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REVIEW

The insecticidal potential of venom peptides

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Abstract Pest insect species are a burden to humans as they destroy crops and serve as vectors for a wide range of diseases including malaria and dengue. Chemical insecticides are currently the dominant approach for combating these pests. However, the de-registration of key classes of chemical insecticides due to their perceived ecological and human health risks in combination with the development of insecticide resistance in many pest insect populations has created an urgent need for improved methods of insect pest control. The venoms of arthropod predators such as spiders and scorpions are a promising source of novel insecticidal peptides that often have different modes of action to extant chemical insecticides. These peptides have been optimized via a prey–predator arms race spanning hundreds of millions of years to target specific types of insect ion channels and receptors. Here we review the current literature on insecticidal venom peptides, with a particular focus on their structural and pharmacological diversity, and discuss their potential for deployment as insecticides.

Keywords Insecticidal toxins · Venom peptides · Toxin structural folds · Spider · Scorpion · Cone snail · Sea anemone · Snake · Voltage-gated ion channels

Abbreviations

3D	Three-dimensional
BK_{Ca}	Maxi-K calcium-activated potassium channel
Ca_V	Voltage-gated calcium channel
CSα/β	Cystine-stabilized α/β
DDH	Disulfide-directed β-hairpin
$DmNa_V$	*Drosophila* voltage-gated sodium channel
GABA	γ-aminobutyric acid
HVA	High-voltage activated
HXTX	Hexatoxin
ICK	Inhibitor cystine knot
K_V	Voltage-gated potassium channel
MATP	Myoactive tetradecapeptide
Na_V	Voltage-gated sodium channel
LVA	Low-voltage activated
MVA	Mid-voltage activated
nAChR	Nicotinic acetylcholine receptor
NMDA	*N*-methyl-D-aspartic acid
RyR	Ryanodine receptor
TRTX	Theraphotoxin
tLVA	Transient low-voltage activated
TTX-R	Tetrodotoxin-resistant
TTX-S	Tetrodotoxin-sensitive

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Introduction

Although only a small minority of insects are classified as pests, they nevertheless destroy 10–14 % of the world's food supply [1, 2] and transmit a diverse array of human and animal pathogens [3, 4]. Despite the introduction of transgenic crops and other biological control methods, chemical insecticides remain the dominant approach for combating insect pests. The major classes of chemical insecticides act on only six molecular targets in the insect nervous system, namely acetylcholinesterase, voltage-gated sodium (Na_V) channels, nicotinic acetylcholine receptors, GABA- and glutamate-gated chloride channels, and ryanodine receptors (RyRs) [3, 5]. With the exception of the latter, resistance to

these classes of insecticides has already developed in over 600 arthropod pest species [6]. Insecticides that target RyRs have only been on the market since 2008 and resistance has not yet emerged in the field. However, the appearance of insects resistant to RyR insecticides is inevitable, as suggested by a recent controlled study in a lab environment that found a 12-fold increase in the LC_{50} of the RyR insecticide chlorantraniliprole against lepidopteran pests after 22 generations under insecticidal selection pressure [5]. The increasing incidence of insecticide-resistant pest species, together with the limited range of molecular targets for extant insecticides, has created an urgent need for improved strategies for insect pest management [3, 7].

In addition, mounting evidence suggests that long-term exposure to certain insecticides may be detrimental to the health of humans and other vertebrates. Chronic exposure to organophosphates, which were widely used until recently, as well as pyrethroids have been linked to decreased male fertility and neurodevelopmental problems in children [8, 9]. These concerns have resulted in restrictions in use or de-registration of a number of insecticidal compounds. The US Environmental Protection Agency cancelled registrations for 169 insecticidal compounds during the 5-year period between January 2005 and December 2009, with only nine new insecticides registered during the same period [4]. With an increasing world population applying further pressure on the agricultural sector and the alarming decrease in commercially available insecticidal compounds, new ligands for existing molecular targets and the discovery of novel insect-specific targets are urgently required. There is no time to waste, as it can take 7–10 years to develop and register a new insecticide [10].

Fortunately, nature has provided us with a treasure trove of insecticidal toxins that have evolved within a diverse range of venomous animals, such as scorpions [11], spiders [12], centipedes [13], cone-snails [14], insects [15, 16], cnidarians (sea-anemone and jellyfish; [17, 18]), and snakes [19]. Due to millions of years of evolutionary fine-tuning, many of these insecticidal toxins exhibit remarkable selectivity and potency for their molecular targets. Since insecticidal toxins have arisen independently in various classes of venomous animals, there is a huge diversity of structural scaffolds for insecticidal toxins present in these organisms [20, 21]. In the venom of spiders alone, which are among the world's most successful insect predators, it is estimated that millions of insecticidal toxins are yet to be discovered [12]. Consequently, this review can only provide a glimpse into the huge diversity of structural scaffolds found in insecticidal toxins derived from venomous animals (Table 1). We have therefore focused this review on peptide-toxin scaffolds that have the potential to be developed into novel insecticides with better properties than our current armamentarium of chemical insecticides.

ICK fold

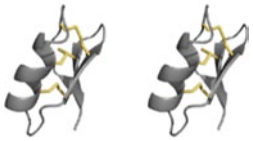
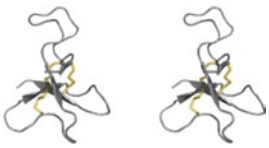
The inhibitor cystine knot motif is a structural scaffold composed of a ring formed by two disulfide bonds and the intervening peptide backbone, which is penetrated by a third disulfide to create a pseudo-knot [22] (Fig. 1a, b). This structural topology provides ICK-folded peptides with a high degree of stability and increased resistance to enzymatic degradation [23, 24], which are desirable features when engineering pesticides. Peptides from a wide range of evolutionarily unrelated organisms including plants, cone snails, and scorpions have been found to adopt the ICK motif [25]. Of all the organisms that utilize the ICK fold, spiders have indisputably produced the most numerous and chemically diverse array of ICK peptides [26, 27]. In spider venom peptides, the ICK configuration has been evolutionarily conserved as a framework on which a multitude of pharmacologically varied motifs have been grafted (Fig. 1c). As a result, the majority of spider-venom peptides contain an ICK-fold. Approximately 900 of the ~2,100 known ICK peptides are from spider venoms, of which about 116 are insect-selective [28]. Pharmacological targets have been identified for many of the insecticidal ICK toxins and include Na_v channels, voltage-gated calcium (Ca_v) channels, Maxi-K calcium-activated potassium channels (BK_{ca}), and the NMDA-subtype of glutamate receptors. The structure–activity relationships for insect-selective ICK toxins at various receptors are discussed in the following sections.

Spider ICK toxins targeting insect Na_v channels

Na_v channels are essential transmembrane proteins that mediate the intracellular influx of sodium ions during the initiation and propagation of action potentials [29]. Toxins that target Na_v channels are therefore present in venoms from a variety of animals for the purposes of prey subjugation and self-defense [30]. Na_v channels consist primarily of a single pore-forming α subunit composed of four homologous domains (I–IV). Each domain is comprised of six transmembrane helical segments (S1–S6), with voltage sensitivity conferred by the S4 segments (Fig. 2a). Ion selectivity is mediated by an inner and outer ring of amino acid residues located between the transmembrane segments five and six of each domain. While the overall domain architecture of insect and vertebrate Na_v channels are very similar, there are considerable sequence differences; insect Na_v channels are only ~60 % homologous to their human counterparts (Table 2) [31], which provides ample opportunity for producing Na_v channel insecticides that are insect-specific.

Although Na_v channels are the target of extant insecticides such as pyrethroids, dihydropyrazoles, and oxadiazines [32], there is still significant potential for the development of

Table 1 Overview of structural folds adopted by insecticidal toxins

	Structural fold	Venomous animals	Targets
Inhibitor cystine knot		Spiders, scorpions, cone snails	Na_v, Ca_v, BK_{Ca} Cl (?) channels
Cystine-stabilized αβ		Scorpions	Na_v, K_v channels
Disulfide-directed β-hairpin		Spiders, scorpions	Unknown
Defensin-like		Sea anemones	Na_v channels
Neuro-toxin III		Sea anemones	Na_v channels
Con-tryphan		Cone snails	K_v, BK_{Ca} channels
Three-finger toxins		Snakes	Nicotinic acetylcholine receptor

Stereoimages of ribbon representations of each fold are shown, with disulfide bonds highlighted in *yellow*

The representative toxins shown for each structural class are as follows, with PDB code given in *parentheses*: inhibitor cystine knot: κ-TRTX-Pg1a (2WH9); cystine-stabilized αβ: charybdotoxin (2CRD); disulfide-directed β-hairpin: U_1-LITX-Lw1a (2KYJ); defensin-like: anthopleurin-A (1AHL); neurotoxin III: Av3 (1ANS); contryphan: contryphan-Vn (1NXN); three-finger toxins: cobratoxin (1COE)

bioinsecticides acting on Na_v channels. Arachnid toxins that target Na_v channel could even be useful in situations where an insect population has developed resistance to a Na_v channel insecticide. This seemingly counterintuitive scenario is possible because arachnid venom peptides act at different sites to chemical insecticides. Thus, even though the scorpion toxin AaIT and pyrethroids both target Na_v channels, a pyrethroid-resistant strain of *H.virescens* is more susceptible than non-resistant strains to a recombinant baculovirus expressing AaIT [33].

There are numerous spider-venom ICK toxins that selectively target insect Na_v channels. Seven neurotoxin binding sites have been identified on Na_v channels, with the site at which the toxin binds usually determining its effect on the channel. Spider ICK insecticidal toxins interact almost exclusively with neurotoxin receptor sites 3 and 4, which cause effects on channel inactivation and activation, respectively [4]. In the following sections, we review some of the insecticidal spider-venom peptides that act on Na_v channels.

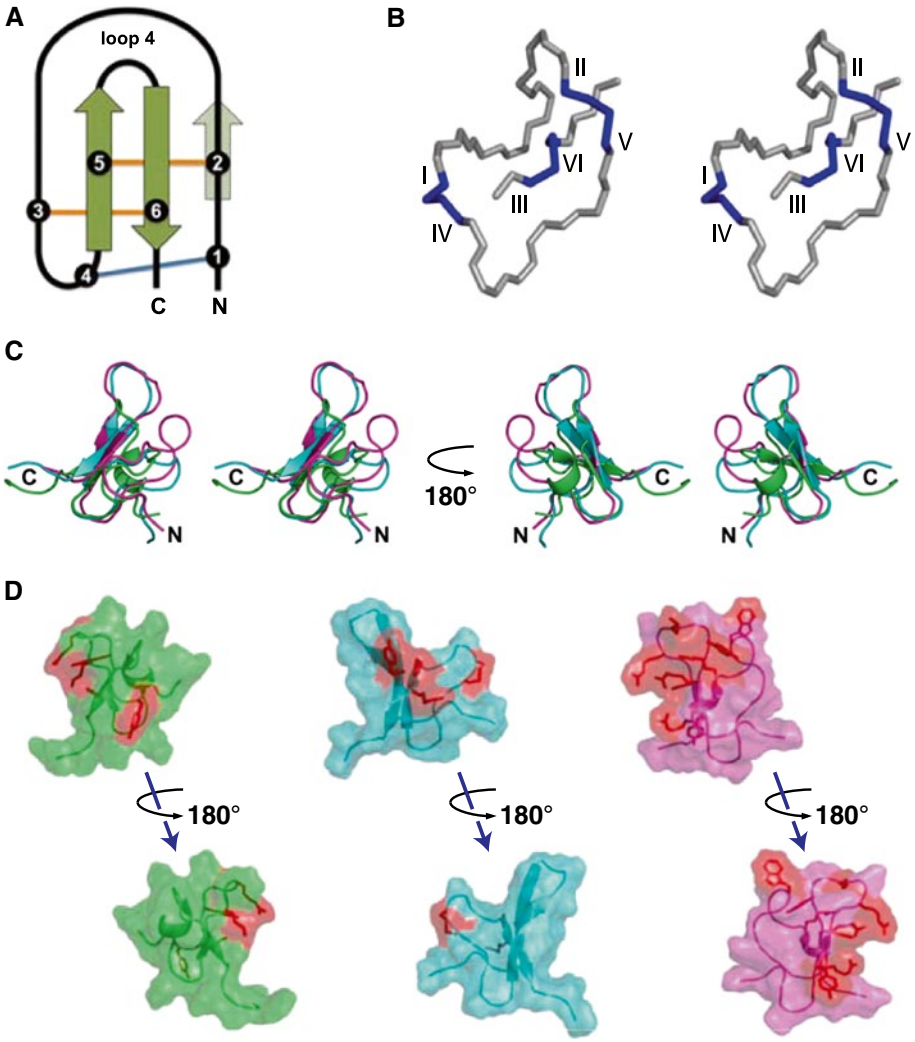


Fig. 1 Inhibitor cystine knot (ICK) fold. **a** Schematic of the ICK fold. The two disulfides present in the disulfide-directed β-hairpin (DDH) fold, the proposed precursor of the ICK motif, are colored *orange*, with the third disulfide necessary for the formation of the ICK fold colored *blue*. *Solid green arrows* represent the two requisite β strands, while the *translucent arrow* represents the third β strand that is present in some ICK peptides. Adapted from [65, 143]. **b** Stereoimage of the disulfide-bond configuration in the ICK motif. The disulfides between the first and fourth, and the second and fifth cystines, along with the intervening peptide backbone, create a ring that is pierced by the disulfide formed between the third and sixth cystines. Adapted from [4]. **c** Stereoview of an overlay of the ICK spider toxins κ-TRTX-Gr1a (K_V channel blocker, PDB 1D1H, *green*), κ-HXTX-Hv1c (BK_{Ca} channel blocker, PDB1DL0, *cyan*), and δ-amaurobitoxin-Pl1b (Na_V channel modulator, PDB 1V91, *magenta*). **d** Surface representations of the same three toxins showing the conserved ICK framework and the location of the toxin pharmacophores (*red*)

Spider ICK toxins potentially targeting neurotoxin receptor site 1

This recently described family of spider toxins is comprised of several members of the huwentoxin and hainantoxin groups isolated from the Chinese tarantulas *Haplopelma huwenum* and *Haplopelma hainanum*, respectively (Table 3). In contrast to all other spider toxins that target Na_v channels, these toxins do not alter the kinetics of channel inactivation or the voltage-dependence of channel activation, but instead are proposed to be pore blockers that inhibit channel current [34].

These toxins inhibit tetrodotoxin-sensitive (TTX-S) but not tetrodotoxin-resistant (TTX-R) currents in rat neurons. Due to their similar action to TTX, it was proposed that these toxins bind to the same neurotoxin receptor site on Na_v channels as TTX (i.e., site 1) [34]. However, competition binding studies with TTX/saxitoxin have not been performed to confirm this hypothesis. Moreover, recent experiments suggest that these toxins may in fact bind to neurotoxin receptor site 4 [35, 36]. It was previously shown that the sensitivity of the vertebrate $Na_v1.7$ channel to TTX was greatly decreased by a single Y632S mutation in site 1 [35] but the putative site 1-binding spider toxin μ-theraphotoxin-Hh2a (μ-TRTX-Hh2a; huwentoxin-IV) remained completely active at the mutant receptor. In contrast, the activity of μ-TRTX-Hh2a was almost abolished at channels with mutations in site 4, revealing an interaction between the toxin and this site. Thus, further studies probing the interaction of these toxins with vertebrate and invertebrate Na_v channels may ultimately lead to their reclassification as site 4 ligands.

All toxins in this family are also active on vertebrate Na_v channels, although two members (μ-TRTX-Hhn2b; hainantoxin-I, and μ/ω-theraphotoxin-Hh1a; huwentoxin-1) are over tenfold more potent on insect channels than rat channels. μ-TRTX-Hhn2b is 15-fold more potent on the insect Na_v channel compared with the rat $Na_v1.2$ channel when expressed in *Xenopus laevis* oocytes (IC_{50} values of 4.3 μM and 68 μM, respectively) [34]. μ/ω-theraphotoxin-Hh1a is 14 times more potent on sodium currents in cockroach DUM

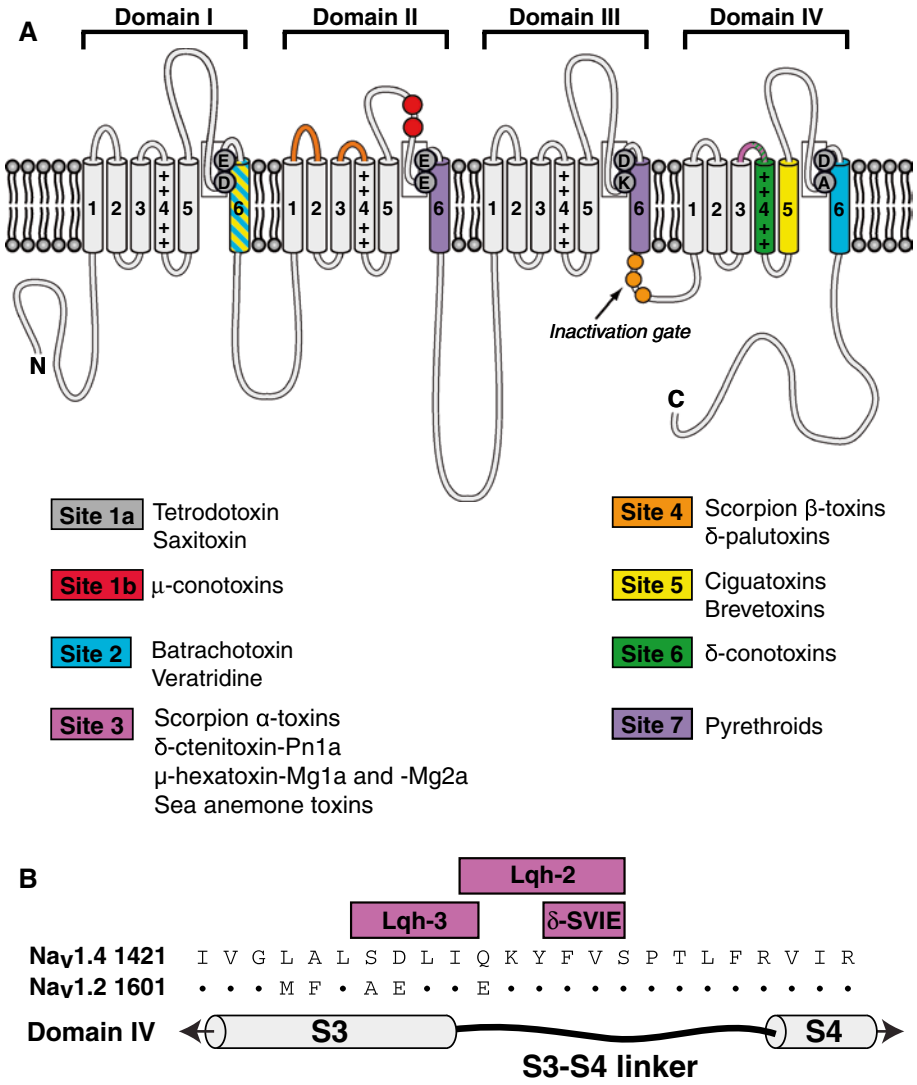


Fig. 2 Structure of voltage-gated sodium (Na_V) channel. **a** Graphical representation of Na_V channel showing the different neurotoxin receptor binding sites. Adapted from [31, 212]. **b** Sequence alignment of the S3–S4 region of Na_V channel domain IV (i.e., neurotoxin receptor site 3) showing residues important for interaction with the scorpion toxins Lqh-3 and Lqh-2, and conotoxin δ-SVIE. Adapted from [95]

neurons than the TTX-S sodium currents of rat hippocampal neurons (IC_{50} values of 4.80 and 66.1 nM, respectively) [37]. μ/ω-theraphotoxin-Hh1a also inhibits high-voltage-activated Ca^{2+} channels in differentiated NG-108-15 rat glioma x mouse neuroblastoma hybrid cells, however the toxin has not been tested on insect Ca^{2+} currents [38]. Comparison of the sequences of μ-TRTX-Hhn2b and μ/ω-theraphotoxin-Hh1a with other toxins in this family suggest that variation in acidic residues and local structural differences play a role in determining phylum selectivity [34, 37]. However, mutational studies to explore the molecular epitopes underlying the preference of μ-TRTX-Hhn2b for insect Na_v channels are still awaited.

Spider ICK toxins and toxins of unknown fold targeting neurotoxin receptor site 3

Spider toxins that bind to neurotoxin receptor site 3 inhibit the fast inactivation of Na_v channels, thereby prolonging action potentials. They produce neuroexcitatory effects, leading to muscle fatigue and paralysis. δ-Ctenitoxin-Pn1a (Tx4(6-1)) (LD_{50} in *Musca domestica* of 36 pmol/g) is a disulfide-rich toxin that adopts an undefined fold from the venom of the highly venomous Brazilian spider *Phoneutria nigriventer*. It is lethal to a range of insects but has no effect on oocyte-expressed rat Na_v channels or on mice when injected intracerebroventricularly [39]. δ-Ctenitoxin-Pn1a competes with scorpion α-like toxin Bom IV for binding to site 3 of cockroach axonal Na_v channels [40]. Two additional homologues named δ-ctenitoxin-Pn1b (PnTx4-3, LD_{50} 192 pmol g^{-1} in *Musca domestica*) and Γ-ctenitoxin-Pn1a (Tx4(5–5), LD_{50} 90 pmol g^{-1} in *Musca domestica*) have been isolated from the same spider species (Fig. 3) [41, 42]. Although their molecular target has not been determined, their similarity in sequence to δ-ctenitoxin-Pn1a, excitatory effects upon injection into insects, and lack of toxicity in mice suggest both peptides probably also act on insect Na_v channels.

Table 2 Homology between various insect Na_V channels and between insect Na_V channels and the nine human Na_V channel subtypes

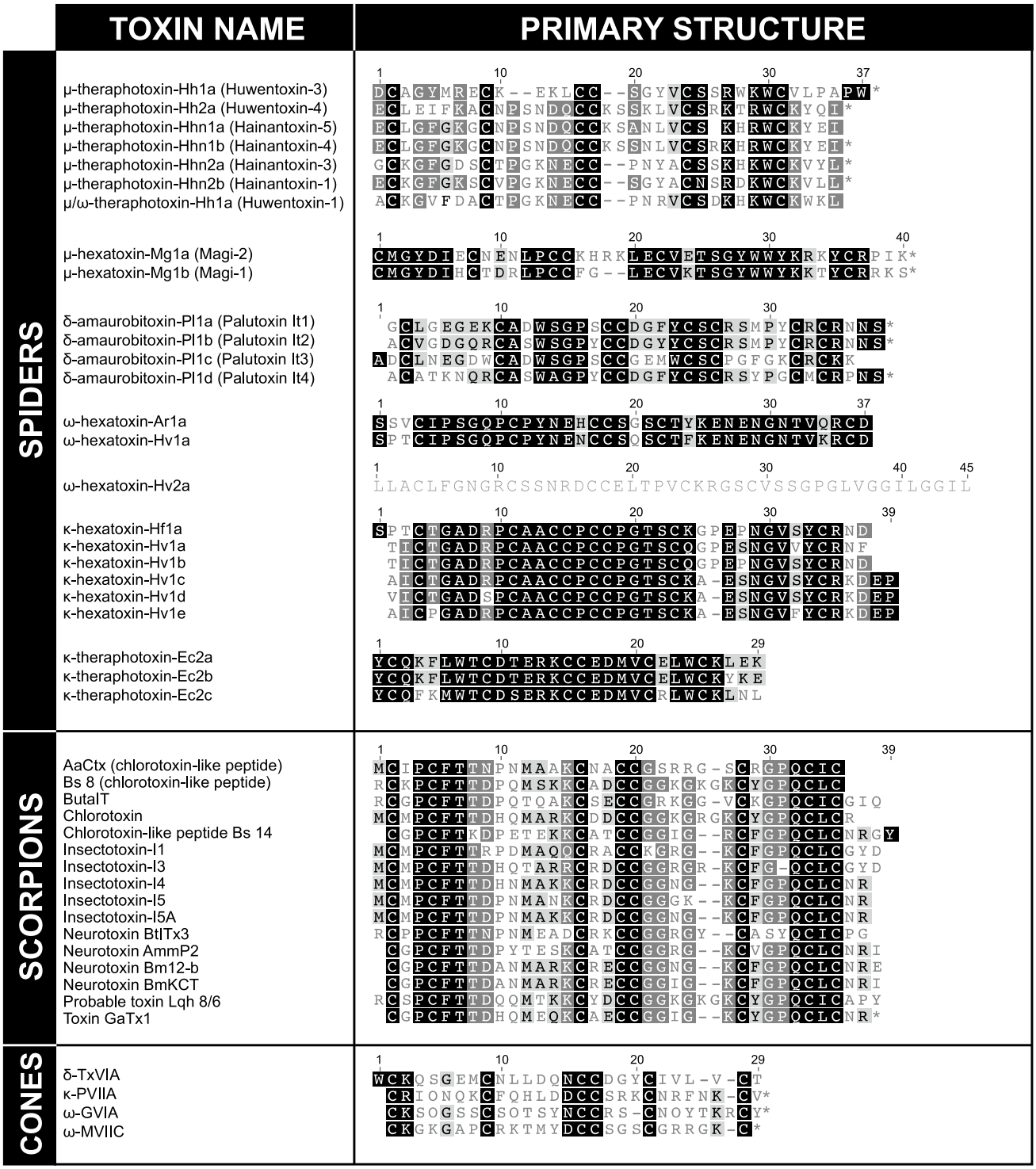
		Insect Nav channels									
		Drosophila melanogaster P3500	*Musca domestica* Q94615	*Anopheles gambiae* A5I843	*Bombyx mori* A5A2M4	*Blattella germanica* O01307	*Heliothis virescens* Q94584	*Nasonia vitripennis* A5I9E9	*Pediculus h corpis* Q86M38	*Tribolium castaneum* GLEAN_04126	*Apis mellifera* hmm6170
Insect Nav channels	*Musca domestica* Q94615	95%	-	-	-	-	-	-	-	-	-
	Anopheles gambiae A5I843	92%	90%	-	-	-	-	-	-	-	-
	Bombyx mori A5A2M4	91%	88%	93%	-	-	-	-	-	-	-
	Blattella germanica O01307	90%	87%	92%	93%	-	-	-	-	-	-
	Heliothis virescens Q94584	89%	87%	92%	98%	91%	-	-	-	-	-
	Nasonia vitripennis A5I9E9	90%	88%	93%	92%	93%	91%	-	-	-	-
	Pediculus h corpis Q86M38	89%	87%	91%	91%	90%	89%	90%	-	-	-
	Tribolium castaneum GLEAN_04126	89%	87%	92%	91%	91%	91%	93%	90%	-	-
	Apis mellifera hmm6170	91%	88%	93%	94%	94%	93%	97%	91%	93%	-
Human Nav channels	Nav 1.1 P35498	59%	58%	59%	59%	59%	58%	59%	59%	58%	59%
	Nav 1.2 Q99250	59%	59%	59%	59%	59%	58%	59%	59%	59%	59%
	Nav 1.3 Q9N746	59%	59%	59%	60%	59%	58%	59%	59%	59%	59%
	Nav 1.4 P35499	57%	57%	58%	60%	58%	57%	57%	58%	57%	58%
	Nav 1.5 Q14524	58%	58%	58%	59%	59%	57%	58%	58%	58%	58%
	Nav 1.6 Q9UQD0	58%	58%	59%	60%	60%	58%	59%	59%	59%	59%
	Nav 1.7 Q15858	58%	59%	59%	58%	58%	57%	58%	58%	58%	59%
	Nav 1.8 Q9Y5Y9	56%	56%	56%	56%	56%	55%	56%	55%	56%	56%
	Nav 1.9 Q9UI33	51%	51%	50%	52%	51%	50%	51%	51%	51%	51%

UniProt identifiers or genome accession numbers are given for each entry. Adapted from [31].

An additional action of this family of toxins is their ability to partially inhibit glutamate uptake in rat brain synaptosomes via an as-yet-unknown mechanism. They are also likely inhibitors of the NMDA-subtype of the ionotropic glutamate receptor as Γ-ctenitoxin-Pn1a inhibits NMDA-elicited currents without affecting GABA-, AMPA-, or kainate-induced currents [41, 42]. No experiments have been performed to ascertain structure–activity relationships for this family of toxins. However, inhibition of glutamate uptake by δ-ctenitoxin-Pn1b is 2.5-fold greater than seen with δ-ctenitoxin-Pn1a, which differs by only one amino acid at position 27 (Ser and Lys, respectively) [42]. It remains to be determined whether the chemical pharmacophore conferring insecticidal activity is distinct from that responsible for the inhibition of glutamate uptake in vertebrates.

Numerous Na_v toxins have been isolated from the Japanese funnel-web spider *Macrothele gigas*, two of which have insecticidal activity and no toxic effects in mice. One toxin adopts the ICK motif (μ-hexatoxin-Mg1a; Magi-2, LD_{50} 17,600 pmol g^{-1} in *Spodoptera litura*), while the 3D fold of the other toxin (μ-hexatoxin-Mg2a; Magi-3) remains to be determined [43]. Both toxins compete with scorpion toxin LqhαIT for binding to Na_v channel site 3 in cockroach synaptosomes, whereas neither toxin competes with radiolabeled toxins for binding to site 4 in cockroach synaptosomes, or sites 3, 4, and 6 in rat brain Na_v channels. Interestingly, μ-hexatoxin-Mg1a

Table 3 Insecticidal venom peptides with an ICK fold



Asterisks indicate an amidated C-terminus

shares 68 % sequence identity with μ-hexatoxin-Mg1b (Magi-1), a peptide with no activity in insects or mice (Table 3) [43]. The other insecticidal toxin from *M.gigas*, μ-hexatoxin-Mg2a, may also act on insect Ca_V channels since it is 43 % homologous to the insect Ca_V channel blocker ω-plectoxin-Pt1a (PLTX-II); however, this has not been experimentally verified [43]. Further investigations, including mutagenesis studies, are required to

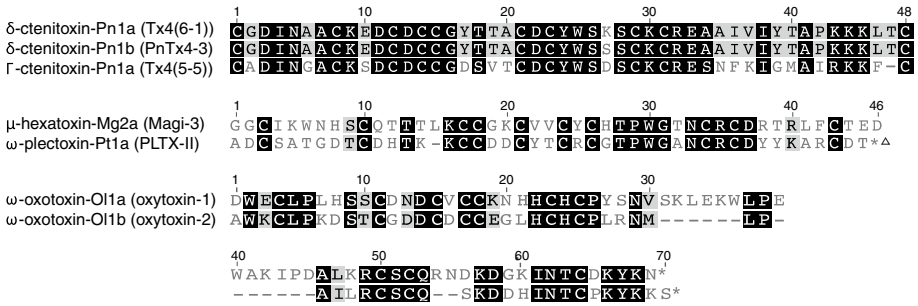


Fig. 3 Insecticidal spider venom peptides with an undefined fold. *Asterisks* indicate an amidated C-terminus. *Triangle* indicates an *O*-palmitoyl group

elucidate the residues necessary for activity and selectivity of these site 3 toxins.

Spider ICK toxins targeting neurotoxin receptor site 4

Toxins that target neurotoxin receptor site 4 classically affect Na_v channels by causing a hyperpolarizing shift in the voltage dependence of activation, leading to a decrease in the threshold potential required for the generation of action potentials and the occurrence of spontaneous transient activity. This is the primary mode of action of spider toxins that target vertebrate Na_V channels [44]. However, only four insecticidal spider toxins, the δ-palutoxins, have been shown to bind at site 4 of insect Na_v channels (Table 3). They compete with the site-4 scorpion toxin Bj-xtrIT and do not compete with the site-3 scorpion toxin LqhαIT for binding to cockroach neuronal membranes [45]. However, the δ-palutoxins are unusual as they slow channel inactivation in a manner similar to toxins that bind to site 3. δ-Amaurobitoxin-Pl1b (δ-palutoxin IT2) is the only δ-palutoxin that also causes toxicity in mice, with the three other members of this family being specific for insects [46]. Although there are few sequence differences between δ-palutoxin paralogues, the residues conferring mammalian activity to δ-amaurobitoxin-Pl1b are not obviously apparent and remain to be determined. Alanine scanning mutagenesis of δ-amaurobitoxin-Pl1b revealed that the bioactive surface is comprised of a basic region that may help localize the toxin to the receptor, plus a hydrophobic cluster and a key aspartic acid residue at position 19 (Fig. 4a). Substitution of D19 with alanine resulted in a considerable decrease in toxin activity, without a concurrent reduction in binding affinity [45]. E15 mutants of the insect-selective scorpion toxin Bj-xtrIT presented a similar disconnect between binding and activity. Consequently, the acidic residue was proposed to be involved in voltage sensor trapping but not receptor binding [47].

Comparison of the three-dimensional (3D) structures of δ-amaurobitoxin-Pl1b and Bj-xtrIT highlights the resemblance in spatial orientation of several key hydrophobic/aromatic residues that are important for activity [45, 48] (Fig. 4b). These hydrophobic residues, as well as many of the other residues comprising the bioactive surface of δ-amaurobitoxin-Pl1b, are highly conserved in other insect-selective spider toxins, namely the β/δ- and μ-agatoxins. Based on their sequence homology with the δ-palutoxins, the β/δ- and μ-agatoxins are thought to bind to receptor site 4, however confirmation is required via binding assays. The β/δ-agatoxins also have an unusual pharmacological profile, since they inhibit channel inactivation similar to site-3 toxins, as well as shifting the voltage dependence of channel activation to more hyperpolarized potentials analogous to classical site-4 toxins. Idiosyncratically, both of these effects are voltage dependent and display a bell-shaped curve between −80 and 0 mV. Although the effects of μ-agatoxins have not been as extensively explored, μ-agatoxin-Aa1a and -Aa1d also affect channel activation and inactivation in a manner qualitatively resembling β/δ-agatoxins [49, 50]. Additional studies into the mode of action of μ-agatoxins may result in their reclassification as β/δ-agatoxins. The diverse effects of these site-4 spider toxins have challenged the long-held presumption of a correlation between binding site and activity. Future studies are needed to uncover the molecular determinants of these various actions.

Spider ICK toxins and toxins of unknown fold targeting insect Ca_v channels

Like Na_v channels, Ca_v channels play an essential role in action potential generation. However, Ca_v channel subtypes are not as highly conserved between different insect orders as Na_v channels, with identity levels between 76–90 % compared to above 90 % for Na_v channels [31]. This theoretically reduces the likelihood of finding toxins active against a broad spectrum of insect Ca_v channels, which may explain the lack of chemical insecticides that target Ca_v channels. However, this attribute could potentially be exploited to develop insecticides that target only pest insects without harming beneficial species.

While modulation of Ca_v channels is one of the dominant pharmacologies of spider-venom toxins, in-depth functional and biophysical characterization of insect-selective Ca_v toxins has been difficult due to the lack of a system by which to study insect Ca_v channels discretely [51]. Recombinant

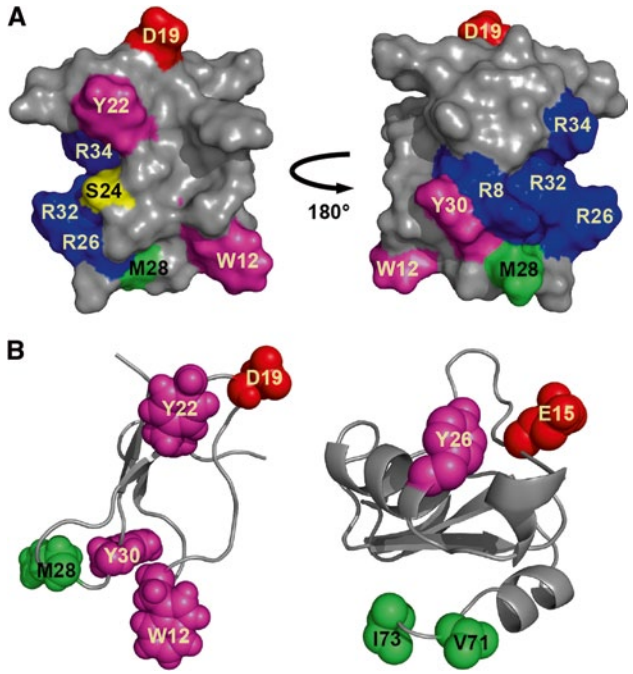


Fig. 4 Structure of site-4 Na_V channel toxins. **a** Surface representation of the spider toxin δ-amaurobitoxin-Pl1b (PDB 1V91) showing key pharmacophore residues. Adapted from [45]. **b** Ribbon representation of δ-amaurobitoxin-Pl1b (*left*) and the scorpion toxin Bj-xtrIT (PDB 1BCG, *right*) showing the similar spatial positioning of key functional residues. In each panel, the chemical nature of amino acid residues is color coded as follows: aromatic, *magenta*; aliphatic, *green*; basic, *blue*; acidic, *red*; polar but uncharged, *yellow*

expression of a functional insect Ca_V channel has been a considerable challenge, with the only example described in a recent patent [52]. Despite this obstacle, numerous insect-selective Ca_V channel spider ICK toxins have been characterized via their ability to block Ca^{2+} currents in insect neurons.

Cockroach dorsal unpaired median (DUM) neurons have been a valuable tool and the archetypal system for the study of Ca^{2+} currents in insects. There are four different types of Ca^{2+} currents that can be evoked in DUM neurons: low-voltage activated currents that are either transient (tLVA) or maintained (mLVA), mid- and high-voltage activated currents (MVA and HVA, respectively). These four current types are thought to be mediated by channels belonging to the three subtype groups Ca_V1, Ca_V2 and Ca_V3, which are orthologues of the respective vertebrate families. Although the subtypes responsible for each current type have not been conclusively established, the pharmacological properties of the subtypes in conjunction with studies of the *Drosophila* Ca_V2 channels have lead to the proposal that Ca_V3 channels cause tLVA/mLVA currents, while MVA and HVA currents are produced primarily through Ca_V2 channels, with a minor component contributed by Ca_V1 channels [31, 51, 53].

To date, all characterized spider toxins that target insect Ca_V channels act by blocking MVA and HVA currents, thus most likely targeting Ca_V1 and/or Ca_V2 channels. The most extensively analyzed of these toxins is ω-hexatoxin-Hv1a (ω-HXTX-Hv1a) from the Australian funnel-web *Hadronyche versuta* [54, 55], which has been proposed to act primarily on insect Ca_V1 channels [51]. ω-HXTX-Hv1a is lethal to a wide range of insect orders (LD_{50} 77 pmol g^{-1} in *Musca domestica*, LD_{50} 89 pmol g^{-1} in *Acheta domestica*) but does not affect vertebrates [54–56]. A panel of alanine mutants of ω-HXTX-Hv1a revealed that three spatially contiguous residues (P10, N27 and R35) form a major pharmacophore crucial for both insecticidal activity and binding to cockroach neurons. Two other residues (Q9 and Y13) comprising a minor pharmacophore also contribute to toxin binding and activity (Fig. 5) [57, 58]. Both the major and minor pharmacophores are highly conserved in ω-HXTX-Hv1a paralogues and orthologues. Whereas the major pharmacophore may bestow activity at insect Ca_V1 channels in general, the slight variations in primary structure between family members might confer selectivity between different insect orders. ω-HXTX-Ar1a is 84 % identical to ω-hexatoxin-Hv1a, however it is 2–3 times less potent in crickets (LD_{50} 236 pmol g^{-1} in *Acheta domestica*) (Table 3) [59]. It is likely that this decrease in toxicity is due to the presence of His rather than Asn at position 16 since an N16A mutation in ω-HXTX-Hv1a caused a ~4-fold reduction in toxicity to crickets. Notably, the N16A mutation did not affect the activity of ω-HXTX-Hv1a in flies or its binding to cockroach neurons [58]. Testing other ω-HXTX-Hv1a orthologues for selectivity over different insect orders would be a worthwhile future study, as it may enable the development of insecticides that target specific pest orders, while leaving neutral or beneficial insect species unharmed.

Another insect-selective toxin from *Hadronyche versuta*, ω-hexatoxin-Hv2a (ω-HXTX-Hv2a), blocks Ca_V channel currents in bee brain neurons and is toxic to insects from a wide array of orders including Lepidoptera, Orthoptera and Diptera [60]. ω-HXTX-Hv2a shows no homology to the ω-HXTX-Hv1a toxin family and does not posses the ω-HXTX-Hv1a pharmacophore (Table 3). While both toxins contain an ICK motif, ω-HXTX-Hv2a differs considerably from ω-HXTX-Hv1a in containing a long, unstructured C-terminal "tail" [60]. Although the residues responsible for insecticidal activity have not been ascertained, synthetic analogues of ω-HXTX-Hv2a without the 12-residue tail were unable to inhibit Ca_V channels [60]. The C-terminal region is also important for the activity of other Ca_V channel spider toxins. The eight C-terminal residues of ω-agatoxin-Aa4a, the terminal Phe and Ser residues in ω-TRTX-Hh2a (huwentoxin-V, $LD_{50} \geq 24{,}339$ pmol g^{-1} in *Locusta migratoria manilensis*), and an unusual *O*-palmitoyl

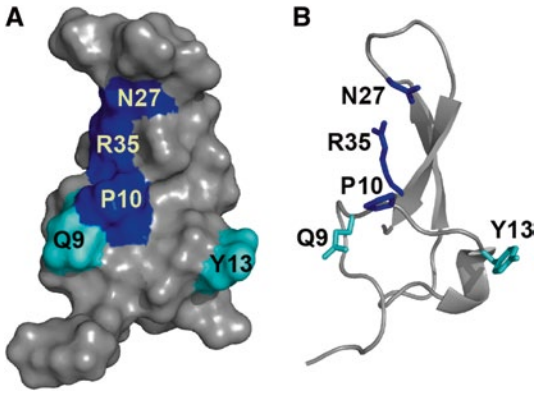


Fig. 5 Pharmacophore of ω-HXTX-Hv1a (PDB 1AXH). **a** Surface and **b** ribbon representations of ω-HXTX-Hv1a with the major pharmacophore residues shown in *blue* and less critical pharmacophore residues in *cyan*

threonine amide at the C-terminus of the non-ICK folded ω-plectoxin-Pt1a (PLTX-II) are essential for the activity of these toxins [60–63]. Although no significant sequence homology exists between these toxins, their C-terminal regions are lipophilic and structurally disordered. Since insect Ca_v channels have a largely polar surface that is unlikely to have any extensive favorable interaction with the hydrophobic tails of these toxins, it was proposed that the C-termini anchor the toxins in the cell membrane and direct the requisite toxin regions into the channel. It is also possible that the C-termini somehow cause a conformational change in the channel, thereby exposing a previously inaccessible high-affinity binding site to which other areas of the toxin bind [60]. Although a hydrophobic C-terminus is vital for the activity of these Ca_v channel blockers, it is most likely not a determinant of vertebrate versus invertebrate selectivity as ω-agatoxin-Aa4a targets both mammalian and insect channels. Much work remains to establish the structure–activity relationships between these spider toxins and Ca_v channels; nevertheless the ICK fold is a sound framework for engineering bioinsecticides that are specifically targeted against insect Ca_v channels.

The most recently characterized spider-venom peptides that inhibit insect Ca_v channels are the ω-oxytoxins, which have low sequence identity with the other spider-derived Ca_v channel toxins, do not adopt the ICK fold, nor possess lipophilic C-termini (Fig. 3). ω-Oxytoxins paralyze larvae of the lepidopteran pest *Spodoptera frugiperda* (army worms) with an ED_{50} of 5,000–6,200 pmol g^{-1} but they are non-toxic to mice. They do, however, block HVA currents of expressed rabbit Ca_v channels, and therefore additional toxicity assays in other vertebrate species are required to assess their suitability as prospective insecticides [64].

Spider ICK toxins targeting insect BK_{Ca} channels

BK_{Ca} channels, also known as Maxi-K or Slo1, are calcium-activated potassium channels important in the control of neuronal and muscle excitability. A lack of insect-selective ligands has meant the BK_{Ca} channel has not previously been considered a potential pesticidal target. However, in recent years, it has been established that two families of insecticidal spider toxins are selective for insect BK_{Ca} channels. The κ-hexatoxins (formerly J-ACTXs) are lethal to insects from an extensive array of taxonomic orders (LD_{50} 167 pmol g^{-1} in *Acheta domestica*, LD_{50} 91 pmol g^{-1} in *Musca domestica*), while being inactive on mouse, chick, rat and rabbit preparations [65, 66]. The prototypic family member, κ-HXTX-Hv1c, potently inhibits BK_{Ca} currents in cockroach DUM neurons with an IC_{50} of ~3 nM [67, 68]. κ-Hexatoxins act as blockers of channel current, probably interacting with the pore or turret residues located between transmembrane segment S5 and S6 of the BK_{Ca} channel [67]. A comparison of the region between S5 and S6 in insect and vertebrate BK_{Ca} channel reveals several amino acid differences that possibly underlie the phyletic discrimination of the κ-hexatoxins (Table 3) [67, 69]. It has been demonstrated that the sensitivity of insect BK_{Ca} channels to charybdotoxin, a pore blocking scorpion toxin with a preference for vertebrates, can be increased upon substitution of single residues in the S5-S6 region with the corresponding residues in vertebrate BK_{Ca} channels [70]. Therefore, there is ample variation in the amino acid sequences of the pore region between insect and vertebrate channels to warrant the insect-selectivity of the κ-hexatoxins.

Mutagenesis studies have shown that the residues responsible for the activity of κ-hexatoxins are Ile2, Arg8, Pro9, Val29, Tyr31 and a rare vicinal disulfide bond between Cys13 and Cys14 (Fig. 6a) [65, 66]. Numerous vertebrate K^+ channel toxins from different phyla interact with the channel via a functional dyad consisting of a Lys and Tyr/Phe 6.6 ± 1.0 Å apart [71]. The Arg8 and Tyr31 in κ-hexatoxins spatially overlay well with the functional dyad in other toxins, and it was thought that the Arg might have been analogous to Lys in the dyad (Fig. 6b). However, substitution of Arg8 with Lys in κ-HXTX-Hv1c resulted in a dramatic decrease in binding and activity [67]. Consequently, the essential Arg8 and Tyr31 are not likely to be synonymous to the dyad of other K^+ channel toxins. The mode of interaction of κ-hexatoxins with BK_{Ca} channels therefore cannot be assumed to be similar to that of dyad-containing Kv channel toxins.

The second group of spider toxins that block BK_{Ca} channels are the κ-TRTX-Ec2 family from the African tarantula *Eucratoscelus constrictus* (Table 3). κ-TRTX-Ec2a and -Ec2b are insect-selective (LD_{50} 1,100 pmol g^{-1} in *Gryllus bimaculatus*), while κ-TRTX-Ec2c is toxic to both insects

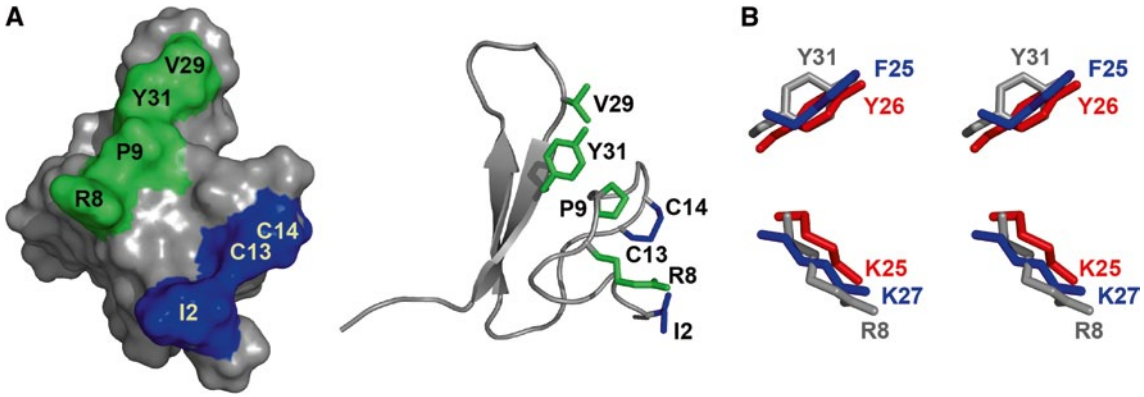


Fig. 6 Pharmacophore of κ-HXTX-Hv1c**. a** Surface view (*left*) and ribbon representation (*right*) of κ-HXTX-Hv1c (PDB 1DL0) showing the bipartite pharmacophore in *green* and *blue*. **b** Stereoimage of the overlay of the side-chains forming the functional Kv-channel dyad of agitoxin 2 (*blue*, PDB 1AGT), BgK (*red*, PDB 1BGK) and the specious Kv-channel dyad of κ-hexatoxin-Hv1c (*gray*). Adapted from [67]

and mice [21]. Although the pharmacophore of the κ-TRTX-Ec2 family has not been elucidated, sequence comparisons with homologous but functionally unrelated spider toxins have identified several residues that may determine their selectivity for insect BK_{Ca} channels. It is hypothesized that a Glu residue in the C-terminal region is responsible for the insect-selectivity of κ-TRTX-Ec2a and -Ec2b, since homologous toxins are mammalian-active and have a basic or hydrophobic residue at the corresponding location. A highly conserved Trp5 and Met6 motif in homologous toxins is not present in the κ-TRTX-Ec2 toxins and this difference may serve as the basis for their affinity at the BK_{Ca} channel [21].

Scorpion ICK toxins targeting unknown insect targets

Several scorpion toxins have been found to adopt the ICK fold, although this fold is not as abundant in the venom of scorpions in comparison to spiders. Many of the scorpion ICK toxins target mammalian RyRs [72], however their toxicity in insects have not been ascertained. Others appear to be insect selective as they are lethal to insects but not toxic to mice, though their molecular targets are unknown [73]. These putatively insect-specific toxins, termed small insectotoxins, share a conserved spatial arrangement of cystines and sequence homology with chlorotoxin, a scorpion ICK toxin that induces paralysis in crayfish and cockroaches and inhibits small conductance Cl^- channels isolated from rat epithelia and brain. Due to the similarities with chlorotoxin (Table 3), it was hypothesized that the short insectotoxins also target Cl^- channels, but this has never been experimentally validated [74, 75]. Furthermore, subsequent studies have revealed that chlorotoxin binds to the cell-surface molecules annexin A2 and matrix metalloprotease-2, and therefore these could also be molecular targets for the short insectotoxins [76, 77]. Based on the action of insectotoxin I5A, an additional target of the insectotoxins may be a glutamate receptor located on postsynaptic membranes [78]. The actual receptors with which the short insectotoxins interact therefore remain to be ascertained and it may eventuate that these toxins target different receptors to chlorotoxin despite their structural homology. Although detailed pharmacological analyses of the short insectotoxins are lacking, a considerable array of bioassays have been performed with the most studied of these toxins, ButaIT. This toxin is lethal to insect pests from a wide range of orders including Lepidoptera (a dose of 2593 pmol g^{-1} was lethal to *Heliothis virescens*), Coleoptera, Diptera, and Dictyoptera, making it a promising candidate for bioinsecticide development [73, 79].

Cone snail ICK toxins targeting various insect channels

While terrestrial venomous organisms produce insecticidal toxins for the benefit of prey capture and/or defense, the presence of such toxins in the venom of marine invertebrates such as cone snails seems paradoxical, since there are no marine insects described. However, a certain degree of homology is likely to exist between insect ion channels and those of the natural molluscan and crustacean prey targets of venomous marine invertebrates, as all belong to the phylum Arthropoda. Indeed, a BLAST search of the putative Na_v channel sequence from the crab *Cancer borealis* reveals its closest homologue to be the para sodium channel from the German cockroach *Blattella germanica*, with which it is 69 % identical [80]. The activity of toxins from marine invertebrates on insects is thus almost certainly incidental, with their intended targets probably being molluscs, crustaceans, and/or annelids.

Although conotoxins have been studied for over 30 years, most research has focused on mammalian-active toxins and their potential as drug leads for human diseases [81–83].

There have been very few investigations into their activity on insects and, as a result, only a handful of conotoxins have been found to be insecticidal. The ω-conotoxins GVIA and MVIIC both inhibit Ca_v channels in cockroach DUM neurons [84] and κ-conotoxin PVIIA is a blocker of the insect shaker K_V channel [85]. However, these conotoxins are not insect-specific, with ω-GVIA and ω-MVIIC potently active on rat Ca_v channels [86, 87] and κ-PVIIA causing hyperexcitability in mice when injected i.c.v. and 'fin popping' activity in fish [85, 88]. To date, the only conotoxin that seems inactive in vertebrates is δ-TxVIA, which is non-toxic in mouse, rat and fish [89, 90] while being lethal to the housefly *Musca domestica* (283436 pmol g^{-1} caused lethality in 40 % of tested insects) and larvae of the cabbage moth *Mamestra brassicae* (247,183 pmol g^{-1} caused lethality in 20 % of tested insects) [91]. δ-TxVIA is also known as the 'King-Kong' toxin as it has the uncanny ability of making submissive lobsters assume a dominant posture [89]. Similar to site-3 binding toxins, δ-TxVIA slows the rate of inactivation of Na_v channels. However, δ-TxVIA does not compete with the site-3 toxin Av2 in channel binding assays, nor does it compete with toxins that bind site 1, site 4 or site 5. Thus, δ-conotoxins were deemed to bind at a novel location, termed site 6 [92, 93]. Scanning mutagenesis of residues in the rat $Na_v1.4$ channel revealed that the main Na_v channel interaction site of the mammalian active δ-conotoxin SVIA is a triad of hydrophobic residues (Y1433, F1434, V1435) in the S3/S4 linker of domain four. This triad of residues is also part of the channel epitope important for activity of the site-3 scorpion α-toxin Lqh-2, but not of α-toxin Lqh-3 (Fig. 2b) [94]. Based on these observations, it was proposed that site 6 overlaps with or may in fact be part of site 3. Moreover, site 3 may be considered a 'macrosite' since different toxins interacting with distinct regions within site 3 are all regarded as site-3 binders [95]. Additional studies are necessary to locate the binding epitope of other δ-conotoxins in order to unequivocally determine whether neurotoxin site 6 is distinct from site 3.

δ-TxVIA is an unusual toxin since despite its lack of activity on vertebrates, it nevertheless binds with high affinity to vertebrate Na_v channels [92]. δ-conotoxins have a high proportion of hydrophobic residues and the solution structure of δ-TxVIA reveals that they are clustered in a hydrophobic patch on the surface of the molecule [96]. It is believed that this hydrophobic region mediates binding of δ-conotoxins to Na_v channels [96], with phyletic selectivity determined by other residues as yet unknown.

CSαβ fold

The cystine-stabilized αβ (CSαβ) motif consists of a short α-helix connected to two or three antiparallel β-strands via three or four disulfide bonds (Table 1) [97, 98]. This fold is adopted by one of two structural classes of defensin molecules. CSαβ defensins are antimicrobial peptides involved in innate immunity in an assortment of plants, fungi, insects and arachnids [99, 100]. CSαβ peptides also dominate the venom peptidome of scorpions, with the majority of known scorpion toxins adopting this fold [101]. It is believed the CSαβ defensins were recruited and neofunctionalized in scorpion venoms, prompting an explosive proliferation of toxin genes [20]. The CSαβ motif serves as a framework for scorpion toxins that are ligands of Na^+ and K^+ channels, many of which are insect-selective. These toxins are discussed in the following sections.

Scorpion CSαβ toxins targeting insect Na_v channels

The potential application of scorpion toxins as insecticides was first proposed more than 40 years ago, with the Na_v channel toxin AahIT (PD_{50} 0.64 pmol g^{-1} in *Sarcophaga argyrostoma*) being the first venom peptide seriously considered as a bioinsecticide lead [102]. The characterization of AahIT prompted the search for more insect-selective scorpion toxins, leading to the discovery of insecticidal toxins such as LqhαIT (PD_{50} 16 pmol g^{-1} in *Sarcophaga falculata*), BjIT2, Cn10 (onset of toxicity at 4,047 pmol g^{-1} in *Acheta spp.*) and BotIT2 (LD_{50} 196 pmol g^{-1} in *Blattella germanica*) [103–108]. Akin to spider ICK toxins, the majority of scorpion toxins that target Na_v channels have been found to bind at either neurotoxin receptor site 3 or 4, with site-3 binders inhibiting fast inactivation and site-4 binders lowering the threshold potential required for channel activation [109].

CSαβ toxins targeting neurotoxin receptor site 3

Scorpion toxins that bind neurotoxin receptor site 3 on Na_v channels are also known as α-toxins. They are divided into three classes based on their activity on vertebrate or invertebrate channels, namely classical, anti-insect, and α-like α-toxins. The classical α-toxins are highly active in mammals and exhibit low toxicity in insects. Anti-insect α-toxins are highly toxic to insects and also display low toxicity to mammals when injected i.c.v. α-Like toxins are toxic to both mammals and insects [110]. Numerous studies attempting to ascertain the pharmacophore of α-toxins have revealed two distinct regions pertinent to the interaction of the toxins with Na_v channels and the partiality of toxins for insect or mammalian receptors. The first of these regions is a highly conserved core-domain, consisting of 4–5 positively charged and aromatic residues located on the loops between the secondary structure elements (Fig. 7a) [111, 112]. It is thought that the core-domain mediates the interaction of all α-toxins with a region of Na_v channels conserved between

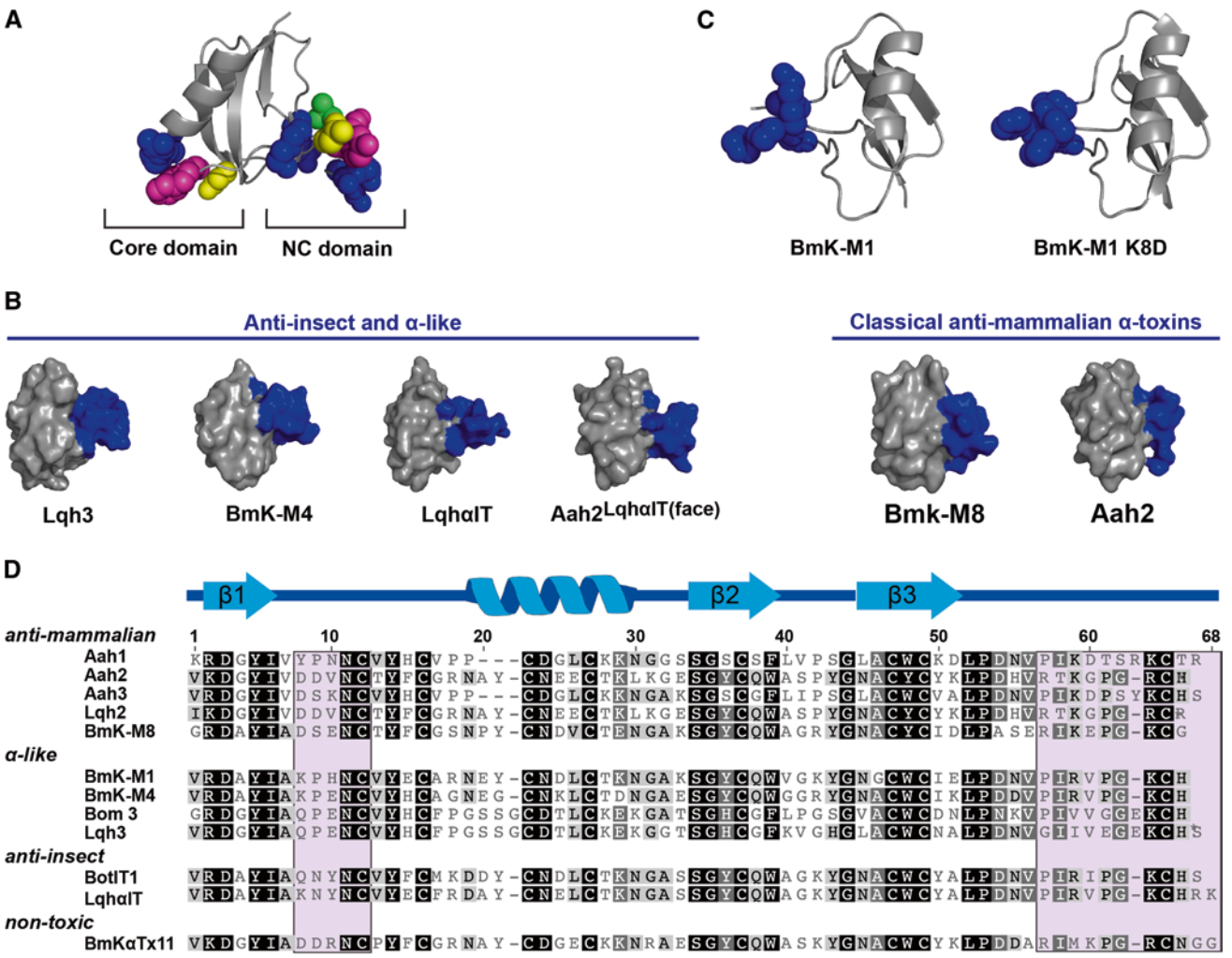


Fig. 7 Scorpion α-toxins**. a** Location of the NC and Core domains in α-toxins. The chemical nature of amino acid residues is color coded as follows: aromatic, *magenta*; aliphatic, *green*; basic, *blue*; polar but uncharged, *yellow*. Adapted from [213]. **b** Spatial orientation of the NC-domains (colored *blue*) in anti-insect and mammalian-active scorpion α-toxins. **c** Orientation of the NC-domain (colored *blue*) of the α-like toxin BmK-M1 and a K8D mutant. **d** Representative sequences of scorpion α-toxins. Residues comprising the NC-domain are shaded in *pink*, and secondary structure elements are shown above the sequences. Adapted from [115]

insects and mammals. The indiscriminate interaction of this domain with phylogenetically different Na_v channels may explain the ability of all α-toxins to bind at both insect and mammalian Na_v channels, albeit with vastly different affinities [111].

The phyletic and channel subtype selectivity of many α-toxins is believed to arise from a second toxin region, termed the NC-domain (Fig. 7). This domain is comprised of the five-residue turn between the first β-strand and the α-helix of the CSαβ structure and the C-terminal tail [111]. In a key experiment, the entire NC-domain and residue 17 of the core-domain of the classical α-toxin Aah2 was replaced with that of the archetypal anti-insect α-toxin LqhαIT. The resulting chimeric toxin, named $Aah2^{LqhαIT(face)}$, was only threefold less active on blowfly larvae than LqhαIT, representing a 380-fold increase in insecticidal activity compared to Aah2. Additionally, the chimera did not bind to rat brain synaptosomes, the high affinity binding of which is characteristic of Aah2. It should be noted that a chimera in which only the NC-domain of Aah2 was substituted with that of LqhαIT was twofold less potent in insects than $Aah2^{LqhαIT(face)}$, hence phyletic selectivity cannot solely be attributed to residues of the NC-domain [111].

A comparison of the 3D structures of various α-toxins revealed that the spatial orientation of the NC-domain differs between insect- and mammalian-active toxins. In anti-insect and α-like α-toxins, the NC-domain protrudes into the solvent, while it is flat in classical α-toxins (Fig. 6b) [111, 113]. The protrusion may present residues of the NC-domain to a region or binding pocket on the Na_v channel specific to

insects. A protruding NC-domain is often associated with an uncommon non-proline *cis* peptide bond between residues nine and ten. In the flat configuration, this peptide bond is consistently *trans* [111]. Mutagenesis studies of the α-like toxin BmK M1 have highlighted residue eight as a potential 'molecular switch' that affects the conformation of this peptide bond in some α-toxins [114]. It was noticed that residue eight in many classical α-toxins is Asp, whereas Lys or Gln occupies this position in nearly all of the α-like and anti-insect α-toxins [115]. Residue eight of wild-type BmK M1 is Lys and a *cis* peptide bond exists between residues nine and ten. Upon substitution of Lys8 with Asp, the 9–10 peptide bond 'switched' to the *trans* conformation. While the NC-domain did not completely assume a flat topology, several residues of the five-residue turn in the NC-domain that protrude in the wild-type toxin adopted a flatter surface topology similar to classical mammalian-specific α-toxins (Fig. 6c) [114]. The flatter topology of the BmK M1 K8D mutant did not result in increased activity in mammals; in fact, the mutant completely lost mammalian activity, with no symptoms observed in mice at 47 times the LD_{50} of native BmK M1 [115]. The mutant was also 12-fold less toxic in insects compared to the wild-type toxin. Moreover, a K8Q mutant of BmK M1 that retained the *cis* 9–10 peptide bond conformation and protruding topology of the wild-type toxin was 200-fold less potent at insect Na_v channels than the native toxin. The activities of the BmK M1 mutants demonstrate that phyletic selectivity is not solely determined by the conformation of the NC-domain, as suitable side-chain chemistries are required in order for the toxin to be able to interact with the target Na_v channel.

Although the topology of the NC-domain is usually indicative of the residue at position 8 and the conformation of the 9–10 peptide bond, this is not always the case, as exemplified by the venom peptide BmKαTx11. The NC-domain of this peptide is protruding, even though the Asp at position 8 and the 9-10 *trans* peptide bond usually gives rise to a flat topology [116]. Closer examination of the 3D structure of BmKαTx11 and other α-toxins revealed that the hydrogen bonding properties of residue 58 (or equivalent position in relation to BmKαTx11) is another factor that can determine the orientation of the NC-domain. In anti-insect α-toxins and some α-like toxins, residue 58 is Arg compared to Lys in all classical α-toxins. A non-polar Val or Ile is also found in this position in various α-like toxins [116]. The side chains of Lys and Arg are both able to form hydrogen bonds and they indeed do so with the backbone of a Gly residue that is highly conserved within the C-terminus of all α-toxins. However, the slightly shorter side chain of Lys draws in the Gly residue to a greater extent than Arg, thus bringing the entire C-terminus closer to the bulk of the toxin [111, 113, 117]. This results in the flat topology of the classical α-toxins, while the less restrained C-terminus of toxins with Arg58 presents as a protrusion. Since Val or Ile cannot form hydrogen bonds, many α-like toxins with these residues at position 58 also have a protruding NC-domain [118].

Aside from the core and NC-domain, several additional functionally important sites have been identified. Many of these sites are toxin-specific and are not part of the functional surface of other α-toxins [119]. Furthermore, the five-residue turn in the NC-domain is not involved in the activity of some α-toxins. In the α-like toxin Lqh3, neutral or charge-inverted substitutions in the five-residue turn did not significantly decrease insecticidal activity, with the residues essential for activity located in the core and C-terminal segment [112]. While the functional surface of α-toxins are similar, these subtle site-specific differences may underlie the variation in activity between family members. Through further research, a more thorough understanding of the sites that increase toxin preference for insect rather than mammalian channels will hopefully allow molecular tuning of α-toxins to improve their selectivity for insects, thereby rendering them suitable for bioinsecticide development.

Recent mutagenesis studies [120] have identified residues within Na_V channel site 3 that appear to be important for sensitivity toward either anti-insect or anti-mammalian α-toxins. Substitution of Glu1613 in the DIV/S3-S4 loop of the rat $Na_V1.2$ channel with Asp, the residue in the equivalent position (1,701) of the *Drosophila* $DmNa_v$ channel, rendered the channel ~1,000-fold more sensitive to LqhαIT without any loss of sensitivity to the classical α-toxin Lqh2. However, the reciprocal replacement of Asp1701 in $DmNa_V$ with Glu did not increase the sensitivity of the channel to Lqh2. $DmNa_V$ gained sensitivity to Lqh2 only upon replacement of external loops between segments of DIV and/or DI. These results suggest receptor site 3 is similar but not identical between mammalian and insect Na_V channels. Further studies are required to pinpoint the insect receptor residues that are involved in interacting with anti-insect α-toxins and to determine whether these receptor residues are important for the activity of other site 3 toxins from scorpions and other organisms. Thus, only the DIV/S3-S4 loop in Fig. 2 is currently assigned as site 3. Future designations of site 3 may include regions of the external loops between segments of DI.

CSαβ toxins targeting neurotoxin receptor site 4

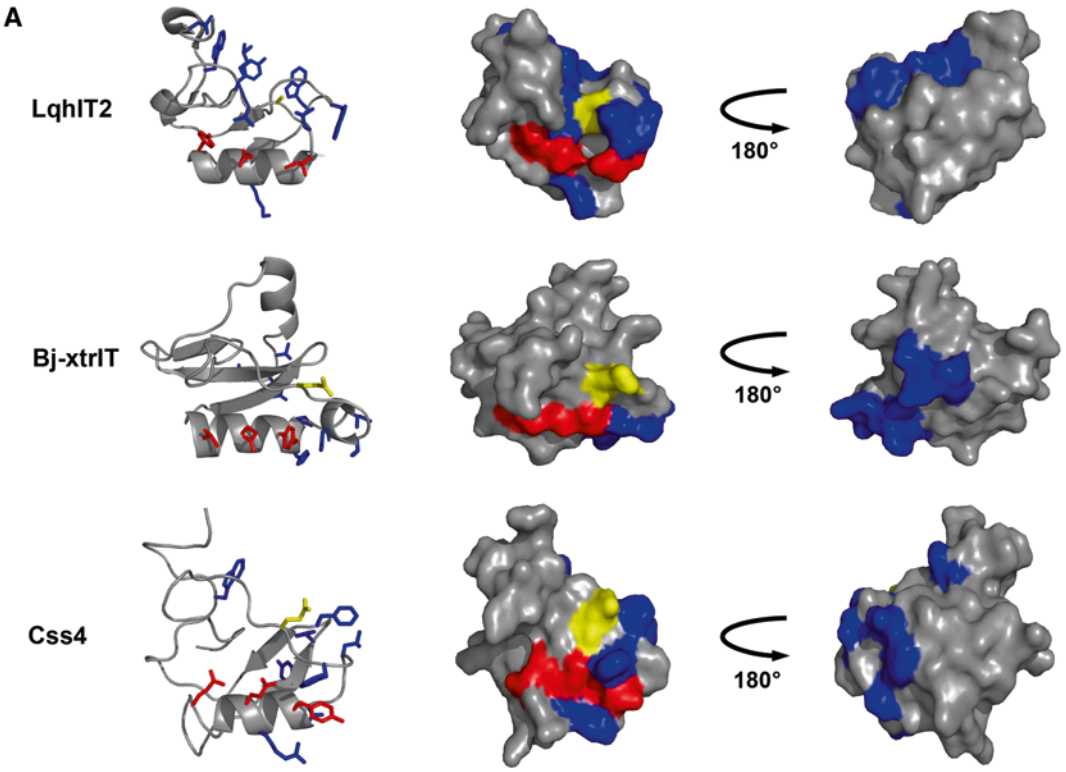
Also known as β-toxins, scorpion toxins that bind site 4 of Na_v channels are divided into four categories based on their activity: (i) anti-mammalian toxins; (ii) β-like toxins that are active on both insect and mammalian channels; (iii) depressant anti-insect toxins; and (iv) excitatory anti-insect toxins. Depressant anti-insect β-toxins induce flaccid paralysis in insects, while excitatory anti-insect β-toxins cause

immediate contractile paralysis upon injection [11]. Similar to the mode of channel interaction proposed for scorpion α-toxins, it is believed channel binding is mediated by a primary pharmacophore common to nearly all β-toxins, while a second cluster of variable amino acids determines phyletic specificity [48]. The solvent-exposed primary pharmacophore consists of a spatially conserved Glu residue located on the core α helix that is flanked by hydrophobic residues (Fig. 8a, b). Site-directed mutagenesis of the Glu and hydrophobic residues in this 'hot spot' region has been shown to severely decrease the binding affinity of various β-toxins [48, 121, 122]. It is thought that the hydrophobic residues act as a gasket, occluding bulk solvent and sealing the point of interaction between Glu and interacting residues on the channel, which are yet to be determined. This pharmacophore, which is common to most β-toxins, may explain their ability to competitively bind Na_v channel types at which they show no activity. For example, the mammalian-specific β-toxin Cn2 is not toxic to insects, yet it competes with the excitatory insect toxin Bj-xtrIT (PD_{50} 4.9 pmol g^{-1} in *Sarcophaga falculata*) for binding at insect Na_v channels [48, 123]. It should be noted that several β-toxins do not have an acidic residue at the 'hot spot' region and further investigations are necessary to elucidate the functional surface of these toxins.

The second bioactive surface believed to be responsible for phyletic selectivity is formed by a group of hydrophobic residues. Based on mutagenesis of Bj-xtrIT and Css4, this surface is proposed to be located at the C-terminus of excitatory anti-insect β-toxins and the β2 strand and loops connecting secondary structures in anti-mammalian toxins (Fig. 8a, b) [48, 121, 123]. In the depressant insect-selective toxin LqhIT2, this hydrophobic region is positioned in a groove preceding the α-helix near the N-terminus, termed the 'N-groove' [122]. Residue Ala13 in the N-groove of LqhIT2 has a particularly interesting role. Substitution of Ala13 with a charged residue (Glu or Arg) was detrimental to binding, however substitution with Trp dramatically increased toxin activity at the insect Na_v channel with very little effect on binding affinity [122]. Glu15 in Bj-xtrIT and Css4, the residue in the same spatial position as Ala13, appears to play a similar functional role. An uncoupling of activity from binding was seen in E15R mutants of Bj-xtrIT and Css4, as both toxins were rendered inactive by this mutation while incurring only a slight decrease in binding affinity [48, 121]. As mentioned in the section above on ICK spider toxins that bind site 4 of Na_v channels, Glu15 may be functionally analogous to Asp19 in δ-amaurobitoxin-Pl1b [45].

The chemical nature of residue 13 in LqhIT2 and its equivalent position in other toxins can also affect the overall topology of the N-groove. A structural comparison of potent and weak β-toxins revealed that highly active β-toxins possess a deep N-groove about 8 Å in width and depth, while the groove is shallow and less defined in weakly active toxins [122]. Both binding and activity were abrogated in an E15Q mutant of Bj-xtrIT, whose N-groove was distorted from the deep hollow of the wild-type toxin to a more protruding structure; thus, the loss of activity in toxins with mutations at this position might be attributed to structural perturbances of the N-groove [48, 112]. Additionally, subtle differences within the N-groove of potent β-toxins may contribute to phyletic selectivity. In the anti-insect toxin LqhIT2 and β-like toxin Ts1, a Lys side chain lies at the bed of the N-groove, while the equivalent Lys in the anti-mammalian toxin Cn2 forms an ionic interaction with Glu15 and bends back, revealing a narrow cavity [122, 124, 125]. A mutant of the Cn2 homologue Css4 was made in which Glu15 was replaced with Ala, thereby removing the interaction restraining Lys and allowing its side chain to assume a similar position in the N-groove as LqhIT2 and Ts1. While wild-type Css4 is non-toxic to insects, the E15A mutant gained activity at the insect Na_v channel and induced contractile paralysis in blowfly larvae. The E15A Css4 mutant also retained high binding affinity and activity at mammalian Na_v channels, suggesting that the residue at the bed of the N-groove may be important for activity in insects but not mammals [121, 122].

Excitatory and depressant insect β-toxins interact with different regions of Na_v channel site 4. Depressant toxins bind at two non-overlapping regions, one with high affinity and low capacity, and the other with low affinity and high capacity [126, 127]. Excitatory toxins interact solely with the high affinity site, as demonstrated by competition binding assays using locust and cockroach membranes which revealed that excitatory toxins are only able to compete with depressant toxins for binding to the high affinity site [127–129]. The location of the common high affinity binding site is suggested by Na_v channel mutagenesis studies that have shown both the excitatory toxin AahIT and the depressant toxin BmK IT2 interact with a region located on domain two of insect Na_v channels, with the receptor site of BmK IT2 further isolated to the S3-S4 linker [130, 131]. This region is essential for the response of the channel to toxin as mutations of G904, E896 or L899 in the S3-S4 linker of domain two rendered the channel insensitive to BmK IT2 [131]. However, these residues are well conserved in mammalian Na_v channels upon which BmK IT2 does not act, and therefore these residues likely mediate the interaction of the toxin with the voltage sensor after initial binding but not the phyletic preference of β-toxins. BmK IT2 also interacts with the N-part of the domain 3 SS2-S6 loop and residues Ile1529 and Arg1530 of the channel, and it is this region that may be the recognition epitope for insect-selective depressant β-toxins [131]. The DII S3-S4 and DIII SS2-S6 linkers of mammalian channels are also important



B

anti-mammalian

```
                    10        20        30
Css4     KEGYLVNSYTGCKFECFKLG--DNDYCLRECRQQYGKG
Css2     KEGYLVSKSTGCKYECLKLG--DNDYCLRECKQQYGKS
Cn2      KEGYLVDKNTGCKYECLKLG--DNDYCLRECKQQYGKG
```

anti-insect excitatory

```
Bj-xtrIT KKNGYPLDRN-GKTTECSGVNAIAPHYCNSECTKVYY-A
AahIT1   KKNGYAVDSS-GKAPECL-----LSNYCNNECTKVHY-A
BmKIT-AP KKNGYAVDSS-GKVAECL-----FNNYCNNECTKVYY-A
```

anti-insect depressant

```
LqhIT2   DGYIKRRD-GCKVACL----IGNEGCDKECKA--YGG
BmKIT2   DGYIKGKS-GCRVACL----IGNQGCLKDCRA--YGA
BotIT4   DGYIRRRD-GCKVSCL----FGNEGCDKECKA--YGG
BmKITc   DGYIRGSD-GCKVSCL----WGNDFCDKVCKK--SGG
BotIT6   DGYPKQKN-GCKYDCI----INNKWCNGICKM--HGG
Bs-dprIT1 DGYILMRN-GCKIPCL----FGNDGCNKECKA--YGG
OsI1     DGYPKQKD-GCKYSCT----INHKFCNSVCKS--NGG
```

```
                    40        50        60        70        81
Css4     SGGYCYAFG--CWCTHLYEQAVVWPLPNKTCN*
Css2     SGGYCYAF--ACWCTHLYEQAVVWPLPNKTCN*
Cn2      AGGYCYAF--ACWCTHLYEQAIVWPLPNKRCS*

Bj-xtrIT ESGYCC-WG-ACYCFGLEDDKPIGPMKDITKKYCDVQIIPS
AahIT1   DKGYCCL--LSCYCFGLNDDKKVLEISDTRKSYCDTTIIN
BmKIT-AP DKGYCCL--LKCYCFGLADDKPVLDIWDSTKNYCDVQIIDLS

LqhIT2   SYGYCWTWGLACWCEGLPDDKT-WKSETNTCG*
BmKIT2   SYGYCWTWGLACWCEGLPDNKT-WKSESNTCG*
BotIT4   SYGYCWTWGLACWCEGLPDDKT-WKSETNTCG*
BmKITc   SYGYCWTWGLACWCEGLPDNEK-WKYESNTCGS
BotIT6   YYGYCWGWGLACWCEGLPEDKK-WWYETNKCGR
Bs-dprIT1 SYGYCWTYGLACACEGQPEDKKHLNYHKKTC
OsI1     DYGYCWFWGLACWCEGLPDNKM-WKYETNTCG*
```

◀ **Fig. 8** Scorpion β-toxins. **a** Structures of representative anti-mammalian (Css4, homology model from [48]), anti-insect excitatory (Bj-xtrIT, PDB 1BCG), and anti-insect depressant (LqhIT2, PDB 2I61) scorpion β-toxins. *Red* denotes the primary pharmacophore located on the α-helix consisting of a central Glu flanked by two hydrophobic residues. *Blue residues* comprise the secondary pharmacophore. Glu15 in Bj-xtrIT and Css4, and Ala13 in LqhIT2 are colored *yellow*. **b** Primary structures of representative scorpion β-toxins

for their interaction with anti-mammalian β-toxins, as well as the DII S1-S2 linker [132, 133]. However, the involvement of the DII S1-S2 linker in binding of scorpion toxins by insect Na_v channels remains to be established. The endeavor to dissect the structure–activity relationships between β-toxins and the insect Na_v channel has uncovered details that aid our understanding of the functional surfaces required for insect-specificity. Nevertheless, much remains unknown, including the molecular basis underlying the differences in sensitivity of various insect species to some β-toxins. For example, AahIT is at least 100-fold more toxic to *Sarcophaga falculata* blowflies (LD_{50} 18 pmol g^{-1}) than the larvae of the cotton leafworm *Spodoptera littoralis* (LD_{50} 1791 pmol g^{-1}), and it appears to be non-toxic to the tenebrionid beetle *Trachyderma philistina* [134–136]. This variable toxicity towards insects of different families may be employed to develop insecticides targeting pest species while reducing the negative impact on beneficial insects such as pollinators and parasitoids.

Scorpion CSαβ toxins targeting insect K_v channels

K_v channel blockers are the second most abundant peptides characterized from scorpion venoms after Na_v channel toxins, with over 200 sequences described to date [80]. Despite such a large pool of scorpion-derived K_v channel toxins, only one shows high preference for insect over mammalian K_v channels. Named BoiTx1, this toxin is at least 100-fold more active on the *Drosophila* shaker K_v channel than a range of human and rat K_v channels, including the mammalian shaker homologues $K_v1.1$ and $K_v1.3$ [137]. The shaker channel is involved in cellular repolarization after the initial depolarization phase of an action potential [138], and its blockage leads to repetitive firing and persistent action potentials. The resulting phenotype includes sustained muscle contraction, which is observed in *Drosophila* larvae upon injection with BoiTx1 [137]. The closest orthologue of BoiTx1 with 84 % sequence identity is agitoxin 1, which is only ten times more potent on shaker than $K_v1.3$ [139]. Scorpion toxins that are active on Shaker and mammalian shaker-like channels are classified as members of the α-KTx3 family [140]. Scanning mutagenesis of agitoxin 2 revealed that residues Arg24, Lys27, and Asn30 are crucial for toxin binding to the shaker channel and that these residues are conserved throughout the α-KTx3 family (Fig. 9a, b) [141]. Although the structure–activity relationship between the insect Shaker channel and BoiTx1 has not been fully dissected, sequence comparison with other α-KTx3 toxins has highlighted a few residues that may provide BoiTx1 with its relatively marked preference for insect channels. This includes the lack of a C-terminal basic residue that is present in most other α-KTx3 toxins and which is hypothesized to affect the spatial position of the critical Arg24 and its predicted interaction with a Glu residue on the Shaker channel [137, 142]. The discovery of BoiTx1 and its preference for insect K_v channels has presented the Shaker channel as a novel insecticidal target. Simultaneous deployment of Shaker channel blockers and sodium channel modulators could potentiate their insecticidal actions [137] and reduce the likelihood of resistance arising. Future mutagenesis studies coupled with the discovery of insect-specific α-KTx3 toxins may reveal the functional surfaces required for Shaker channel specificity.

DDH fold

Composed of a double-stranded β-sheet core stabilized by two disulfide bonds, the disulfide-directed hairpin, or DDH fold, is considered the evolutionary precursor to the ICK fold (Table 1) [65, 143]. Several proteins from evolutionarily diverse organisms, such as the cellulose-binding domain of cellobiohydrolase I from the *Trichoderma reesei* fungus and the pancreatic lipase cofactor colipase from various vertebrates either contain or appear to have arisen through elaborations of the DDH fold [65]. In venomous species, it is hypothesized that the ICK fold arose from the addition of a single disulfide bond to the DDH fold (Fig. 1a), resulting in the characteristic disulfide-pierced ring structure [65, 143]. The dominance of the ICK fold over the DDH fold in venoms presumably results from the extraordinary chemical and thermal stability and greater resistance to enzymatic degradation conferred by the cystine knot [23]. Nevertheless, toxins that adopt the simpler DDH fold have been found in the venom of spiders and scorpions, though they are uncommon. ω-TRTX-Ba1a and ω-TRTX-Ba1b are two insect-selective DDH toxins from the venom of the Mexican golden redrump tarantula *Brachypelma albiceps* that are lethal to crickets (Ba1a: LD_{50} 2,451 pmol g^{-1}, Ba1b: 2,072 pmol g^{-1} in *Acheta domestica*) but non-toxic to mice when injected intracranially or intraperitoneally [144]. Although these toxins contain three disulfide bonds, they are not arranged in the ICK motif [144]. The molecular target for these toxins have not been elucidated, however they have been provisionally assigned the ω prefix based on sequence homology to the ICK toxin ω-TRTX-Asp1a which is active on vertebrate Ca_v channels (Fig. 10) [145]. Two-disulfide DDH peptides with activity across various insect families

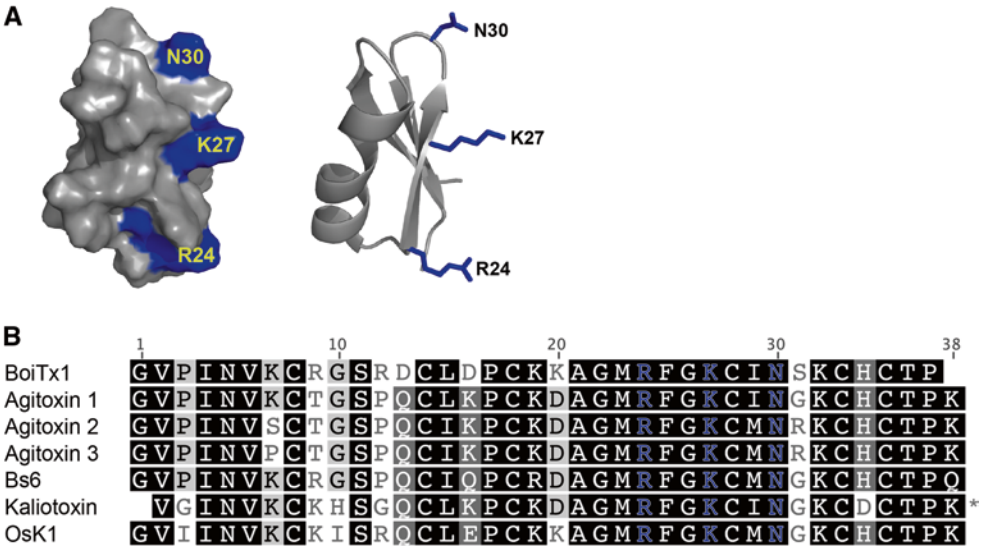


Fig. 9 Scorpion K_V toxins. **a** Surface (*left*) and ribbon (*right*) representations of agitoxin 2 (PDB 1AGT), with residues crucial for binding to $K_V1.3$ channels highlighted in *blue*. **b** Representative sequences of members of the α-KTx3 scorpion toxin family

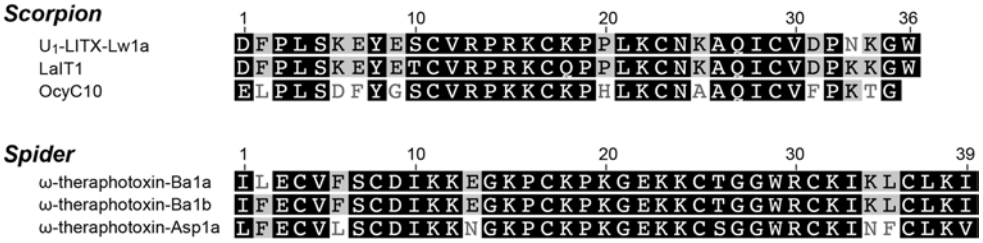


Fig. 10 Arachnid venom peptides with a disulfide-directed β-hairpin (DDH) fold

have also been isolated from the venom of *Liocheles* and *Opisthacanthus sp.* scorpions [143, 146, 147].

Linear peptides

Non-disulfide bonded peptides are ubiquitous in nature and serve many purposes, from neurotransmission [148, 149] to forming spider silk [150]. A major property of linear peptides in venoms appears to be antimicrobial activity and it is has been proposed they may protect venom glands from microbial infection [151], although this seems unlikely in the case of spiders [152]. In communal arthropods such as ants and bees, antimicrobial excretions may protect the colony from pathogens and prevent fungal and bacterial outbreaks in the nest or hive [153, 154].

Linear venom peptides are usually amphipathic and although they are structurally disordered in aqueous solution, they typically adopt an α-helical conformation in the presence of membranes containing negatively charged lipids [24], thereby forming pores that result in cell lysis. It is unlikely that they target a specific receptor as they generally display broad-spectrum antimicrobial, antifungal, and cytolytic activity, though their activity profile varies [155–157]. Some linear antimicrobial venom peptides also have low levels of insecticidal activity, presumably due to local tissue damage caused by lysis [158, 159]. Their weak insecticidal activity and lack of a specific molecular target renders the linear venom peptides unsuitable as standalone insecticides.

It has been proposed that their primary role of cytolytic venom peptides is to potentiate the action of the disulfide-rich neurotoxins by breaking down anatomical barriers, dissipating transmembrane ion gradients, and/or perturbing the membrane potential across excitable cells [152]. Thus, they could potentially be used in combination with other venom peptides to enhance bioactivity. Consistent with this idea, it was demonstrated that the combined injection of a disulfide-rich spider-venom neurotoxin and the linear spider-venom peptide M-oxotoxin-Ot1a into tobacco cutworms resulted in over tenfold reduction in IC_{50} compared with injection of the neurotoxin alone [160]. Furthermore, the time required for the neurotoxin to exert its paralytic and lethal effects on the larvae was greatly reduced upon co-injection with the linear peptide. A synergistic effect was also observed between the linear scorpion-venom peptide pandinin-2 and spider neurotoxins [160]. These amphipathic linear peptides therefore have the potential to augment the efficacy of insecticidal neurotoxins. However, a targeted delivery approach may need to be implemented to ensure their cytotoxic effects are localized to

Fig. 11 Homology between linear conomap venom peptides and myoactive tetradecapeptides (MATPs). *Asterisks* indicates an amidated C-terminus, while *lowercase letters* indicate D-amino acids

pest invertebrates and do not adversely affect vertebrates or the crop that is being produced.

An unusual linear peptide named conomap-Vt from the venom of *Conus vitulinus* was found to be homologous to myoactive tetradecapeptides (MATPs) found in annelids, molluscs and insects (Fig. 11) [161]. MATPs are short linear peptides with excitatory or inhibitory activity in invertebrate muscles, however their pharmacological targets have not been ascertained [162, 163]. Conomap-Vt exerted a potent excitatory contractile effect on a range of molluscan muscle preparations without being active on isolated rat atria and ileum [161]. While the activity of conomap-Vt in insects is yet to be determined, its similarity to MATPs suggests it will be active in arthropods. Further characterization of this interesting peptide is required to determine its suitability as a candidate for insecticide development.

Defensin-like and neurotoxin III fold

Peptides of the defensin-like structural fold are disulfide-rich and consist primarily of β strands (Table 1). Included in this fold are the human β-defensins involved in innate immunity and melanogenesis [164, 165], as well as the related bovine and murine defensins. Akin to the CSαβ defensins, the crotamine myotoxins in rattlesnake venoms and the β-defensin-like peptides in platypus venom arose from duplication and functional diversification of β-defensin genes [20, 166, 167]. Defensin-like toxins are also one of the major components of sea anemone venoms and comprise the type 1 class of anemone toxins active on Na_v channels [168]. Although rich in structurally diverse peptides that target various Na_v and K_v channels, the only sea anemone toxins found to show significant selectivity for insect over mammalian channels are the Na_v channel ligands Nv1, BgII and Av3 (Fig. 12) [169–172]. Nv1 and BgII are defensin-like toxins belonging to the type 1 class of anemone toxins. Av3 is comprised of four reverse turns and two chain reversals with no α-helix or β-sheet structures [169, 173, 174]. Av3 has no structural homologues and thus defines the monoclastic neurotoxin III fold. Nv1 is hypothesized to bind Na_v channels at site 3 as it presents the typical site 3 modulatory action of inhibiting channel inactivation [169]. Av3 and BgII also inhibit inactivation of insect Na_v channels and were shown to compete with the site-3 scorpion toxins LqhαIT, Aah II, and the sea anemone toxin CgNa, respectively [171–173]. As mentioned in the previous section on ICK toxins from cone snails, the ability of sea anemone toxins to target insects may be due to conservation between insect and marine arthropod ion channels. Additionally, many insect larval stages subsist in water where they are likely to encounter sea anemones. Indeed, the anemone *Nematostella vectensis* from which the toxin Nv1 was discovered includes insect larvae in its diet [175].

Of the three described insecticidal sea anemone toxins, Av3 has the highest preference for insects, being at least 300-fold more toxic to blowfly larvae (PD_{50} 11.5 pmol g^{-1} in *Sarcophaga falculata*) than mice and having a negligible effect at 10 μM on mammalian $Na_v1.2$, $Na_v1.3$, $Na_v1.5$, and $Na_v1.6$ channels expressed in *Xenopus* oocytes [172]. Although the activity of Nv1 in mammals has not been established, it had an insignificant effect on rat $Na_v1.2$ and $Na_v1.4$, as well as human $Na_v1.5$ channels at 25 μM, while 1 μM completely abolished inactivation of the *Drosophila* $DmNa_v$ channel [169]. BgII is at least 15-fold more potent on $DmNa_v$ channels than $Na_v1.2$, the most sensitive mammalian channel tested [171]. However, BgII is potent in mice upon i.c.v. injection, causing toxicity at 80 pmol/kg, and it binds rat brain synaptosomes with a K_d of 9 nM [173]. Consequently, BgII is unlikely to be a useful insecticidal lead, although ascertaining the molecular epitopes that confer its toxicity to both insects and mammals might allow engineering of more insect-selective analogues. APETx3 is the most recently discovered insect selective sea anemone toxin; it is eightfold less potent at the most sensitive mammalian channel tested ($Na_v1.6$) than at $DmNa_v1$ and the cockroach $BgNa_v1.1$ channel [170]. Additional structure–function studies of this group of toxins and testing over a more comprehensive array of channels, tissues, and whole organisms are necessary to better understand their mechanism of action and phyletic selectivity.

Most research on sea anemone toxins has focused on their interaction with mammalian channels, with very

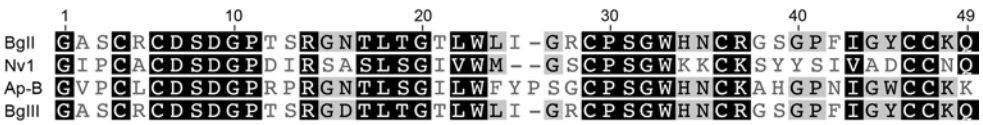


Fig. 12 Sea anemone toxins with defensin-like and neurotoxin III folds

few studies investigating the basis of toxin preference for insect or mammalian targets. Alanine mutagenesis of surface residues of the insect and mammalian active type 1 toxin Av2 revealed that the anti-insect bioactive surface is comprised of residues Val2, Leu5, Asp9, Asn16, Leu18 and Ile41. Five of these residues were also important for activity of Av2 on the human $Na_v1.5$ channel, and therefore the toxin surfaces involved in the insect and mammalian bioactivity of Av2 appear to be similar [176]. Additionally, the residues equivalent to Asp9, Asn16 and Leu18 in the highly potent mammalian type 1 toxin Anthopleurin-B (Ap-B) are also functionally significant [177–179]. The role of Asn16 in insect selectivity is further highlighted by comparison of the activities BgII and BgIII. BgII is at least 180-fold more active on insect Na_v channels than BgIII, though differing by only a single residue at position 16; Asn in BgII and Asp in BgIII [171, 173]. The six residues important to the insecticidal activity of Av2 are not, however, conserved throughout all type 1 toxins that are able to affect insects. Four of the six residues are conserved in BgII while only three are present in Nv1 [169, 173, 176]. This suggests the toxin face that interacts with the receptor, as well as the exact location of the toxin binding site within neurotoxin receptor site 3 on the Na_v channel varies between different type 1 toxins.

APETx3 differs from APETx1 by only one residue (position 3 in APETx1 is Thr, while it is a Pro in APETx3) (Fig. 12), yet the two toxins have vastly dissimilar pharmacological profiles. APETx1 is a promiscuous toxin, acting on the human ether-á-go-go related gene (hERG) K^+ channel ($K_V11.1$) and mammalian Na_v channel subtypes 1.2, 1.4, 1.5, 1.6 and 1.8 [170]. Unlike APETx3 however, APETx1 does not affect the insect Na_v channels $DmNa_v1$ and $BgNa_v1.1$. Moreover, APETx1 inhibits Na_v channel conductance and interacts with neurotoxin binding site 1, while APETx3 locks the channel in the open state and binds to site 3 [170]. The manifestation of such remarkably different functional profiles caused by a single amino acid change is a structure–activity relationship enigma. It is thought the Pro3 residue of APETx3 may introduce a structural kink that is not present in APETx1, resulting in a conformational change that may explain the functional differences [170]; however, detailed structural analyses are required to examine this hypothesis.

Other potential insecticidal leads

The sections above highlight the folds adopted by the majority of venom-derived insecticidal toxins known to date. There are also numerous other venom components with insecticidal properties, however they have not been studied in as much detail or they are non-discriminant in their action towards vertebrates and invertebrates. Thus, in the following sections, we provide only a brief discussion of these toxins.

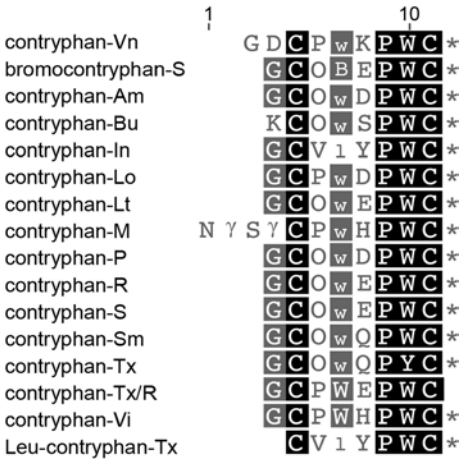


Fig. 13 Contryphan peptides. *Asterisks* indicate amidated C-termini and *lowercase letters* indicate D-amino acids. B and γ denote bromotryptophan and γ-carboxyglutamic acid, respectively

Contryphan fold

The contryphans are a group of 7–12-residue peptides from the venom of marine cone snails. Their 3D structure consists of a well-defined loop constrained by a single disulfide bridge (Table 1) and they are rich in unusual post-translational modifications including tryptophan bromination, proline hydroxylation and, characteristically, D-tryptophan or D-leucine [180]. Less than 20 contryphans have been discovered, with pharmacologically characterized members displaying mammalian toxicity (Fig. 13) [181]. However, Contryphan-Vn also affects voltage-gated and calcium-dependent K^+ channel currents in cockroach DUM neurons at a concentration of 20 μM [182]. While Contryphan-Vn modulates K^+ channels in cultured rat fetal chromaffin cells and very weakly binds to human $K_V1.1$ and 1.2 channels [182], further investigation into contryphans may uncover members with insect selectivity.

Three-finger snake toxins

The three-finger toxins found in snake venoms contain 60–74 amino acid residues and are crosslinked by 4–5 disulfide bonds (Table 1) [183]. They are named after their 3D characteristic structure composed of three β-stranded loops resembling fingers extending from a hand formed by the small hydrophobic peptide core [184]. While these toxins have primarily evolved to target nicotinic acetylcholine receptors (nAChRs) in vertebrate prey, a recent study demonstrated that six members can also block cockroach

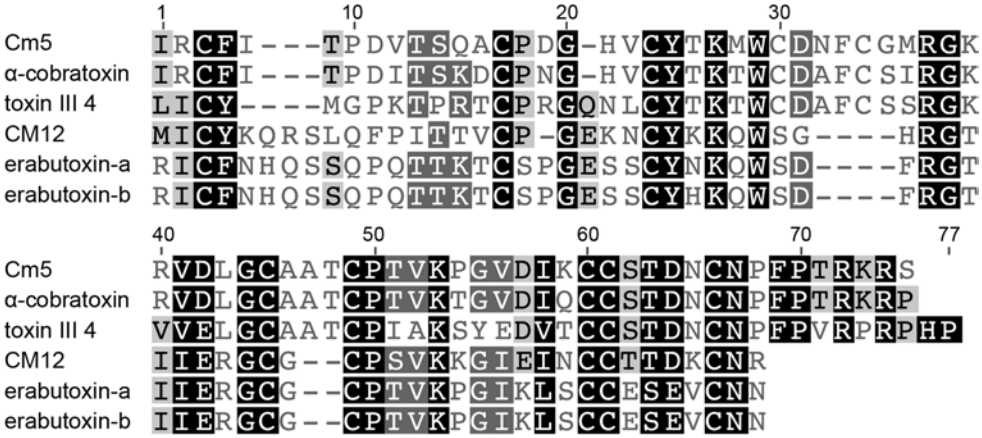


Fig. 14 Insecticidal three-finger toxins from snake venoms

nAChRs at low concentration (0.1 μM) (Fig. 14) [185]. With additional studies to dissect the molecular basis for their activity at invertebrate nAChRs, the three-finger toxin fold may become a useful scaffold for engineering insecticides.

Proteins

In addition to small neurotoxins, venoms from many organisms also contain larger proteins and enzymes that often have insecticidal activity. For example, the large latroinsectotoxins found in the venom of widow spiders (genera *Latrodectus* and *Steatoda*) are the most potent insecticidal toxins isolated to date from spider venoms. Latroinsectotoxins induce exhaustive neurotransmitter release at insect neuromuscular junctions, and they have extremely low LD_{50} values of <1 pmol g^{-1} in both lepidopterans and dipterans [186]. However, they have not been pursued as bioinsecticide leads due to their large size (110–140 kDa), complex mode of action, and the difficulty of producing them using synthetic or recombinant methods [24].

A number of proteins of mass 16 kDa and larger isolated from the venom of the small ectoparasitic wasp *Bracon hebetor* cause flaccid paralysis in invertebrate pest species including the tobacco budworm [187]. Many venoms also contain enzymes that are likely to be intrinsically insecticidal in addition to potentiating the activity of disulfide-rich neurotoxins found in venom [24]. For example, sphingomyelinase found in the venom of sicariid siders was recently shown to be lethal to crickets [188]. However, these enzymes are unlikely to be useful insecticidal leads as their activity often extends to vertebrates. For example, sphingomyelinase is responsible for the dermonecrotic lesions and serious systemic effects in humans (loxoscelism) that sometimes result from bites by sicariid spiders [189]. Moreover, unlike peptides, enzymes have the disadvantage of being more difficult and costly to produce on a commercial scale.

Deployment of insecticidal venom peptides

Orally active fusion proteins

In contrast with chemical insecticides, venom peptides are unlikely to be topically active since they would have to penetrate the insect exoskeleton in order to access their molecular targets in the insect nervous system. Thus, in order to be effective they must be delivered to insect pests via a vector such as an entomopathogen or ingested by the targeted insect pest if they have oral activity.

While some insecticidal venom peptides are orally active [190], most are ~90-fold less potent when fed to insects compared to when they are injected [24]. This lower activity results primarily from a slow rate of absorption in the insect gut, as observed for disulfide-rich peptides from scorpion and snake venoms [191]. Thus, the commercial potential of insecticidal venom peptides would be enhanced by any approach that significantly improved their oral activity. One promising option is to fuse venom peptides with a carrier protein that facilitates their transport across the insect gut. The best studied fusion protein for this purpose is *Galanthus nivalis* agglutinin (GNA), a mannose-specific lectin from the snowdrop plant. When ingested by insects, GNA binds to glycoproteins in the digestive tract and is subsequently transported across the gut epithelium into the hemolymph; over a period of several hours, the protein accumulates in the insect gut, Malpighian tubules, hemolymph, and central nervous system [192, 193]. Thus, GNA can be fused to insecticidal peptides to enhance their transport across the gut to sites of action in the nervous system, thereby enhancing their oral activity. This approach has been used to massively

enhance the oral insecticidal activity of a variety of venom peptides from both spiders and scorpions [79, 193–195].

Enhanced entomopathogens

Since venom peptides are genetically encoded mini-proteins, an alternative method of peptide delivery is to engineer entomopathogens to express transgenes encoding these toxins. For example, transgenes encoding a variety of insecticidal arachnid and sea anemone toxins have been engineered into lepidopteran-specific baculoviruses. Wild-type baculovirus are generally not competitive with chemical insecticides because they typically take a week or longer to kill their host, during which time the insect continues to feed and cause crop damage [196]. In contrast, the time between virus application and the cessation of feeding was dramatically reduced in transgenic viruses engineered to express a variety of insecticidal venom peptides [12].

The potency and speed of kill of the entomopathogenic fungus *Metarhizium anisopliae* can also be enhanced by engineering it to express insecticidal venom peptides. For example, a transgenic fungus expressing the scorpion-venom peptide AahIT reduced the kill time as well as the dose required to kill the tobacco hornworm *Manduca sexta* and the dengue vector *Aedes aegypti* [197]. Moreover, mosquitoes infected with transgenic fungus had a reduced tendency to blood feed [197].

Engineering entompathogens to express insecticidal venom-peptide transgenes mitigates two of the potential disadvantages of venom peptides as bioinsecticides. First, in this scenario, the venom peptides are produced systemically in the insect host after viral/fungal infection, and hence lack of oral activity is not an impediment to toxin deployment. Second, the phylum selectivity of the venom peptide becomes unimportant as the range of affected insects will be determined largely by the host range of the entomopathogen. This should limit off-target effects, particularly on predators and parasitoids, since entomopathogens can be chosen that have a very restricted host range; for example, *Metarhizium acridum* exclusively infects grasshoppers in the suborder Caelifera [198] and hence it is ideal as a locust-specific bioinsecticide.

Transgenic plants

Transgenes encoding insecticidal venom peptides can also be engineered into crop plants. In 2010, 148 million hectares of genetically modified (GM) crops were planted in 29 countries, representing 10 % of all cropland [199]. The active transgene in all extant insect-resistant GM crops encodes an insecticidal protein known as δ-endotoxin, Cry toxin, or simply *Bt* from the bacterium *Bacillus thuringiensis*. Insecticidal venom peptides might represent viable alternatives to *Bt* transgenes as they have vastly different modes of action and different phylum selectivities.

A number of plants have been engineered to express insecticidal spider-venom peptides, beginning in 1996 with the demonstration that transgenic tobacco expressing ω-HXTX-Ar1a from the Australian funnel-web spider *Atrax robustus* have enhanced resistance to larvae (cotton bollworms) of the recalcitrant lepidopteran pest *Helicoverpa armigera* [200]. Transgenes encoding this venom peptide (or its orthologue ω-HXTX-Hv1a) have subsequently been engineered into cotton [201], tobacco [202], and poplar [203]. Tobacco plants have also been engineered to express Magi-6, a 36-residue insecticidal peptide from the venom of the related hexathelid spider *Macrothele gigas* [204]. All of these transgenic plants have enhanced resistance to lepidopteran pests and it has even been claimed that transgenic cotton expressing ω-HXTX-Hv1a is as effective as Monsanto's pyramided Bollgard II® cotton for controlling major cotton pests [201].

Thus, transgenes encoding insecticidal venom peptides hold promise as a standalone insect-resistant plant traits. Moreover, these transgenes might be good candidates for trait stacking with *Bt* since they have completely different mechanisms of action and are likely to be synergized by *Bt*, which should facilitate movement of venom peptides into the insect hemocoel by virtue of its ability to induce lysis of midgut epithelial cells [205].

Future prospects and concluding remarks

The armamentarium of insecticides for control of insect pests is rapidly diminishing due to the evolution of insecticide resistance and the de-registration of key insecticide classes due to concerns about their impact on human health and the environment. Consequently, there is a pressing need for the development of novel ligands for current insecticide targets or, better still, ligands with novel modes of insecticidal action. Due to their high potency, stability, and molecular and organismal selectivity, a number of venom peptides are being used as leads for bioinsecticide development. The current review has showcased the multitude of insecticidal toxins present in animal venoms and their wide range of molecular targets. Although the putative functional surfaces of many insecticidal toxins have been deduced, there are clear knowledge gaps regarding how slight differences between toxins can result in large variations in channel selectivity. The answers to these questions undoubtedly lie in the further mapping of sites on receptors where toxins bind as well as studying the structural changes that occur in receptors upon toxin binding.

With few exceptions [206–211], methods to elucidate the structure of receptor–toxin complexes are mostly limited to computational studies. Technical advancements of existing

methodologies such as NMR spectroscopy and X-ray crystallography are needed to expedite the study of such complexes. A better understanding of the dynamic interactions between toxins and receptors would bring us closer to the ultimate goal of predicting the selectivity of toxins from their sequence or structure alone, bypassing the often laborious and time consuming process of biological screens. Despite these current limitations, the numerous insecticidal venom peptides discussed herein provide ample leads for insecticidal development. While these peptides could be used in a similar manner as chemical insecticides, for example as foliar sprays, the fact that they are genetically encoded mini-proteins opens the door to a wider variety of deployment methods, such as incorporation of transgenes encoding these peptides into crop plants and entomopathogens. Thus, venom-derived insecticidal peptides appear to have immense potential for the development of novel bioinsecticides.

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