisation