Review

Peptide toxins that selectively target insect Na_v and Ca_v channels

Glenn F. King,1,* Pierre Escoubas2 and Graham M. Nicholson3

1Institute for Molecular Bioscience; University of Queensland; St Lucia, Queensland Australia; 2Institut de Pharmacologie Moléculaire et Cellulaire; Centre National de la Recherche Scientifique; Valbonne, France; 3Department of Medical and Molecular Biosciences; University of Technology—Sydney; Broadway, Sydney, Australia

Key words: Na_v channel, Ca_v channel, peptide toxin, venom, insecticide

Numerous metazoans express venoms for the purpose of defense, competitor deterrence or prey capture. Peptide neurotoxins are particularly well represented in the venoms of arachnids, cnidarians and mollusks and these toxins often possess high affinity and specificity for particular classes of ion channels. Some of these toxins have become the defining pharmacology for certain vertebrate ion channel subtypes. Unfortunately, due to differences in the structure, pharmacology and ion selectivity of insect voltage-gated sodium (Na_v) and calcium (Ca_v) channels compared with their vertebrate counterparts, these peptide toxins have proven less useful for the characterization of insect ion channels. Despite these disparities in channel structure and function, the armament of peptide toxins that specifically modulate the activity of insect ion channels is slowly expanding. This review focuses on insect-selective peptide toxins and their utility for the study of insect Na_v and Ca_v channels. The high affinity and selectivity of some of these neurotoxins means that they have the potential to become the defining pharmacology for specific subtypes of insect ion channels. In addition, it might be possible to exploit the phylogenetic specificity of these toxins as the basis for rational development of novel classes of insect channel insecticides.

Introduction

The range of metazoans that produce venoms for prey capture or defense is remarkably diverse and includes arthropods, cnidarians, mollusks and vertebrates.1 Cone snails and arachnids are especially remarkable because of the incredible diversity of peptide toxins expressed in their venom glands, with some venoms comprising more than 1000 unique peptides.2,3 These venomous animals have proved particularly useful from a pharmacological perspective because the majority of peptide toxins that they express appear to target specific subtypes of ion channels, receptors or transporters in the peripheral or central nervous system.1 These neurotoxic functions are perhaps not surprising given that the primary role of these venoms is to paralyze or kill venemated prey.4

Venom toxins have a long and storied history in the isolation and characterization of vertebrate ion channels. Tetrodotoxin, for example, was critical for Bernard Katz’s pioneering studies on the mechanism of synaptic transmission5 and a key reagent along with scorpion α-toxins for the subsequent purification and characterization of voltage-gated sodium (Na_v) channels.6,7 More recently, peptide toxins from cone snails, scorpions and spiders have proved to be the defining pharmacology for specific subtypes of vertebrate voltage-gated calcium (Ca_v) channels,8 calcium-activated potassium (K_{Ca}^+) channels,9 nicotinic receptors,10 and acid-sensing ion channels.11

Despite the impressive repertoire of peptide toxins that have been assembled for characterization of vertebrate ion channels, it turns out that insect Na_v and Ca_v channels are sufficiently different from their vertebrate counterparts that these pharmacological tools are often not useful for insect studies. Thus, although insects have a smaller repertoire of both Na_v and Ca_v channels than vertebrates, the isolation of specific pharmacological reagents for studying these channels has lagged well behind developments in the vertebrate arena. There are numerous reasons for this deficiency including the smaller community of researchers working on insect channels, the lack of functional cloned insect Ca_v channels,12 and a limited number of readily-accessible and well-characterized insect neuron preparations. However, despite these hurdles, the armament of peptide toxins that specifically modulate the activity of insect Na_v and Ca_v channels has slowly expanded in recent years. As outlined below, some of these toxins may ultimately prove to be the defining pharmacology for certain subtypes of insect ion channels.

An Overview of the Na_v/Ca_v Channel Superfamily

The pore-forming α subunit of Na_v channels and the α subunit of Ca_v channels together constitute a superfamily of structurally related voltage-gated ion channels. Na_v channels are transmembrane proteins that provide the current pathway for the rapid depolarization of excitable cells that is required to initiate action potential generation.13 Neurotoxin labeling, purification and subsequent functional expression showed that they are comprised principally of a single pore-forming 220–260 kDa glycoprotein known as the α-subunit.14 The α-subunit is composed of four internally homologous but non-identical domains (designated I–IV) connected by intracellular linkers (Fig. 1A).15–17 Each of these domains contains six putative transmembrane (TM) segments (S1–S6) joined by intracellular or extracellular loops. The four domains fold together in a clockwise orientation such that domains I and IV are brought
Figure 1. Schematic representation of the molecular structure and membrane topology of Na_v and HVA Ca_v channels. In each case, the pore-forming subunit (centre) comprises four homologous domains designated I–IV. Within each domain, the putative transmembrane-helical segments (S1–S6) are represented by cylinders, with the S4 voltage-sensor shown in red. The pore-lining segments S5 and S6 and the intervening P loop that form the walls of the ion-conducting pathway are shown in blue. (A) The Na_v channel inactivation gate [magenta] is represented by the inactivation particle (hydrophobic residues IFM in mammals) with magenta arrows indicating the sites thought to form the inactivation gate receptor. The extracellular domains of the 1 and 2 subunits are represented as immunoglobulin-like folds similar to myelin protein P_0, indicates sites of probable N-linked glycosylation. (B) HVA Ca_v channels typically comprises a single copy of each of the 1, 2-, and 3 subunits, whereas LVA Ca_v channels consist of only the pore-forming 1 subunit. The intracellular subunit comprises an N-terminal SH3 domain (blue) and a C-terminal guanylate kinase domain (cyan). The guanylate kinase domain binds the interaction domain (AID; purple) located in the cytoplasmic loop linking domains I and II of 1, and this interaction modulates the rate of channel inactivation. Calmodulin (CaM) interacts with Ca_v1 and Ca_v2 channels via a conserved sequence motif (the "IQ motif") located in the cytoplasmic C-terminal region of 1. The N- and C-terminal lobes of CaM (green) bind the IQ domain (red) at different sites and with different affinities, making this interaction sensitive to both global calcium levels and the concentration of calcium in the vicinity of the Ca_v channel pore. Calcium ions bound to CaM in the CaM-IQ complex are indicated by orange spheres. Adapted from refs. 12 and 66.
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Figure 2. Location of known neurotoxin receptor sites on Na\textsubscript{v} channels. Green circles represent the outer (EEDD) and inner (DEKA) rings of amino acid residues that form the ion selectivity filter and the proposed neurotoxin receptor site-1 for the water-soluble guanidinium toxins, tetrodotoxin (TTX) and saxitoxin (STX). Some \(\mu\)-conotoxin binding sites overlap with those of TTX and are omitted for clarity. In the case of receptor sites 3 and 4, only areas where mutagenesis of key residues leads to more than a 5-fold decrease in binding affinity are highlighted. Insect-selective toxins are highlighted in red text. Adapted from ref. 80.

into close proximity to form the outer pore vestibule. Membrane re-entrant loops (P-loops) between TM segments S5 and S6 from each domain dip into the transmembrane region of the protein and form the narrow ion-selectivity filter at the extracellular end of the pore (Fig. 1A).\textsuperscript{17,18} The S4 segments, which are the most highly conserved, contain a positively charged amino acid (Arg or Lys) at every third position; this segment serves as a ‘voltage sensor’ that initiates voltage-dependent activation by moving outward under the influence of changes in the electric field.\textsuperscript{19-23} In response to membrane depolarization the channel undergoes a conformational change to allow selective influx of Na\textsuperscript{+} ions through the pore. Sodium channel inactivation is mediated by a short intracellular loop connecting domains III and IV that contains a conserved stretch of three consecutive hydrophobic residues, namely Ile-Phe-Met in vertebrates or Met-Phe-Met in insects (Fig. 1A).\textsuperscript{24,25} In mammals, the \(\alpha\)-subunit is also associated with one or two smaller auxiliary subunits (\(Na_{\beta}1-\beta_{4}\)) of approximately 33–36 kDa that are required for normal kinetics and voltage-dependence of gating but are not required for ion flux, ionic selectivity and pharmacological modulation (Fig. 1A).\textsuperscript{26-28}

Ca\textsubscript{v} channels, which belong to the same ion channel superfamily, are key signal transducers that convert depolarization of the cell membrane into an influx of extracellular calcium ions. This ion influx mediates a wide range of critical intracellular processes since calcium is a second messenger that triggers muscle contraction, hormone and neurotransmitter release, regulation of enzymatic activities and altered patterns of gene expression.\textsuperscript{29} Ca\textsubscript{v} channels are divided into two broad families based on their voltage-dependence of activation: low-voltage-activated (LVA) Ca\textsubscript{v} channels are activated by small membrane depolarizations and show rapid voltage-dependent inactivation, whereas high-voltage-activated (HVA) Ca\textsubscript{v} channels are only activated by larger depolarizations and inactivate more slowly. The Ca\textsubscript{v}\(\alpha_1\) subunit is a protein of ~2000 amino acid residues and, as for the Na\textsubscript{v} channel, the Ca\textsubscript{v}\(\alpha_1\) subunit is sufficient to form a voltage-gated calcium-selective pore. The Ca\textsubscript{v}\(\alpha_1\) subunit is also analogous to the Na\textsubscript{v} channel \(\alpha\) subunit in structural organization\textsuperscript{30} being organized in four repeat domains (I–IV), each with six TM segments (S1–S6), and a membrane-associated re-entrant loop between S5 and S6 (Fig. 1B). Unlike the Na\textsubscript{v} channel, however, the HVA Ca\textsubscript{v}\(\alpha_1\) subunit is associated with auxiliary subunits that are unrelated to the various auxiliary mammalian Na\textsubscript{v}\(\beta\) subunits. The quaternary structure of HVA Ca\textsubscript{v} channels typically comprises 4–5 subunits (Fig. 1B): (i) a pore-forming \(\alpha_1\) subunit of 170–250 kDa; (ii) an extracellular \(\alpha_2\) subunit; (iii) a transmembrane \(\delta\) subunit that is covalently linked to \(\alpha_2\) via a disulfide bond to form an 170 kDa \(\alpha_2-\delta\) complex;\textsuperscript{31} (iv) an intracellular 50–78 kDa \(\beta\) subunit; and (v) in some cases, a transmembrane \(\gamma\) subunit of 25–36 kDa.\textsuperscript{32,34} In contrast, LVA Ca\textsubscript{v} channels appear to consist primarily of just the pore-forming \(\alpha_1\)
subunit, with little evidence of regulation by additional subunits.\(^{34-36}\)
In HVA Ca\(_V\) channels, the \(\alpha2\)-\(\delta\), \(\beta\) and \(\gamma\) subunits modulate properties of the \(\alpha\) subunit such as activation/inactivation kinetics and the voltage-dependence of activation.\(^{37,38}\) The \(\beta\) subunit additionally helps traffic the \(\alpha1\) subunit to the plasma membrane.\(^{39}\)

### An Overview of Insect Na\(_V\) Channels

While at least 18 genes encoding Na\(_V\)-like channels have been cloned from invertebrate species, most have not been functionally expressed.\(^{40,41}\) The first putative insect Na\(_V\) channel gene, para (encoding the \(\alpha\)-subunit of the DmNa\(_V\)1 channel), was isolated from the fruit fly Drosophila melanogaster in the late 1980s using temperature-sensitive paralysis phenotypes displayed by mutant alleles on the Drosophila X chromosome.\(^{40,42}\) The overall domain organization and amino acid sequence of DmNa\(_V\)1 is highly homologous to that of mammalian Na\(_V\) channel \(\alpha\)-subunits. The structural features critical for mammalian Na\(_V\) channel function, including the D, E, K and A residues in the pore regions of domains I–IV that are crucial for sodium selectivity (Figs. 2 and 3), are conserved in DmNa\(_V\)1.\(^{43,44}\) Most importantly, expression and subsequent electrophysiological characterization in Xenopus laevis oocytes conclusively demonstrated that the para gene encodes a functional Na\(_V\) channel.\(^{43,44}\) More recently, the orthologous channels MdNa\(_V\)1 from the housefly Musca domestica and BgNa\(_V\)1 from the German cockroach Blattella germanica have been identified\(^{45,46}\) and functionally expressed in Xenopus oocytes.\(^{47,48}\)

Around the same time as identification of para as an additional gene, DSC1, was isolated from a Drosophila genomic DNA library using an cel Na\(_V\) channel probe.\(^{49}\) BSC1, an ortholog of DSC1, was later identified in B. germanica.\(^{46}\) Based on sequence identities with para Na\(_V\) channels of around 43–53% (Table 1) and a similar domain organization, DSC1 and BSC1 were predicted to encode a Na\(_V\) channel.\(^{50}\) However, it was recently shown that Blattella BSC1 encodes a novel Ca\(_{2+}\)-selective cation channel, with different functional properties compared to Na\(_V\) channels,\(^{51}\) that may play a role in olfaction rather than neurotransmission.\(^{52}\)

The ion selectivity of both Na\(_V\) and Ca\(_V\) channels is strongly affected by a ring of amino acids formed by the pore regions of domains I–IV.\(^{53,54}\) All Na\(_V\) channels, including insect para-like Na\(_V\) channels, contain the signature sequence DEKA in the four pore regions, whereas Ca\(_V\)1 channels contain four acidic glutamate residues (EEEE) at the corresponding positions (Fig. 3). Substituting the critical Glu for Lys in the pore region of domain III of the rat Na\(_V\)1.2 channel results in a channel that is more selective for calcium ions,\(^{55}\) while substitution of Lys for Glu in the corresponding region of L-type CaV channels results in channels with selectivity for monovalent cations.\(^{56}\) Blattella BSC1 has the signature sequence DEEA, identical to that of Na\(_V\) channels except in domain III, which has a Ca\(_{2+}\)-like Glu residue instead (Fig. 3). Site-directed mutagenesis of the Glu residue (E1497K) in the pore region of domain III of BSC1 demonstrated that this residue is a critical determinant of the Ca\(_{2+}\) selectivity of BSC1.\(^{51}\) Thus, orthologous invertebrate BSC1/ DSC1-like channels that have Glu in domain III but which have not yet been functionally expressed, such as ApNa\(_V\)1 (Aiptasia pallida, sea anemone), CcNa\(_V\)1 (Cyanea capillata scyphozoan jellyfish), LbNa\(_V\)1 (Loligo bleekeri, squid), and PpNa\(_V\)1 (Polyorchis penicillatus, hydrozoan jellyfish), are likely to encode calcium-selective cation channels.
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Drosophila appears to lack homologs of the mammalian auxiliary Na\textsubscript{V}\textbeta subunits.\textsuperscript{50} Nevertheless, fully functional expression of insect para Na\textsubscript{V} channels in Xenopus oocytes can be achieved by co-injection with TipE, the protein product of the Drosophila ‘temperature-induced paralysis locus E’ (tipE) on chromosome 3L.\textsuperscript{43} This 65-kDa glycosylated protein has two TM segments connected by an extracellular loop with intracellular N- and C-termini;\textsuperscript{43} there do not appear to be any close sequence homologs in mammals.\textsuperscript{50} Coexpression of TipE with Drosophila DmNaV1 in Xenopus oocytes increases $I_{Na}$ amplitude, causes a more rapid inactivation, modulates channel trafficking and alters its pharmacology.\textsuperscript{43,44,57} This implies that TipE protein is functionally analogous to the mammalian Na\textsubscript{V}\textbeta subunits. It also appears to be important for trafficking the DmNaV1 $\alpha$-subunit from the endoplasmic reticulum to the plasma membrane\textsuperscript{57} and it also enhances the expression of the BgNaV1 channel in oocytes.\textsuperscript{58} Recently, four TipE structural homologs (TEH1, 2, 3, 4) were identified in D. melanogaster.\textsuperscript{59} Like TipE, TEH1-3 (but not TEH4) expression in Xenopus oocytes increases $I_{Na}$ most likely by enhancing membrane trafficking of the Drosophila DmNaV1 channel protein.\textsuperscript{59} TEH1 also shifts the voltage-dependence of inactivation and slows the kinetics and rate of recovery from inactivation of Drosophila DmNaV1, making it functionally analogous to TipE and mammalian $\beta$-subunits.\textsuperscript{59} Interestingly, TEH2-4 are widely expressed in both neuronal and non-neuronal tissues and thus TEH auxiliary subunits may be widely involved in specific regulation of sodium or other ion currents in a variety of insect cells.

para is not similar to any other genes in the Drosophila or other insect genomes, apart from moderate sequence homology with DSC1 and genes encoding Ca\textsubscript{V} $\alpha_1$ subunits. Therefore, at present, it appears that the DmNaV1 channel encoded by para is the only bona fide Na\textsubscript{V} channel in Drosophila and other insects.

A Comparison of Insect and Vertebrate Na\textsubscript{V} Channels

The para-type Na\textsubscript{V} channel is exceptionally well conserved across diverse orders of insects, with the level of identity ranging from 87 to 98\% (Table 1). This is one reason why insecticides that target the insect Na\textsubscript{V} channel have broad activity across many insect orders. In striking contrast to the high level of sequence conservation across diverse insect species, para-type Na\textsubscript{V} channels have significantly lower levels of identity (typically 50–60\%) with the various types of mammalian Na\textsubscript{V} channels (Table 2). This explains why a high degree of phylogenetic specificity can be achieved with both Na\textsubscript{V} channel toxins and insecticides that target the Na\textsubscript{V} channel. Interestingly, the para-type insect Na\textsubscript{V} channel is not more closely related to any one of the human Na\textsubscript{V} channels than the others. Identity with Na\textsubscript{V}1.1–Na\textsubscript{V}1.7 is around 57–60\% and with Na\textsubscript{V}1.8 it is 55–56\%. The para-type Na\textsubscript{V} channels are least homologous to human Na\textsubscript{V}1.9 channels, with identity levels of 50–52\%.

