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Review

Structure and function of δ-atracotoxins: lethal neurotoxins targeting the voltage-gated sodium channel

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Abstract

δ-Atracotoxins (δ-ACTX), isolated from the venom of Australian funnel-web spiders, are responsible for the potentially lethal envenomation syndrome seen following funnel-web spider envenomation. They are 42-residue polypeptides with four disulfides and an 'inhibitor cystine-knot' motif with structural but not sequence homology to a variety of other spider and marine snail toxins. δ-Atracotoxins induce spontaneous repetitive firing and prolongation of action potentials resulting in neurotransmitter release from somatic and autonomic nerve endings. This results from a slowing of voltage-gated sodium channel inactivation and a hyperpolarizing shift of the voltage-dependence of activation. This action is due to voltage-dependent binding to neurotoxin receptor site-3 in a similar, but not identical, fashion to scorpion α-toxins and sea anemone toxins. Unlike other site-3 neurotoxins, however, δ-ACTX bind with high affinity to both cockroach and mammalian sodium channels but low affinity to locust sodium channels. At present the pharmacophore of δ-ACTX is unknown but is believed to involve a number of basic residues distributed in a topologically similar manner to scorpion α-toxins and sea anemone toxins despite distinctly different protein scaffolds. As such, δ-ACTX provide us with specific tools with which to study sodium channel structure and function and determinants for phyla- and tissue-specific actions of neurotoxins interacting with site-3.

Keywords: Funnel-web spider, δ-atracotoxin, voltage-gated sodium channel, inhibitor cystine-knot, scorpion α-toxin, sea anemone toxin

1. Introduction

Spider venoms contain potent neurotoxins and other bioactive compounds, many of which are lethal to humans or selectively insecticidal. Many of these neurotoxins are highly potent and specifically target ion channels, receptors or other targets involved in important physiological functions (for a review of other spider toxins see Escoubas et al., 2000; Rash and Hodgson, 2002). In particular, some toxins, such as ω-agatoxin IVA and α-latrotoxin, have been invaluable molecular tools for the identification and characterization of ion channel subtypes and the process of neurotransmitter exocytosis, respectively. Others are now being investigated for their possible use as bioinsecticidal agents for the control of phytophagous pests.

The focus of this review is the primate lethal neurotoxins isolated from Australian funnel-web spiders, commonly regarded as the World's most dangerous spider. The review will detail the site and mechanism of action of these neurotoxins and discuss structure-function with relevance to the application of these neurotoxins in the fields of pharmacology and neuroscience and the development of novel insecticides.

2. Taxonomy and distribution of Australian funnel-web spiders

Australian funnel-web spiders (Araneae:Mygalomorphae:Hexathelidae:Atracinae) are a diverse group of large aggressive, nocturnal spiders that are classified into two genera, *Atrax* (Cambridge) and *Hadronyche* (Koch). They comprise of at least 40 species, based on morphological and electrophoretic data, however many species remain undescribed (Gray, 1988). These spiders form the sub-family Atracinae and are found primarily along the eastern seaboard of Australia reaching from southeastern Queensland to Tasmania and in a small pocket of the Flinders Ranges in South Australia. Despite being classified into separate genera, all spiders in this sub-family are thought to have venom potentially toxic to primates (White et al., 1995; Graudins et al., 2002). The apparent resistance of non-primates to funnel-web venom is thought to be due to the presence of IgG, and possibly cross-linked IgG and IgM, inactivating factors in their plasma that bind to the toxins responsible for the envenomation syndrome, or it may simply involve a non-specific reaction due to the highly basic nature of the toxins (Sheumack et al., 1991).

3. Clinical features of funnel-web spider envenomation

Funnel-web spider venom arguably represents the most toxic spider venom known to affect humans. When significant envenomation does occur, a unique syndrome develops which can cause death within 15 minutes. Between 1927 and the introduction of an antivenom for clinical use in 1980, fourteen deaths were reported in the medical literature (Sutherland, 1980). All deaths were caused by bites from the male Sydney funnel-web spider *Atrax robustus*. Nevertheless, additional species of funnel-web spider from varied locations have caused serious systemic illness in humans. They include male *H. versuta* (Blue Mountains funnel-web), *H. formidabilis* (Northern tree-dwelling funnel-web), *H. cerberea* (Southern tree-dwelling funnel-web) and *H. infensa* (Toowoomba funnel-web) (Dieckmann et al., 1989; Harrington et al., 1999; Miller et al., 2000). Envenomation resulting from the bite of these species appears to be becoming more common as the human population spreads up and down the eastern seaboard of Australia.