To date, nine functionally distinct mammalian Na\textsubscript{V} channel $\alpha$ subunits (Na\textsubscript{V}1.1–Na\textsubscript{V}1.9) have been cloned, functionally expressed and characterized.\textsuperscript{50} Thus the structural, functional and pharmacological diversity of mammalian Na\textsubscript{V} channels is achieved primarily through expression of multiple genes. In contrast, insects appear to rely upon extensive alternative splicing and RNA editing of a single para Na\textsubscript{V} channel gene to provide channels with different functional properties. To date, a total of nine alternative splice sites (a, b, c/d, i, j, e, f, h, l/k) have been identified in para genes that potentially generate >100 different tissue/cell type-specific

### Table 1 Conservation of the para-type Na\textsubscript{V} channel in insects

| Insect para Na\textsubscript{V} channels | Drosophila melanogaster P36500 | Musca domestica Q94615 | Anopheles gambiae AS843 | Bombyx mori A512M4 | Blattella germanica C01307 | Helicthys virescens Q94584 | Nasonia vitripennis A510E9 | Pediculus h. corporis Q89M38 | Tribolium castaneum GLEAN Q4126 |
|-----------------------------------------|-------------------------------|--------------------------|--------------------------|---------------------------|----------------------------|--------------------------|---------------------------|--------------------------|--------------------------|
| Musca domestica                        | Q94615                        |                          |                          |                           |                           |                          |                           |                          |                          |
| Anopheles gambiae                      | 92%                           | 90%                      | 93%                      | 92%                       | 93%                       | 91%                      | 91%                       | 91%                      | 91%                      |
| Bombyx mori                            | 91%                           | 88%                      | 93%                      | 92%                       | 93%                       | 91%                      | 91%                       | 91%                      | 91%                      |
| Blattella germanica                     | 90%                           | 87%                      | 92%                      | 93%                       | -                         | -                        | -                         | -                        | -                        |
| Heliocthus virescens                   | 89%                           | 87%                      | 92%                      | 93%                       | -                         | -                        | -                         | -                        | -                        |
| Nasonia vitripennis                    | 90%                           | 88%                      | 93%                      | 92%                       | 93%                       | 91%                      | -                         | -                        | -                        |
| Pediculus h. corporis                  | 89%                           | 87%                      | 91%                      | 91%                       | 99%                       | 89%                      | 90%                       | -                        | -                        |
| Tribolium castaneum                    | 89%                           | 87%                      | 92%                      | 91%                       | 91%                       | 91%                      | 93%                       | 93%                      | 90%                      |
| Apis mellifera                         | 91%                           | 88%                      | 93%                      | 94%                       | 94%                       | 93%                      | 97%                       | 91%                      | 93%                      |

Percent identity between para-type Na\textsubscript{V} $\alpha$ subunits encoded in various insect genomes. The sequence of the Drosophila calcium-selective cation channel DSC1 is included for comparison. Pairwise alignments were performed with SIM\textsuperscript{149} using only the four homologous repeat regions (i.e., the cytoplasmic N- and C-terminal segments and cytoplasmic interdomain linkers were excluded). SWISS-PROT or genome accession numbers are indicated. Alternative splicing leads to multiple isoforms for several insect Na\textsubscript{V} $\alpha$ subunits, but the percent identities are similar for all variants.
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Table 2  Percent identity between the α subunits of insect para Naᵥ channels and human Naᵥ1 channels

| Insect para Naᵥ channels                  | Human Naᵥ1 channels |
|------------------------------------------|---------------------|
|                                          | Naᵥ 1.1  | Naᵥ 1.2  | Naᵥ 1.3  | Naᵥ 1.4  | Naᵥ 1.5  | Naᵥ 1.6  | Naᵥ 1.7  | Naᵥ 1.8  | Naᵥ 1.9  |
| Drosophila melanogaster P36500            | 59%      | 59%      | 59%      | 57%      | 58%      | 56%      | 58%      | 56%      | 51%      |
| Musca domestica Q94615                   | 58%      | 59%      | 59%      | 57%      | 58%      | 58%      | 59%      | 56%      | 51%      |
| Anopheles gambiae A51843                 | 59%      | 59%      | 59%      | 58%      | 58%      | 59%      | 59%      | 56%      | 50%      |
| Bombyx mori A5A2M4                       | 59%      | 59%      | 60%      | 60%      | 59%      | 60%      | 58%      | 56%      | 52%      |
| Blattella germanica Q01307                | 59%      | 59%      | 59%      | 58%      | 59%      | 60%      | 58%      | 56%      | 51%      |
| Heliolthis virescens Q94584               | 58%      | 58%      | 58%      | 57%      | 57%      | 58%      | 57%      | 55%      | 50%      |
| Nasonia vitripennis A519E9                | 59%      | 59%      | 59%      | 57%      | 58%      | 59%      | 56%      | 51%      | 51%      |
| Pediculus h. corporis Q96M38              | 59%      | 59%      | 59%      | 58%      | 59%      | 59%      | 55%      | 51%      | 51%      |
| Tribolium castaneum GLEAN_04126          | 58%      | 58%      | 58%      | 57%      | 57%      | 58%      | 58%      | 56%      | 51%      |
| Apis mellifera hmm8170                    | 59%      | 59%      | 59%      | 58%      | 58%      | 59%      | 58%      | 56%      | 51%      |

Pairwise alignments were performed with SIM149 using only the four homologous repeat regions (i.e., the cytoplasmic N- and C-terminal segments and interdomain linkers were excluded). SWISS-PROT or genome accession numbers are indicated. Alternative splicing leads to multiple isoforms for several Naᵥ α subunits, but the percent identities are similar for all variants.

and functionally distinct insect Naᵥ channels.25,48,61 Interestingly, the majority of these alternative splice sites are conserved in para orthologs from house flies,62 mosquitoes25 and cockroaches.48,61 Post-transcriptional RNA editing, including both A-to-I and U-to-C editing events, is also used to increase the diversity of insect Naᵥ channels.63‑65 Electrophysiological characterization of variants of the cockroach Naᵥ channel gene BgNaV revealed that single RNA editing events, which occur in a tissue- and development-specific manner, can modify gating properties such as the voltage-dependence of activation and/or inactivation.48

The mammalian Naᵥ channel is the primary molecular target of numerous therapeutic drugs (e.g., local anaesthetics, anticonvulsants and antiarrhythmics). However, much of its structure and function has been elucidated using guanidinium, peptide and small alkaloid toxins derived from various plants and animals. These molecular probes alter voltage-dependent activation, conductance and inactivation and they have enabled identification of at least seven allosterically coupled neurotoxin binding sites, referred to as neurotoxin receptor sites 1–7 (Table 3 and Fig. 2). For an overview of neurotoxin receptor sites, the reader should consult recent reviews.29,66 Ligands are associated with a receptor site if they compete in radioligand competition binding assays, often with specific allosteric interactions with other sites, or elicit similar electrophysiological effects.

Given the structural and sequence homology between metazoan Naᵥ channels, it is not surprising that analogous neurotoxin receptor sites have been identified in insect and mammalian Naᵥ channels.67 Nevertheless, the significant primary structure differences between mammalian and arthropod Naᵥ channels are reflected in complex differences in the affinity for, or the allosteric modulation of, neurotoxin receptor sites in addition to the phylogenetically selective actions of certain Naᵥ channel toxins. As a result, insect Naᵥ channels are often more or less sensitive to the actions of particular neurotoxins.44,68 For example, brevetoxin-1 (site-5) induces a negative allosteric modulation of site-3 on rat brain Naᵥ channels, a positive modulation of locust Naᵥ channels, and it has no effect on cockroach Naᵥ channels.69 Importantly, insect and mammalian Naᵥ channels are distinguishable pharmacologically by the selective action of several classes of compounds that have been widely exploited as insecticides, including DDT, pyrethroids, N-alkylamides, oxadiazines and dihydropyrazoles,70‑72 as well as a growing range of insect-selective Naᵥ channel toxins derived from arachnid and sea anemone venoms.

The Naᵥ channel is a key structural element that controls cellular excitability in a wide range of phyla. Not surprisingly, therefore, it has evolved as one of the major targets of toxins from metazoa to assist them in combating predators or in capturing prey. In particular, peptide neurotoxins that target Naᵥ and other ion channels are disproportionately represented in the venoms of arachnids, cnidarians and molluscs that prey on invertebrates, no doubt due to their ability to induce rapid paralysis following envenomation. It is becoming clear that a number of these peptide toxins are selective for insect voltage-activated ion channels. Thus, in addition to being useful molecular tools for defining the pharmacology of insect ion
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channels, these toxins might prove to be useful lead compounds for the development of novel classes of insecticides. Insect-selective toxins have been found that selectively inhibit three of the four known peptide neurotoxin sites (sites 1, 3 and 4 but not 6) on insect Na\textsubscript{v} channels, thus validating these sites as potential insecticide targets (Table 3). Some Na\textsubscript{v} channel toxins such as the δ-atractotoxins\textsuperscript{73} and scorpion α-like toxins\textsuperscript{74} are taxonomically promiscuous, with activity on both vertebrates and insects, and thus are not covered in this review.

### Pore Blocking Na\textsubscript{v} Channel Toxins: Potential Site-1 Ligands

The hainantoxins and huwentoxins are a family of 33–35-residue toxins with three disulfide bonds isolated from the venom of Chinese bird spiders (Ornithoctonus spp.). Hainantoxin-I (HNTX-I) from O. hainana has a 15-fold-selectivity for block of Drosophila DmNa\textsubscript{v}1 compared with rat Na\textsubscript{v}1,2, and it has no effect on a variety of other Na\textsubscript{v} and Ca\textsubscript{v} channels.\textsuperscript{75} This action occurs in the absence of any alterations in channel inactivation kinetics or the voltage dependence of channel activation. However, it is associated with a hyperpolarizing shift in the voltage dependence of steady-state channel inactivation to stabilize the channel in the inactivated (closed) state and inhibit Na\textsuperscript{+} conductance.\textsuperscript{75–78} It has been claimed that this group of polypeptides is the first family of spider toxins to selectively block Na\textsuperscript{+} conductance via an interaction with site-1 of the Na\textsubscript{v} channel. However, competition radioligand binding studies using [3H]-STX to confirm this interaction are still awaited and the significant alteration in steady-state inactivation points to potentially remote allosteric actions to inhibit conductance. Thus the binding site for these toxins could be distinct from site-1, but near the pore, or potentially at a remote site that allosterically leads to a conformational change in the channel protein resulting in a block of ion conductance. Nevertheless, HNTX-I represents the first insect-selective spider toxin interacting with site-1 or a novel site on the insect Na\textsubscript{v} channel. The 3D structure and pharmacophore of several of these toxins have been determined. Using a panel of alanine mutants, it was found that the key residues responsible for the interaction of HNTX-I with mammalian Na\textsubscript{v} channels are Lys27, Arg29, His28, Lys32, Phe5 and Trp30.\textsuperscript{79} Interestingly, His28 is substituted by the negatively charged Asp26 in HNTX-I, thus providing a possible molecular basis for the selectivity of HNTX-I for the insect Na\textsubscript{v} channel.

### Toxins Interacting with Neurotoxin Site-3: Gating Modifiers of Inactivation

Several distinct classes of peptide toxins bind Na\textsubscript{v} channel site 3 and induce a slowing or blockade of channel inactivation. These include spider toxins, sea anemone toxins and α-type scorpion toxins. The latter class of toxins was used to define neurotoxin receptor site 3. The binding affinity of these toxins is reduced by depolarization of channels in rat brain, and the binding correlates with the voltage dependence of channel activation.\textsuperscript{29} Toxin binding affects the coupling of activation and inactivation and therefore the conformational changes associated with fast inactivation of the channel.

### Site-3: Gating Modifiers of Inactivation

Toxin binding affects the coupling of activation and inactivation and therefore the conformational changes associated with fast inactivation of the channel. Receptor site 3 has been localized primarily to the extracellular S5–S6 loops of domains I and IV, with some involvement of residues in the S3–S4 loop of domain IV (Fig. 2). Since these domains form
part of the voltage sensor, it has been hypothesized that toxin binding may prevent outward movement of the S4 segment of domain IV.80

**Alpha-insect and α-like scorpion toxins.** Many scorpion toxins display high selectivity for insect NaV channels and have been designated “insecticidal scorpion toxins” (reviewed in ref. 74). Based on toxicity symptoms, electrophysiological properties and binding assays, they have been classified into two groups: α-insect toxins, which are insect-selective, and α-like toxins that have similar activity on insect and mammalian NaV channels.74 Both classes of toxins comprise 61–76 amino acid residues crosslinked by four disulfide bridges. However, since the α-like toxins are not selective for insect channels, they fall outside of the scope of this review and will not be discussed further. The prototypic α-insect toxin is LqhαIT from the scorpion *Leurus quinquestriatus hudsonius*. LqhαIT slows axonal current inactivation, leading to prolonged action potentials. In contrast to mammalian-active scorpion α-toxins, the insecticidal α-toxins bind to site-3 on insect NaV channels in a voltage-independent manner. Toxins from this group bind with high affinity to insect neuronal preparations (K<sub>d</sub> = 0.06–1 nM) but they only affect the rat brain Na<sub>V</sub>1.2a channel at very high concentrations and they compete weakly (μM range) for binding to rat brain synaptosomes with anti-mammalian α-toxins such as Aah2.

**Tx4(6-1).** Tx4(6-1) is a novel 48-residue peptide with five disulfide bridges isolated from the venom of the South American ‘armed’ spider *Phoneutria nigriventer*.81 It is not toxic to mammals but is lethal to a variety of insects. The toxin prolongs action potential duration in cockroach axons via a slowing of NaV channel inactivation. This occurs in the absence of alterations to the voltage dependence of NaV channel activation or steady-state inactivation. Patch clamp experiments on rat Na<sub>V</sub>1.2 and Na<sub>V</sub>1.4 channels expressed in *Xenopus* oocytes revealed that Tx4(6-1) failed to alter any aspects of NaV channel gating or kinetics and thus this toxin appears to be insect-selective.82 The toxin competes with the scorpion α-like toxin Bom IV for site-3 on insect Na<sub>V</sub> channels.82

**Magi 2.** Magi 2, a peptide neurotoxin from the Japanese funnel-web spider *Macrothele gigas*, induces paralysis in insects but lacks mammalian toxicity.83 Magi 2 does not have significant sequence homology with any other known toxin except Magi 1, a peptide that fails to exhibit any overt toxicity in insects or mammals. Magi 2 displaces the site-3 ligand LqhαIT from cockroach synaptosomes with a K<sub>d</sub> of 21 nM, whereas the K<sub>d</sub> for displacement by Magi 1 is around 83-fold higher. Importantly, both Magi 1 and 2 fail to inhibit binding of radiolabelled neurotoxins to site-4 on insect Na<sub>V</sub> channels, or site-3, -4 or -6 on mammalian Na<sub>V</sub> channels. Like other site-3 spider toxins, Magi 2 awaits delineation of its structure-activity relationships in order to reveal the molecular determinants of its specificity for insect Na<sub>V</sub> channels.

**Sea anemone toxins.** Although sea anemones are not naturally in contact with insects, their ecology involves predation on crustacean arthropods and therefore it has been hypothesized that toxins in their venoms could be of interest for the development of insecticides. However, evidence of toxin selectivity is patchy as most studies concerning Na<sub>V</sub> channel toxins from this group have been conducted on mammalian preparations with additional data sometimes obtained using crustaceans.84 ATX-I from *Anemonia sulcata* displays preferential toxicity for crabs over mice.85 Toxins Sh-I from *Stichodactyla gigantea* and CgII from *Condylactis gigantea* are more lethal to crustaceans than cockroaches, and non-toxic to mice.86 On a *Periplaneta americana* giant axon preparation, both of these toxins prolonged action potentials by selectively slowing Na<sub>V</sub> channel inactivation without affecting activation. ATX-II from *A. sulcata* is toxic to crabs and mice, blocks the insect para channel with high potency, and binds with high affinity to cockroach neuronal membranes whereas its binding affinity for rat brain synaptosomes is low.84

In terms of its phylogenetic selectivity, Av3 from the *Anemonia viridis* is perhaps the most pharmacologically interesting sea anemone toxin. Av3 is a site-3 Na<sub>V</sub> channel toxin that is active against crustaceans and insects but not mammals.87 It is highly toxic to blowfly larvae, competes with LqhαIT for binding to cockroach neuronal membranes (K<sub>d</sub> = 0.21 nM), and it inhibits inactivation of the *Drosophila* DmNa<sub>V</sub>1 channel while being inactive on cloned rat Na<sub>V</sub>1.2 and Na<sub>V</sub>1.4 subtypes. Mutagenesis of the toxin suggests that it binds the channel and has a mode of action different from other site-3 toxins, and thus it might provide a template for the development of a novel class of insect-selective Na<sub>V</sub> channel toxins.87

**Toxins Interacting with Neurotoxin Site-4: Gating Modifiers of Activation.**

Toxins interacting with Na<sub>V</sub> channel site 4, whether insecticidal or anti-mammalian, have in common a decrease in the threshold for action potential generation. Site-4 toxins induce a dose-dependent shift of the voltage-dependence of channel activation towards more negative potentials, resulting in spontaneous neuronal activity that is reinforced by stronger membrane polarization. Scorpion β-toxins enhance sodium channel activation by trapping the voltage sensor in its outward activated position via interaction with TM segments S1–S4 of domain II (Fig. 2). Two classes of β-toxins have been recognized on the basis of their different effects on insect larvae: excitatory and depressant toxins, which respectively produce spastic or flaccid paralysis, respectively. Both toxin classes comprise peptides of 70–76 amino acid residues cross-braced by four disulfide bridges. They are distinguished from α-toxins by differences in sequence and disulfide pairing arrangement.

**Excitatory and depressant β-scorpion toxins.** AahIT1 is the prototypic excitatory insect-selective β-toxin from *Androctonus australis*. It induces repetitive firing of action potentials as a result of activation of Na<sub>V</sub> channels at lower membrane potentials, which explains the contractile paralysis induced by AahIT1 and similar toxins such as Bj-xtrIT from *Buthus judaicus* and Lqh-xtrIT from *L. q. hudsonius*. The insecticidal depressant toxins such as LqhIT2 from *L. q. hudsonius* induce a dual effect in toxicity tests and electrophysiological experiments. This includes an action similar to that of excitatory toxins, followed by fast resting depolarization of the axon and a decrease in action potential amplitude, leading to the inability of the axon to generate action potentials even in response to strong depolarisation. This results in flaccid paralysis of insects, but this does not result from blockade of sodium conductance as previously hypothesized. LqhIT2 decreases peak current amplitude but produces an unusually maintained slow activating-deactivating sodium current. The slow component develops at membrane potentials more negative than the control, indicating that LqhIT2 shifts the voltage-dependence of activation, and it has a time constant of activation of tens of milliseconds. Thus, the mechanism
of action of LqhIT2 may be attributed to modified NaV channels that open at more negative potential, activate slowly and do not inactivate normally.

In insect membrane preparations, AahIT and other excitatory toxins define a high affinity (Kd = 0.2–3 nM), low-capacity (1–2 pmol/mg protein) binding site. AahIT competes for binding with mammalian site-4 β-toxins such as TsVII, the prototypical New World β-toxin from Tityus serrulatus, and CsvVII from the Mexican scorpion Centruroides suffusus suffusus. Studies using cockroach neuronal membranes have shown that depressant toxins competitively inhibit the binding of contractile toxins, while the contractile excitatory toxins compete for the high-affinity binding site for depressant toxins, but not the low-affinity binding site.

δ-palutoxins. Toxins interacting with NaV channel site-4, such as scorpion β-toxins, normally act as gating modifiers of activation. A quite different mode of action is seen with the δ-palutoxins (δ-PaluITs), a family of 36–37-residue peptide toxins from the spider Paracoelotes luctuosus that selectively modulate the activity of insect NaV channels. Using an isolated cockroach axon preparation and cloned Drosophila Para/TipE NaV channels expressed in Xenopus oocytes, the δ-PaluITs have been shown to slow insect NaV channel inactivation with no shift in the voltage dependence of activation. They also have no effect on vertebrate NaV1.2 channels at concentrations up to 10 μM. This action is similar to site-3 neurotoxins such as the scorpion α-insect toxin, LqhIT. Despite this, they have been shown to displace the site-4 excitatory scorpion α-toxin, Bjt-xrIT, from cockroach neuronal membranes but they fail to displace LqhIT. In reciprocal experiments, Bjt-xrIT and the depressant scorpion β-toxin LqhIT2 also displaced δ-PaluIT2. Thus, the δ-PaluITs are the first spider toxins that have been definitively shown to bind to site-4 on insect NaV channels but which modulate NaV channel inactivation. To date, only scorpion α-toxins have been shown to compete with this site.

The 3D structures of δ-PaluIT1 and IT2 have been determined using NMR spectroscopy and, like all other spider toxins targeting insect NaV channels, they belong to the inhibitor cystine-knot (ICK) structural family. Alanine scanning mutagenesis experiments revealed that the putative pharmacophore of δ-PaluIT2 is similar to the bipartite bioactive surface of Bjt-xrIT2 despite different protein folds. The differences in the mode of action of δ-PaluIT toxins and scorpion β-toxins provide a novel perspective on the structural relatedness of receptor sites 3 and 4. These sites have previously been considered to be topologically distinct but receptor site-4 may in fact be an extended macrosite that encompasses site-3. Thus, these peptide toxins reveal that modulation of inactivation can be achieved by binding to a site, until now, thought to be associated exclusively with effects on channel activation.

μ-agatoxins and curtatoxins. The μ-agatoxins from the venom of the American funnel-web spider Agelenopsis aperta are a family of C-terminally amidated 36–37 residue peptides containing four disulfide bridges. They show high sequence homology to the curta-toxins from the related agelenid spider Holodera curta. Little data is available as to their actions but it is known that the μ-agatoxins are insect-selective neurotoxins that induce a convulsive paralysis in insects. This action is correlated with repetitive firing in the terminal branches of the insect motor axons resulting in a marked increase in spontaneous neurotransmitter release of the fly Musca domestica. This correlates with a -30 mV hyperpolarizing shift in the voltage-dependence of NaV channel activation causing channels to open at, or close to, the resting membrane potential. The increase in open channel probability leads to repetitive firing and consequently increased Ca2+ entry into nerve terminals resulting in an increased frequency of miniature excitatory junctional potentials. This action is analogous to that reported for scorpion β-toxins and thus it is likely that μ-agatoxins target NaV channel site-4, although confirmation requires further radioligand binding studies. μ-Agatoxins also slow NaV channel inactivation in motor neurons from the moth Heliothis virescens an action similar to that of δ-PaluIT toxins with whom they share considerable sequence homology. The similarities in primary structure and pharmacology of these toxins provide further support for the hypothesis that receptor site-4 on insect NaV channels is a macrosite that may be allosterically linked to channel inactivation. Further experiments using these two classes of spider toxins may reveal the mechanism of this connection.

Toxins Interacting with Unidentified Sites on the Insect NaV Channel

DTX9.2. While a number of spider toxins have been shown to interact with a known neurotoxin receptor site on the NaV channel, several insect-selective toxins are still awaiting identification of their target site. This includes a family of 56–61-residue insecticidal polypeptides, DTX9.2, 10 and 11, isolated from the venom of the primitive weaving spider Diggia canities. These insect-selective neurotoxins cause progressive spastic paralysis in tobacco budworm larvae but they have no effects on mice following intraperitoneal or intracerebroventricular injection. DTX9.2 causes repetitive excitatory postsynaptic potential discharges in housefly larvae neuromuscular and sensory nerve preparations and a depolarization of cockroach axons; these actions are blocked by TTX, indicating that these toxins target insect NaV channels. However preliminary radioligand binding studies revealed only a partial inhibition of AahIT binding to site-3 on housefly head membranes. Further voltage-clamp and binding studies are required to determine the precise target of these toxins on insect NaV channels, but it is unlikely that DTX9.2 interacts with site-3.

Oxytoxin1. Oxytoxin1 (OxyTx1) is a 69-residue insect-selective neurotoxin isolated from the venom of the wolf spider Oxyopes kitabensis. OxyTx1 has limited sequence identity (33%) to the insect-specific toxin Tx4(6–1) from P. nigriventer but further studies are required to determine whether its primary target is also insect NaV channels.

An Overview of Insect CaV Channels

Insects have a much smaller repertoire of CaV channels than vertebrates. For example, whereas the human genome encodes 10 pore-forming α1 subunits, four β subunits, four α2-δ complexes and seven γ subunits, the genome of the fruit fly Drosophila melanogaster appears to encode only three α1 subunits, a single β subunit, three α2-δ subunits and possibly a single γ subunit. However, insects are able to expand their array of functional CaV channels through alternative splicing and RNA editing.

Amino acid sequence comparisons indicate that the three α1 subunits produced by D. melanogaster, designated Dmca1D,
Peptide toxins that selectively target insect Na\textsubscript{V} and Ca\textsubscript{V} channels

Dmca1A and Ca-\(\alpha_1\)\textsubscript{1B} can be classified as Ca\textsubscript{V}1, Ca\textsubscript{V}2, and Ca\textsubscript{V}3-type channels, respectively.\textsuperscript{12} The fact that insects express only a single ortholog of each of these subtypes of Ca\textsubscript{V} channels might explain why they are all apparently essential. For example, loss-of-function mutations in the genes encoding Dmca1D and Dmca1A are embryonic lethal,\textsuperscript{100,101} indicating that these ion channels have distinct physiological roles in fruit flies that cannot be complemented by other Ca\textsubscript{V} channel subtypes. In contrast, the larger repertoire of Ca\textsubscript{V} channels in vertebrates permits at least some functional plasticity since mice that harbour a knockout of the gene encoding the \(\alpha_1\) subunit of the Ca\textsubscript{V}1.3, Ca\textsubscript{V}2.2, Ca\textsubscript{V}2.3, Ca\textsubscript{V}3.1 or Ca\textsubscript{V}3.2 channel are viable.\textsuperscript{102-106}

BLAST searches of sequenced insect genomes indicate that they each encode a single ortholog of the Ca\textsubscript{V}1- to Ca\textsubscript{V}3-type channels encoded in the \textit{Drosophila} genome (Fig. 4). In general, however, Ca\textsubscript{V} channels appear to have been less well conserved throughout the course of insect evolution than Na\textsubscript{V} channels. For example, the percent identity between the Ca\textsubscript{V}1 channel of the fruit fly \textit{Drosophila melanogaster} and the red flour beetle \textit{Tribolium castaneum} is 74\%, which is only slightly higher than the level of identity

![Diagram](image-url)
between the *Drosophila* channel and its closest human ortholog (66% with human CaV1.3) (Fig. 4). Interestingly, it appears that insect CaV1 channels, with an identity level of 74–82% across six sequenced insect genomes representing five different taxonomic Orders, have been less well conserved than the CaV2 and CaV3 subtypes (identity levels of 79–90% and 76–89%, respectively) (Fig. 4). Whether this is indicative of a more diverse physiological role for insect CaV1 channels, or is perhaps the result of a selective pressure driving evolution of the CaV1 but not the CaV2 and CaV3 subtypes, remains an interesting question.

The high level of conservation of insect NaV channels (i.e., typically >90% identity across diverse phyla) explains why they are a widely exploited insecticide target,107 since molecules that block insect NaV channels would be expected to be effective against many different types of insect pests. In contrast, the weaker conservation of insect CaV channels suggests that it might be more difficult to develop blockers of these channels that are broadly active. Of course, the corollary of this hypothesis is that it might be easier to develop CaV channel blockers that target specific groups of insect pests without harming beneficial insects such as bees.

**A Comparison of Insect and Vertebrate CaV Channels**

It should be noted that even when the variable N- and C-terminal cytoplasmic regions of the α1 subunit are excluded from amino acid sequence comparisons, the level of identity between insect CaV channels and their closest human ortholog is only ~66% (Fig. 4).12 This presumably explains why many of the pharmacological agents used to define vertebrate CaV channel subtypes are not as useful for characterizing insect CaV channels.108 For example, insect CaV channel currents are typically less sensitive to dihydropyridines and ω-conotoxin GVIA, which are routinely used to distinguish vertebrate L-type (CaV1) and N-type (CaV2.2) channels, respectively.109,110 Thus, while there is a tendency in the literature to classify the *Drosophila* Dmca1D, Dmca1A and Ca-αIT CaV channels as L-type, N-type and T-type respectively, this classification is confusing given their different electrophysiological properties and sensitivity to various pharmacological reagents compared with the corresponding vertebrate channels. Thus, when referring to these and other insect CaV channels, we will use either the gene name or the broad subtype classification (i.e., CaV1, CaV2 or CaV3).

**Peptide Toxins that Specifically Target Insect CaV Channels**

There are no examples in the scientific literature of the successful heterologous expression of a functional recombinant insect CaV channel, which has severely limited biophysical and pharmacological characterization of these channels. However, a patent recently awarded to FMC Corporation describes the functional expression in Xenopus oocytes of a CaV2 channel cloned from the tobacco budworm Heliothis virescens.111 As anticipated, no voltage-gated currents were obtained when only the α1 subunit was expressed. Modest currents (10–20 nA) were obtained when the α1 subunit was co-expressed with a β3 subunit cloned from *H. virescens*, and these became more stable and more robust (30–40 nA) when an α2-δ subunit cloned from *H. virescens* was co-expressed with both α1 and β3.111 The cloned channel had an activation threshold of ~30 mV and a reversal potential of ~50 mV, consistent with it being a CaV2-type channel. Hopefully this work will mark the beginning of a new era in insect CaV channel research but, for the moment, most labs are still restricted to the use of isolated or cultured neurons for studying these channels.

Dorsal unpaired median (DUM) neurons isolated from abdominal ganglia of the American cockroach *Periplaneta americana* have been the widely used model system for studying the action of insect CaV channel toxins. Since they are central to the discussion that follows, a brief description of CaV currents in these neurons is warranted. Four different kinds of CaV channel currents can be evoked in cockroach DUM neurons, namely transient and maintained low-voltage-activated currents (tLVA and mLVA, respectively), a mid-voltage-activated (MVA) current, and a high-voltage-activated (HVA) current.109,112 These LVA, MVA and HVA currents have activation thresholds of approximately ~70 mV, ~50 mV and ~30 mV, respectively.109,112 There are no known organic compounds that block the tLVA/mLVA currents, while the MVA and HVA currents are blocked (incompletely) by ω-agatoxin IVA/ω-conotoxin MVIIIC and verapamil/diltiazem/ω-conotoxin GVIA, respectively (see below). Thus, although the molecular identity of the CaV channels that underlie these currents remains to be unequivocally determined, it is tempting to speculate based on their pharmacology and activation thresholds112 that the cockroach DUM neuron tLVA/mLVA, MVA and HVA currents are mediated by CaV3, CaV2 and CaV1 channels, respectively. However, the overall pharmacology of these channels is so different to vertebrate channels that such a conclusion is likely to be premature. Indeed, as outlined below, we favour an alternative explanation based on recent studies of the *Drosophila* CaV2 channel.

The *Drosophila* CaV2 channel (Dmca1A) is the primary presynaptic CaV channel in *Drosophila* motor-nerve terminals.113,114 The Dmca1A gene is also known as cacophony (gc) because certain mutant alleles are linked to defects in the male courtship song.115 Although a GC null allele is embryonic lethal,101 various mutant alleles have been isolated that are viable but which lead to reduced synaptic efficiency and altered motor terminal growth at neuromuscular junctions.101,113,116 Cultured *Drosophila* giant neurons contain two HVA CaV currents with threshold potentials of ~40 and ~20 mV, and it was recently shown that both of these currents are significantly diminished in neurons containing a GC mutant allele.117 Moreover, both currents were almost completely eliminated in a GC deficiency line.117 This strongly suggests that both types of HVA CaV currents in these neurons are mediated by isoforms of the *Drosophila* CaV2 channel. A minor component of the current remained in the GC deficiency line, suggesting that these neurons may also contain a CaV1 (Dmca1D)-mediated current.

Thus, in line with these results, we propose that the MVA and HVA currents in cockroach neurons are mediated by different isoforms of the cockroach CaV2 channel, since alternative splicing and editing of RNA transcripts appears to be a major mechanism by which insects increase calcium channel diversity. For example, there are at least four different splice isoforms of the CaV2 channel (Dmca1A) in *Drosophila*118 and RNA editing has been reported not just for Dmca1A119 but also for transcripts encoding the *Drosophila* CaV1 and CaV3 α subunits and an α2-δ auxiliary subunit.120 The fact that ω-agatoxin-IVA (ω-Aga-IVA), the prototypic blocker of vertebrate CaV2.1 channels, blocks cockroach MVA currents, and does so with reasonably high affinity,109 supports the assignment of at least the DUM neuron MVA current to CaV2...
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channels. This is because only the CaV2 insect channel contains a canonical ω-Aga-IVA binding site. The binding site for ω-Aga-IVA has been mapped in part to the extracellular S3–S4 loop in repeat IV of the α1 subunit of vertebrate CaV2.1 channels (i.e., proximal to the S4 sensor domain). An E1658K mutation in this loop in rabbit CaV2.1 caused a 200-fold reduction in the binding affinity for ω-Aga-IVA,121 whereas a two-residue insertion in the S3–S4 loop of domain IV of an alternative spliced form of rat CaV2.1 resulted in an 11-fold reduction in ω-Aga-IVA affinity.122 The inferred binding site is consistent with the primary effect of the toxin, which is not to block the channel pore, but rather to shift the threshold of channel activation to more depolarized potentials.93,123

Alignment of the S3–S4 loop in domain IV of the rabbit CaV2.1 channel with the corresponding sequences of the α1 subunit of insect CaV1, CaV2 and CaV3 channels reveals a good match only with the CaV2 channels (Fig. 5). The CaV1 and CaV3 channels contain insertions in the S3–S4 loop relative to the rabbit sequence, and they show significant deviations in the S4 TM segment. Moreover, the Glu residue implicated in ω-Aga-IVA binding121 is not conserved in insect CaV1 channels, where it is sometimes Asp, Gln or Lys (Fig. 5). In contrast, the S3–S4 loop of insect CaV2 channels is the same or very similar in length to the rabbit CaV2.1 sequence, their S4 sensor elements are identical, and the key Glu residue is strictly conserved (Fig. 5).