The envenomation syndromes observed following bites by all these spiders are identical (Dieckmann et al., 1989; Harrington et al., 1999; Miller et al., 2000). Clinical features of envenomation in primates involve initial local features including muscle fasciculation, sweating, and piloerection. Early symptoms of systemic envenomation include perioral tingling, tongue and facial muscle fasciculation, nausea, vomiting, profuse sweating, salivation, lachrymation, and dyspnoea. Patients may rapidly develop confusion, agitation, and coma associated with marked hypertension, metabolic acidosis, raised intracranial pressure, mydriasis, generalised muscle fasciculation, and non-cardiogenic pulmonary oedema (Torda et al., 1980). Untreated envenomation may result in marked hypotension refractory to fluids and inotropic agents, profound coma, and continuing, persisting muscle fasciculation. Death results from progressive, irreversible hypotension, or possibly raised intracranial pressure resulting from cerebral oedema (Torda et al., 1980). These signs and symptoms suggest involvement of both the somatic and autonomic nervous systems.

4. Neurotoxins isolated from Australian funnel-web spider venom

A variety of peptides, known as atracotoxins (ACTX), have been isolated from the venom of funnel-web spiders. These toxins include two structurally distinct families of ω-ACTX, which

selectively inhibit insect voltage-gated calcium channels (Fletcher et al., 1997b; Wang et al., 1999; Wang et al., 2001), ACTX-Hvf17, a peptide with strong sequence homology to mamba intestinal toxin-1 (Szeto et al., 2000), and more recently the insecticidal J-ACTX (Wang et al., 2000) that inhibit insect target voltage-gated potassium channels (Nicholson, G.M. and Gunning, S.J., unpublished data) (for a review of insecticidal neurotoxins see King et al., 2002). However, the lethal toxins responsible for the major primate-specific symptoms of envenomation have been shown to target the voltage-gated sodium channel and according to the systematic nomenclature system of Fletcher et al. (1997b) have been designated as δ-ACTX. A single primate-specific variant of δ-ACTX-1 is the major component of most Australian funnel-web spider venoms (Sheumack et al., 1985; Brown et al., 1988), but some species produce multiple δ-ACTX-1 homologues. For example, *Hadronyche versuta* venom is known to contains two pharmacologically characterized δ-ACTX-Hv1 homologues purified from crude venom (Sheumack et al., 1985; Szeto et al., 2000). Further screening of *A. robustus*, *H. valida, H. infensa, and H. versuta* venom gland cDNA libraries has also revealed the presence of 12 novel δ-ACTX-like mature toxins, some with single residue changes others with much greater variation in peptide sequence (D. Wilson and P.F. Alewood, personal communication; B. Sollod, personal communication).

5. Isolation and structural characterization of δ-atracotoxins

To date, of the many species of funnel-web spiders identified, only the venom of two species, the Sydney funnel-web spider (*Atrax robustus*) and the Blue Mountains funnel-web spider (*Hadronyche versuta*), have been extensively studied for the presence of δ-ACTX. Using various purification techniques, such as ion-exchange and reverse-phase HPLC, lethal neurotoxins were extracted from the venom of both species, namely δ-ACTX-Ar1a (formerly robustoxin) from *A. robustus* (Sheumack et al., 1984; Sheumack et al., 1985) and δ-ACTX-Hv1a (formerly versutoxin) from *H. versuta* (Brown et al., 1988). Both δ-ACTX-Ar1a and δ-ACTX-Hv1a produce an envenomation syndrome in anaesthetized monkeys indistinguishable from that seen in humans suggesting that they are responsible for the physiological effects seen with crude venom (Phillips et al., 1987; Mylecharane et al., 1989).

Early attempts at isolation of δ-ACTX-Ar1a and subsequent sequence determination gave conflicting and incorrect results (Gregson and Spence, 1983; Sheumack et al., 1984). The advent of more reliable analysis methods resulted in a proposed amino acid sequence of 42 residues containing eight cysteines (Sheumack et al., 1985). Similar peptides have subsequently been found in the venom of other Australian funnel-web spiders including δ-ACTX-Hv1b from *Hadronyche versuta* (Szeto et al., 2000) and δ-ACTX-Hs20.1a from *Hadronyche* sp. 20 (Wilson D, Birinyi-Strachan LC, Alewood PF and Nicholson GM, unpublished data)(Fig. 1).

Figure 1 here

All δ-ACTX are highly homologous 42-residue peptides with no significant sequence homology with any presently known neurotoxins. The toxins have a high proportion of basic residues and are cross-linked by four conserved intramolecular disulfide bonds (Fig. 1). The cysteine framework of δ-ACTX is unique in bioactive peptides due to the occurrence of three contiguous cysteines ($Cys^{14,15,16}$) and disulfide-bridged cysteine residues at both the N- and C-termini. The disulfide connectivity of δ-ACTX-Ar1a and δ-ACTX-Hv1a corresponds to a 1-4, 2-6, 3-7, 5-8 motif that has also been observed in the venom of snakes (echistatin; Calvete et al., 1992) and scorpions (AaHIT, chlorotoxin; Darbon et al., 1982; DeBin et al., 1993) but none of these other toxins have the same cysteine spacings as δ-ACTX. The three-dimensional solution structures of δ-ACTX-Hv1a (Fletcher et al., 1997a) and δ-ACTX-Ar1a (Pallaghy et al., 1997) have been determined using NMR spectroscopy (Fig. 2). They comprise a core β region containing a triple-stranded antiparallel β-sheet and a thumb-like extension protruding from the β region. The C-terminal region (residues 34-42) differs slightly between the two molecules, given that it is the region of lowest homology, with a 3_{10}-helix in δ-ACTX-Hv1a and a series of classic or inverse γ-turns in δ-ACTX-Ar1. However, these structures are positioned in close proximity to the β domain by a disulfide bond between Cys^{42} and Cys^{16}.

Figure 2 here

δ-Atracotoxins have structural similarities, and to a much lesser extent sequence homology, with a number of other related and unrelated spider toxins (μ-ACTX-Hi:0B4219, J-ACTX-Hv1c, ω-agatoxin IVA, ω-agatoxin IVB, μ-agatoxin I, huwentoxin-I, hanatoxin1; Kim et al., 1995; Reily et al., 1995; Omecinsky et al., 1996; Qu et al., 1997; Takahashi et al., 2000; Wang et al., 2000;

Rosengren et al., 2002), marine snail toxins (ω-conotoxin GVIA, conotoxin GS; Hill et al., 1997; Pallaghy and Norton, 1999) and a sweet taste suppressing plant polypeptide (gurmarin; Fletcher et al., 1999), all of which have six conserved cysteine residues. Interestingly, the β region of δ-ACTX contains a 'disulfide-knot' which places them in a class of toxins and inhibitory polypeptides with an 'inhibitor cystine-knot' (ICK) motif (Pallaghy et al., 1994; Norton and Pallaghy, 1998). Within this class however, the biological activities of ICK toxins are quite diverse with activity at voltage-gated sodium, potassium or calcium channels, mechanosensitive channels, nicotinic acetylcholine receptors or ryanodine receptors (Fig. 3). As such, they provide few clues to predict the target site of δ-ACTX from their three-dimensional fold and highlight that different biological functions are often grafted onto the same or similar structural scaffolds. Nevertheless, the cystine-knot no doubt contributes to the high stability and resistance to proteases of δ-ACTX, reducing *in vivo* degradation.

Figure 3 here

6. Electrophysiological characterization of δ-atracotoxins

Electrophysiological studies have identified that δ-ACTX alter neuronal excitability by causing a prolongation of action potential duration. This results in the appearance of plateau potentials, accompanied by spontaneous repetitive firing (Grolleau et al., 2001; Alewood et al., 2003). These actions underlie the intense muscle fasciculation and autonomic disturbances seen clinically during systemic envenomation. Effects on the autonomic nervous system, including vomiting, profuse sweating, salivation, lachrymation, marked hypertension followed by hypotension, together with effects on the somatic nervous system to cause muscle fasciculation and dyspnoea (Sutherland and Tibballs, 2001), are presumably due to excessive neurotransmitter release. These actions are consistent with the effects of δ-ACTX to cause spontaneous and prolonged prejunctional action potentials in efferent nerve fibres. Moreover, it is these effects that ultimately lead to death from respiratory or circulatory failure (Sutherland and Tibballs, 2001) and highlights that δ-ACTX are the toxins primarily responsible for lethality (Phillips et al., 1987; Mylecharane et al., 1989).

In order to determine the underlying cause of this increased neuronal excitability, the effects of δ-ACTX on ion channel gating and kinetics was determined using whole-cell patch-clamp

techniques in dorsal root ganglia and periaqueductal gray neurons. δ-Atracotoxins were shown to produce a selective slowing of tetrodotoxin (TTX)-sensitive voltage-gated sodium current inactivation and a reduction in peak sodium current. These actions were only apparent when toxin was applied to the external bathing solution, indicating an extracellular target on the channel. In addition, the toxins caused a modest hyperpolarising shift in the voltage-dependence of activation (Nicholson et al., 1994; Nicholson et al., 1998; Szeto et al., 2000). Figure 4 shows the effects of a δ-ACTX-Hs20.1a, a previously unreported δ-ACTX isolated from the venom of *Hadronyche* sp. 20, showing modulation of sodium channel gating and kinetics representative of other δ-ACTX. Sodium channels were also observed to have an increased rate of recovery from inactivation to the resting state, allowing channels to be available for activation much earlier than under control conditions (Nicholson et al., 1994; Nicholson et al., 1998). The above actions indicate that δ-ACTX inhibit conversion of the sodium channel from the open to the inactivated state, resulting in sodium current remaining at membrane potentials where inactivation is normally complete. Moreover, the alterations in action potential firing and duration are consistent with these actions to slow sodium channel inactivation and shift the voltage-dependence of activation to potentials closer to the resting membrane potential. Notably, these actions are comparable to those observed with scorpion α-toxins and sea anemone toxins which bind to neurotoxin receptor site-3 on the sodium channel (Strichartz and Wang, 1986; Hanck and Sheets, 1995).

Figure 4 here

7. Neurochemical characterization of the binding site of δ-atracotoxins

Based on apparent similarity in the electrophysiological effects of scorpion α-toxins and δ-ACTX, subsequent studies aimed to determine if the binding site of δ-ACTX on the voltage-gated sodium channel was analogous to scorpion α-toxins, or a novel site. A variety of neurotoxins target different receptor sites on the sodium channel, leading to the characterization of at least seven binding sites, referred to as neurotoxin receptor sites 1-7 (Table 1) (for reviews see Catterall, 1992; Gordon, 1997a,b; Cestèle and Catterall, 2000). Classical scorpion α-toxins, such as AaH-II (Martin-Eauclaire and Courand, 1995) and Lqh-II (Gordon et al., 1998; Sautière et al., 1998), and sea anemone toxins, such as anthopleurin-B (ApB) (Benzinger et al., 1998), bind to receptor site-3 on

mammalian voltage-gated sodium channels. This interaction with site-3 on the S3-S4 extracellular loop in domain IV of the sodium channel α-subunit is voltage dependent. This receptor site has also been shown to have complex allosteric interactions with site-2, shown to bind several lipid soluble alkaloid toxins such as batrachotoxin and veratridine, and site-5 which binds the cyclic polyether toxins brevetoxin and ciguatoxin (Cestèle and Catterall, 2000). The alkaloid toxins induce persistent activation of sodium channels by shifting the voltage-dependence of activation to very negative membrane potentials and inhibiting the inactivation process. Brevetoxins, from marine dinoflagellates, also shift the voltage-dependence of activation to more hyperpolarized potentials to cause repetitive firing of action potentials. Scorpion α-toxins were shown to cooperatively enhance the effect of alkaloid toxins by increasing their binding affinity and activity (Catterall, 1977; Ray et al., 1978) whereas brevetoxins allosterically inhibit the binding of scorpion α-toxins to site-3 (Cestèle et al., 1995) (Table 1).

Table 1 here

Using both $^{22}Na^{+}$ uptake and radiolabelled neurotoxin binding assays, it was found that δ-ACTX bind to site-3 in rat brain synaptosomes but exhibit unusual allosteric interactions with the site-2 alkaloid toxin receptor. δ-ACTX produce up to a 30% increase in $^{22}Na^{+}$ flux and at nanomolar concentrations δ-ACTX also completely inhibit the binding of the classical scorpion α-toxins Lqh-II and AaH-II to site-3 on rat brain synaptosomes (Little et al., 1998a; Little et al., 1998b). Moreover, while they enhance ^{3}H-batrachotoxin binding to site-2 similarly to scorpion α-toxins they differ from scorpion α-toxins in that they inhibit, rather than enhance, the activation of sodium channels by batrachotoxin (Little et al., 1998b).

Voltage-dependent binding to site-3 has recently been confirmed using ^{125}I-labelled δ-ACTX-Hv1a (Gilles et al., 2002). However, depolarizing conditions hinder δ-ACTX-Hv1a association to a lesser extent than Lqh-II, which may be related to their different structures (see section 9). Nevertheless, δ-ACTX-Hv1a binding was inhibited competitively by the classical scorpion α-toxin Lqh-II and allosterically inhibited by brevetoxin-1. In light of the subtle variations in action and binding properties (Little et al., 1998b; Nicholson et al., 1998), it suggests that δ-ACTX interact at a non-identical yet overlapping site to that of scorpion α-toxins at site-3.

8. Phyla-specific actions of δ-atracotoxins

Of great interest has been the finding that δ-ACTX are, in addition to being mammalian toxic, also insecticidal. Both δ-ACTX-Ar1a and δ-ACTX-Hv1a are lethal by lateroventral injection into crickets, showing similar signs of delayed contractile paralysis as the anti-insect scorpion α-toxin LqhαIT (Eitan et al., 1990; Little et al., 1998a). These neurotoxic actions on insects led researchers to investigate the effects of δ-ACTX on insect voltage-gated sodium channels. In isolated giant axon and dorsal unpaired median neurones of the cockroach *Periplaneta americana,* δ-ACTX-Hv1a modified the action potential by prolonging the repolarization phase, causing the development of spontaneous plateau action potentials. Under voltage-clamp conditions, these alterations in neuronal excitability were found to be due to a slowing of sodium channel inactivation and a shift in the voltage-dependence of activation towards more negative potentials (Grolleau et al., 2001). These actions are very similar to LqhαIT (Eitan et al., 1990) and almost identical to that observed in mammalian preparations (Nicholson et al., 1994).

All site-3 toxins that compete for classical scorpion α-toxin binding to rat brain sodium channel have been shown to compete for LqhαIT binding, a well defined ligand of insect sodium channel receptor site-3, albeit at different potencies (Gordon et al., 1996; Little et al., 1998a). For example, 100-fold higher concentrations of classical scorpion α-toxins, such as AaH-II, Lqh-II and Lqq-V (LqTx; Ray et al., 1978), which bind rat brain synaptosomes with high affinity (K_d < 1 nM), are required to compete for LqhαIT binding to cockroach sodium channels. In contrast to the phyla-specific actions of scorpion α-toxins, radioligand binding experiments revealed that δ-ACTX inhibit ^{125}I-labelled LqhαIT binding to cockroach neuronal membranes with a K_d of less than 1 nM (Little et al., 1998a; Gilles et al., 2002). Therefore δ-ACTX are unique in that they bind with equal high affinity to receptor site-3 of both rat brain and cockroach sodium channels (Little et al., 1998a; Little et al., 1998b; Gilles et al., 2002).

Notably, however, δ-ACTX-Hv1a exhibits a low binding affinity to locust sodium channels (Gilles et al., 2002). Thus unlike scorpion toxins, which are only capable of differentiating between mammals and insects, δ-ACTX differentiate between insect voltage-gated sodium channels from different insect orders (Dictyoptera vs. Orthoptera). Structural differences between the two types of insect sodium channels have been previously inferred from allosteric modulations of LqhαIT

binding. For example, brevetoxin (site-5) and veratridine (site-2) have been shown to increase the binding of LqhαIT to locust but not cockroach sodium channels (Gordon and Zlotkin, 1993; Cestèle et al., 1995; Gordon et al., 1996). In addition, it has been shown that the α-like scorpion toxin Lqh-III, which binds to locust and cockroach site-3 with equal affinity, preferentially lost its binding capacity to locust sodium channels upon iodination (Gilles et al., 2000). The ability of site-3 to differentiate between such subtle structural alterations on Lqh-III (addition of an iodine atom) and the differential binding of δ-ACTX suggests structural differences at the binding site between sodium channels from the two insect orders. Hence, employing various toxin probes can expose subtle differences at receptor site-3. However, the structural basis for these selective interactions still awaits elucidation of the contact surfaces between the various toxins and their receptor binding sites in different sodium channel subtypes. This may lead to the development of more efficacious and more selective insecticidal toxins capable of being employed in a recombinant baculovirus model or used to design non-peptide mimetics that could be used in foliar sprays.

9. Structure-function relationships

Despite the difference in three-dimensional structure, size, and primary sequence, the similarity in binding and electrophysiological properties of scorpion α-toxins and δ-ACTX may suggest some structural resemblance at the bioactive surface. Unfortunately, the presence of four disulfide bonds has severely limited the efficient production of synthetic or recombinant δ-ACTX in order to assess the effect of mutations to ascertain the toxin pharmacophore. Therefore, the structure-function relationships of these toxins have not been determined directly.

Nevertheless, attempts have been made to indirectly identify critical residues involved in δ-ACTX binding to site-3 based on knowledge of the binding site on the sodium channel or from mutagenesis studies of related site-3 toxins. This is despite distinctly different protein scaffolds of δ-ACTX in comparison to other site-3 toxins such as AaH-II (Fontecilla–Camps et al., 1982), LqhαIT (Tugarinov et al., 1997) and the sea anemone toxin anthopleurin-B (ApB) (Monks et al., 1995). One model (Fletcher et al., 1997a) proposes that the interaction is based mainly on electrostatic complementarity with a patch of charged residues known to be critical for site-3 toxin binding. These residues are located within the short extracellular S3–S4 loop of domain IV of the

mammalian voltage-gated sodium channel α-subunit (Rogers et al., 1996). Some of these residues identified as critical for binding (Glu^{1613}, Glu^{1616} and Lys^{1617}) are charged, consistent with the identification of several anionic and cationic residues in scorpion α-toxins and sea anemone toxins that are crucial for binding to site-3 (Gordon et al., 1996; Rogers et al., 1996). Fletcher et al. (1997a) found that three cationic and two anionic residues in δ-ACTX-Hv1a can be superimposed on similarly charged residues in the structures of ApB and AaH-II, despite their lack of sequence homology and totally different three-dimensional folds. They proposed that Lys^3, Arg^5 and Asp^{13} in δ-ACTX-Hv1a provide a complementary surface to the residues identified in the S3-S4 loop of domain IV.

In a more recent search for possible resemblance on the molecular exterior of these toxins Gilles et al. (2002) compared LqhαIT, AaH-II and δ-ACTX-Hv1a focusing on residues previously shown by mutagenesis studies to constitute the bioactive surface of LqhαIT (Tugarinov et al., 1997; Zilberberg et al., 1997; Gurevitz et al., 2001). These residues cluster similarly on the counterpart surface of AaH-II and also appear on the surface of δ-ACTX-Hv1a. Although not included in their study, Figure 5 shows a number of residues in δ-ACTX-Ar1a also cluster in a similar fashion due to the homology in sequence and structure between δ-ACTX-Ar1a and δ-ACTX-Hv1a. Accordingly, the conserved positively charged residues Lys^3, Lys^4, Arg^5, and Lys^{10} of δ-ACTX-Hv1a and δ-ACTX-Ar1a seem to occupy a similar surface position to the bioactive Lys^8, Arg^{58}, Lys^{62}, and Arg^{18} of LqhαIT. The aromatic Trp^7 and Tyr^{25} in δ-ACTX-Hv1a and Trp^7 and Tyr^{22} in δ-ACTX-Ar1a resemble to some extent Trp^{38} and Phe^{17} in LqhαIT and Trp^{38} and Phe^{15} in AaH-II. The nonpolar Asn^6 in δ-ACTX-Hv1a and δ-ACTX-Ar1a occupies a similar position to Asn^{44} in LqhαIT or AaH-II. Finally, the negatively charged Glu^{12} in δ-ACTX-Hv1a and δ-ACTX-Ar1a resembles Glu^{24} in both LqhαIT and AaH-II. Also included in this figure are residues known to be important for binding of the sea anemone toxin ApB which selectively binds to site-3 on cardiac sodium channels and also slows channel inactivation (Benzinger et al., 1998). It would also appear that critical residues known to be important for ApB binding (Dias-Kadambi et al., 1996; Khera and Blumenthal, 1996) group together in a similar fashion. Figure 5 shows that bioactive residues in ApB (Asp^9, Arg^{12}, Arg^{14}, Asn^{16}, Leu^{18}, Lys^{37}, Lys^{49}, Trp^{33}) occupy a similar topological position to those residues in the δ-ACTX, as detailed above, and to a lesser extent in scorpion α-toxins.