Thus, the pharmacological evidence supports the contention that the MVA currents in cockroach DUM neurons are mediated by CaV1 channels. We suggest that HVA currents are mediated by an alternatively spliced form of the cockroach CaV2 channel that is sensitive to ω-conotoxin GIVA, but which has significantly attenuated affinity for ω-Aga-IVA. Splice isoforms A and B of the Drosophila CaV2.2 channel (Dmca1A) contain a three-residue insertion (HDD) in the S3–S4 loop of domain IV, two residues after the Glu residue that is critical for ω-Aga-IVA binding. This is the exact same location as the two-residue (NP) insertion in rat CaV2.1 that causes an 11-fold reduction in ω-Aga-IVA affinity.122 Whether HVA currents in cockroach neurons are mediated by a similar CaV2 isoform remains an interesting question for future studies.

Peptide Toxins that Block Insect CaV1 Channels

ω-ACTX-Hv1a. ω-ACTX-Hv1a is the prototypic member of a family of 37-residue insect toxins found in the venom of the lethal Australian funnel-web spider.124-126 These peptides are highly toxic to a wide range of arthropods4,127-129 but harmless to newborn mice and inactive on vertebrate nerve-muscle preparations.124,126 In affected insects, ω-ACTX-Hv1a induces a slow-onset, irreversible spastic paralysis that precedes flaccid paralysis and death.124,130 D. melanogaster that were engineered to express an inducible ω-ACTX-Hv1a transgene often developed a phenotype reminiscent of a hypomorphic allele of the dmca1D gene which encodes the Drosophila CaV1 channel.131 This led to the suggestion that insect CaV1 channels are the primary target of ω-ACTX-Hv1a,131, since similar phenotypes have not been reported for hypomorphic alleles of dmca1A.101,115,132 However, recent electrophysiological studies revealed that ω-ACTX-Hv1a is a moderately potent blocker of both MVA and HVA (putative CaV2) currents in cockroach DUM neurons with IC50 values of 279 nM and 1080 nM, respectively.126

These relatively high IC50 values for block of insect CaV2 channels are difficult to rationalize with the fact that ω-ACTX-Hv1a

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binds to cockroach neuronal membrane preparations with a $K_d$ of $<5$ nM$^{127}$ and that subnanomolar concentrations induce excitatory responses in Drosophila CNS preparations.$^{130}$ However, the genetic, biochemical and biophysical data can be reconciled if one proposes that $\omega$-ACTX-Hv1a has high affinity for insect CaV1.1 channels (which may not be present, or present only at very low levels, in DUM neurons) and only moderate affinity for CaV2.2 channels. Thus, $\omega$-ACTX-Hv1a might be a useful pharmacological agent for simultaneous block of all insect HVA CaV channel subtypes. In contrast to its effect on insect HVA channels, the toxin has no effect on calcium currents in rat trigeminal neurons,$^{124}$ nor does it block cloned rat CaV1.2, CaV2.1 and CaV2.2 HVA channels at concentrations as high as $10$ μM.$^{127}$

**Peptide Toxins that Block Insect CaV2 Channels**

**Omega‑conotoxin GVIA.** This cone snail toxin is the prototypic blocker of vertebrate CaV2.2 (N-type) channels. In contrast, $\omega$‑conotoxin GVIA has much weaker and less specific effects on insect CaV channels. In cockroach DUM neurons, for example, $\omega$‑conotoxin GVIA is a very weak blocker of HVA currents (ED$_{50}$ > $1$ μM)$^{109,133}$ and an even less effective blocker of MVA currents. The toxin also has no effect on HVA CaV currents in locust embryonic neurosecretory cells$^{134}$ or cockroach embryonic brain neurons.$^{135}$ Thus, due to its limited potency, $\omega$‑conotoxin GVIA is likely to have limited utility for the study of insect CaV channels.

**Omega‑conotoxin MVIIC.** This cone snail toxin is a high-affinity blocker of vertebrate CaV2.1 and CaV2.2 channels, with an ED$_{50}$ < $100$ pM in some species.$^{136}$ However, it is a poor blocker of insect CaV2 channels.$^{109,133}$ For example, in cockroach DUM neurons, the ED$_{50}$ for block of MVA (putative CaV2) currents is >$1$ μM.$^{109,126}$ The toxin is also rather nonspecific, since it causes moderate inhibition of HVA currents in cockroach DUM neurons.$^{109,126}$ Thus, this toxin is likely to be of only limited use for studies of insect CaV2 channels.

**Huwentoxin.** Huwentoxin-V is a 35-residue peptide toxin isolated from the venom of the Chinese tarantula Ornithoctonus huwena.$^{137}$ Small amounts of toxin induce a reversible paralysis when injected into locusts and cockroaches (PD$_{50}$ = $16$ μg/g) whereas much larger doses (>100 μg/g) are lethal. The neurotoxic effects of the peptide appear to be insect-specific since mice injected with high doses of toxin (30–200 μg/g) via the intra-abdominal or intracerebroventricular route are unaffected.$^{137}$

Huwentoxin-V has no effect on NaV, K$_V$ and MVA CaV$_V$ currents in cockroach DUM neurons, but it blocks HVA CaV$_V$ currents with an IC$_{50}$ of $219$ nM.$^{135}$ It therefore appears to be a moderately potent but specific blocker of $\omega$-Aga-IVA-insensitive insect CaV2 channels, although its effect on insect CaV1 and CaV3 channels remains to be examined. Nevertheless, huwentoxin-V might prove to be a valuable pharmacological tool for the study of insect CaV2 channels, especially for dissecting out currents mediated by different CaV2 isoforms.

**Omega-Aga-IVA.** This peptide toxin from the venom of the American funnel-web spider Agelenopsis aperta has become the defining pharmacology for vertebrate P/Q-type (CaV2.1) channels.$^{138}$ However, it is less well known that $\omega$-Aga-IVA blocks CaV$_V$ currents in at least some insect neurons with equal or higher potency than its effect on vertebrate CaV$_V$ currents. This is perhaps not surprising since the toxin is derived from spider venom and the physiologically relevant target of the toxin is almost certainly insect rather than vertebrate CaV$_V$ channels.

The effect of $\omega$-Aga-IVA on insect CaV$_V$ currents is highly variable, making it difficult to pinpoint the subtype of insect CaV$_V$ channel that it primarily targets. Of the two HVA CaV$_V$ currents observed in embryonic cockroach neurons, one (representing about 80% of the total I$_{CaV}$) is potently blocked by $\omega$-Aga-IVA (IC$_{50}$ = $9$ nM) whereas the other current is only weakly inhibited by the toxin (IC$_{50}$ = $900$ nM).$^{135}$ An MVA current in bee brain neurons is potently blocked by $\omega$-Aga-IVA$^{139}$ with an IC$_{50}$ value (10 nM) that is very similar to that observed for block of the $\omega$-Aga-IVA-sensitive HVA current in embryonic cockroach neurons. In contrast, HVA CaV$_V$ currents in cockroach DUM neurons are completely insensitive to $\omega$-Aga-IVA whereas MVA currents in the same neurons are blocked by the toxin with moderate potency (IC$_{50}$ > $100$ nM).$^{109}$

As outlined above, CaV2 channels are the only subtype in insects that have a canonical $\omega$-Aga-IVA binding site, and therefore the target of $\omega$-Aga-IVA in all insect neurons where it has a potent effect is almost certainly CaV2 channels. The widely varying effects of the toxin are likely due to different insect neurons expressing different isoforms of CaV2 channels that result from alternative splicing and RNA editing events. Thus, while $\omega$-Aga-IVA is a very useful pharmacological agent for the study of insect CaV2 channels, it needs to borne in mind that certain insect CaV2 isoforms may be insensitive to the toxin just as certain splice isoforms of vertebrate CaV2.1 channels are insensitive to $\omega$-Aga-IVA.$^{122}$

**PLTX-II.** Plectreurys toxin II (PLTX-II) is a 44-residue peptide toxin from the primitive hunting spider Plectreurys tristis that contains an unusual palmitoylated C-terminal Thr residue.$^{140}$ It was shown almost 20 years ago that PLTX-II blocks presynaptic CaV$_V$ currents in Drosophila nerve terminals$^{140,141}$ but only recently has it been demonstrated that this toxin most likely targets insect CaV2.2 channels.$^{142}$

Drosophila that express a temperature-sensitive mutation of cacophony (caT$^{52}$), the gene encoding the CaV2.2 channel, are deficient in synaptic vesicle exocytosis at the non-permissive temperature.$^{116,142}$ This defect can be recapitulated at the permissive temperature by low doses (1 nM) of PLTX-II,$^{142}$ suggesting that PLTX-II selectively blocks the Drosophila CaV2.2 channel (Dmca1A). At concentrations >$5$ nM, PLTX-II begins to also affect endocytosis,$^{142}$ suggesting that it might block additional insect CaV channel subtypes at higher concentrations. Nevertheless, low concentrations of PLTX-II appear to be a defining pharmacology for the Drosophila and possibly other insect CaV2 channels. It will be interesting to determine whether it blocks both the $\omega$-Aga-IVA-sensitive M-LVA currents as well as the $\omega$-Aga-IVA-insensitive HVA currents in cockroach DUM neurons. The effect of PLTX-II on vertebrate CaV2 channels has not been widely explored, but it does not block synaptic transmission at frog neuromuscular junctions.$^{141}$

**Omega-ACTX-Hv2a.** $\omega$-ACTX-Hv2a is the prototypic member of a family of 42–45-residue insecticidal peptides isolated from the venom of Australian funnel-web spiders.$^{139}$ The toxin is also lethal to ticks,$^{129}$ but it causes no adverse effects when injected into newborn mice.$^{139}$ Injection of $\omega$-ACTX-Hv2a into insects induces instantaneous paralysis,$^{139}$ which contrasts with the slow onset of paralysis following injection of $\omega$-ACTX-Hv1a.$^{124}$ At low toxin
doses, the paralysis is reversible, with affected insects recovering in 4–5 h, but injection of a second bolus prior to recovery from the first dose is lethal.\textsuperscript{139} Whether recovery from paralysis results from clearance of the toxin, turnover of affected channels, or some other mechanism remains to be determined.

Omega-\textit{ACTX-Hv2a} is the most potent blocker of insect Ca\textsubscript{\textit{V}} channels reported thus far. It inhibits HVA Ca\textsubscript{\textit{V}} currents in bee brain neurons with an IC\textsubscript{50} of -130 pM, whereas \textit{\omega-Aga-IVA} has an IC\textsubscript{50} value of 10 nM in the same assay.\textsuperscript{139} Moreover, whereas \textit{\omega-Aga-IVA} inhibits \textit{l}_{\textit{Ca}} in mouse sensory neurons with IC\textsubscript{50} = 20 nM, 1 \textmu M \textit{\omega-ACTX-Hv2a} has very little effect.\textsuperscript{139} Thus, \textit{\omega-ACTX-Hv2a} preferentially blocks insect Ca\textsubscript{\textit{V}} channels, whereas \textit{\omega-Aga-IVA} is essentially equipotent against insect and vertebrate Ca\textsubscript{\textit{V}} channels.

The insect Ca\textsubscript{\textit{V}} channel subtype targeted by \textit{\omega-ACTX-Hv2a} has not been determined, but several lines of evidence suggest it is likely to be Ca\textsubscript{\textit{V}}2 (reviewed in ref. 12). The three-dimensional structure of the disulfide-rich core of \textit{\omega-ACTX-Hv2a} resembles that of \textit{\omega-Aga-IVA},\textsuperscript{139} which targets vertebrate Ca\textsubscript{\textit{V}}2.1 channel. Moreover, in addition to their compact disulfide-rich cores, \textit{\omega-ACTX-Hv2a} and \textit{\omega-Aga-IVA} both possess a structurally disordered C-terminal region that is critical for toxin activity.\textsuperscript{139,143} This C-terminal “tail” is reminiscent of the functionally critical palmitoyl group that is covalently attached to the C-terminal Thr residue in PLTX-II.\textsuperscript{144} As outlined above, PLTX-II and \textit{\omega-Aga-IVA} appear to be a specific blockers of insect Ca\textsubscript{\textit{V}}2 channels and thus it seems likely, although it remains to be experimentally proven, that the structurally homologous \textit{\omega-ACTX-Hv2a} also targets insect Ca\textsubscript{\textit{V}}2 channels.

**Peptide Toxins that Block Insect Ca\textsubscript{\textit{V}}3 Channels**

At this stage there are no described peptidic blockers of insect Ca\textsubscript{\textit{V}}3 channels. Indeed, biophysical and pharmacological characterization of these channels is sadly lacking, with not a single study of insect Ca\textsubscript{\textit{V}}3 channels reported in the scientific literature. It remains to be determined whether some of the LVA currents recorded from Na\textsubscript{\textit{V}} channel inactivation in cockroach DUM neurons.\textsuperscript{145} To be determined whether some of the LVA currents recorded from Na\textsubscript{\textit{V}} channel inactivation in cockroach DUM neurons.\textsuperscript{145}

**Promiscuous Toxins with Non-Selective Actions on Insect Na\textsubscript{\textit{V}} and Ca\textsubscript{\textit{V}} Channels**

The promiscuous activity of some toxins on both Na\textsubscript{\textit{V}} and Ca\textsubscript{\textit{V}} channels may arise due to common structural elements shared between these channels, which are members of the same ion channel superfamily. For example, Magi 3 from \textit{M. acrothode giga}s is an insect-selective neurotoxin that causes a reversible paralysis in insects but fails to display any signs of toxicity in mammals. It has been shown to partially inhibit Lqh\textsubscript{TT} binding to Na\textsubscript{\textit{V}} channel site-3 on cockroach synaptosomes.\textsuperscript{85} The primary structure of Magi 3, however, shows significant homology to the insect Ca\textsubscript{\textit{V}} channel blocker PLTX-II. Thus, while Magi 3 has moderate affinity for insect Na\textsubscript{\textit{V}} channels, its major target is more likely to be an insect Ca\textsubscript{\textit{V}} channel. This type of dual activity has been reported for the Ca\textsubscript{\textit{V}} channel blocker \textit{\omega-Aga-IVA}, which also decreases TTX-sensitive \textit{l}_{\textit{Na}} amplitude, enhances \textit{l}_{\textit{Na}} decay, and leads to a slower recovery from Na\textsubscript{\textit{V}} channel inactivation in cockroach DUM neurons.\textsuperscript{145} A limited activity on Na\textsubscript{\textit{V}} channels has also been reported for the insect-selective Ca\textsubscript{\textit{V}} channel blocker \textit{\omega-ACTX-Hv1a}.\textsuperscript{126}

**Concluding Remarks**

Although insects have a smaller repertoire of Na\textsubscript{\textit{V}} and Ca\textsubscript{\textit{V}} channels than vertebrates, the panel of pharmacological tools available for studying these channels is smaller than that available for studying their vertebrate counterparts. Nevertheless, the repertoire of peptide toxins that specifically modulate the activity of insect Na\textsubscript{\textit{V}} channels has grown impressively in recent years. Some of these toxins bind at sites on the insect Na\textsubscript{\textit{V}} channel, or have modes of action, distinct from those of classical chemical insecticides such as DDT and the pyrethroids and therefore they might provide leads for the development of novel classes of Na\textsubscript{\textit{V}} channel insecticides. The situation with respect to insect Ca\textsubscript{\textit{V}} channels is more complex. Although a number of peptide toxins have been isolated that specifically block insect Ca\textsubscript{\textit{V}} channel currents, in most cases it remains to be determined whether these toxins block specific channel subtypes. However, some of these toxins, such as PLTX-II, might ultimately prove to be the defining pharmacology for certain insect Ca\textsubscript{\textit{V}} channel subtypes.

Given the critical role of Ca\textsubscript{\textit{V}} channels in insect physiology it is perhaps not surprising that most spider venoms appear to be rich sources of Ca\textsubscript{\textit{V}} channel toxins. What is surprising, however, is that there are no commercially available insecticides that specifically target insect Ca\textsubscript{\textit{V}} channels. Thus, in addition to being useful pharmacological probes, these toxins might facilitate the development of completely new classes of Ca\textsubscript{\textit{V}} channel insecticides. Alternatively, genes encoding insect-selective Na\textsubscript{\textit{V}} and Ca\textsubscript{\textit{V}} channel toxins could be engineered into plants,\textsuperscript{128} entomopathogenic fungi,\textsuperscript{146} and insect-specific bacteria\textsuperscript{147} and viruses\textsuperscript{148} for biocontrol of insect pests.

**Acknowledgements**

We thank the Australian Research Council (Discovery Grant DP0774245 to Glenn F. King and DP0559396 to Graham M. Nicholson/Glenn F. King), the Australian Department of Education Science and Technology (International Linkage Grant FR50106 to Graham M. Nicholson/Pierre Escoubas), and the Centre National de la Recherche Scientifique (Projets Internationaux de Coopération Scientifique Grant to Pierre Escoubas/Graham M. Nicholson) for financial support. Insect images in Figure 4 are from the University of South Florida Clipart Library (http://etc.usf.edu/cliptart/index.htm).

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