Differences between these residues and those in the scorpion α-toxins may therefore provide clues for the tissue-specific actions of ApB known to selectively target cardiac rather than neuronal sodium channels (Benzinger et al., 1998). In addition, the similar disposition of these residues on the surface of peptide toxins with different three-dimensional structures, particularly the scorpion α-toxins and δ-ACTX, demonstrates a possible route of convergent evolution of site-3 toxins.

Figure 5 here

Structural comparison of δ-ACTX-Hv1a to scorpion α-toxins suggests a similar putative bioactive surface but a slightly 'narrower' face which may explain its accessibility, with high binding affinity, to both rat brain and cockroach receptor site-3 (Gilles et al., 2001). This possibility may also explain the smaller alteration in binding affinity to site-3 between polarized and depolarized rat brain synaptosomes compared to Lqh-II, an AaH-II analogue (Gilles et al., 2001). The conformational change in the sodium channel that is associated with depolarization-induced shift from high to low affinity state of receptor site-3 (Gilles et al., 2001) may involve steric hindrance for toxin access, which is less pronounced for the slimmer δ-ACTX-Hv1a. This hypothesis is in concert with the smaller effect of membrane depolarization on the δ-ACTX-Hv1a association rate, k_{on}, compared to the effect on Lqh-II. However, the variations in k_{on} and unusual selectivity of toxins binding to receptor site-3 may result from either variations at the bioactive surface, or other differences in the entire toxin structure (Gilles et al., 2001).

Recently a homologous δ-ACTX was isolated from the venom of the Blue Mountains funnel-web spider *H. versuta* (Szeto et al., 2000). This 42–residue peptide, designated δ-ACTX-Hv1b, shows 67% sequence identity with δ-ACTX-Hv1a previously isolated from the same spider as shown in Fig. 1 (Szeto et al., 2000). δ-ACTX-Hv1b is unique amongst the δ-ACTX in that it lacks insecticidal activity and shows a 15- to 30-fold reduction in mammalian activity compared to δ-ACTX-Hv1a/Ar1a (Szeto et al., 2000). Close inspection of the primary structure reveals that a number of charged amino acids at the N-terminus are not conserved between δ-ACTX-Hv1b and δ-ACTX-Hv1a/Ar1a (Fig. 1) such that the cationic residues Lys^4 and Arg^5 of δ-ACTX-Hv1a/Ar1a are substituted by Ser^4 and Asp^5 in δ-ACTX-Hv1b. In addition, Asn^6 of δ-ACTX-Hv1a/Ar1a is substituted by Gly^6 and the hydrophobic residue Tyr^{22} in

δ-ACTX-Hv1a/Ar1a is replaced by Lys^{22}. This lends support to the hypothesis that these residues, putatively involved in binding, are important for determining insect and/or mammalian selectivity.

Given that δ-ACTX target both mammalian and insect sodium channels they have considerable potential as tools to aid in the investigation of structural requirements for anti-insect versus anti-mammal activity. The residues that differ between δ-ACTX-Hv1b and δ-ACTX-Hv1a/Ar1a therefore may give us an unexpected insight into the residues that facilitate interaction of δ-ACTX with insect sodium channels. This raises the possibility that manipulation of key residues may enable construction of a functional mirror-image of δ-ACTX-Hv1b, namely a δ-ACTX that binds to insect, but not vertebrate, voltage-gated sodium channels. Such an insect-specific δ-ACTX homologue might be a useful biopesticide.

9. Future directions

Further screening of venom gland cDNA libraries from other funnel-web spider species may provide important clues about the pharmacophore of these toxins by comparison of the primary sequence of toxin homologues. However, in isolation this approach has some drawbacks in that it does not provide any information of biological activity of any novel δ-ACTX homologues, especially insect vs. mammalian toxicity. This is highlighted by δ-ACTX-Hv1b that completely lacks insecticidal activity, an action not predictable from comparison of the primary sequence. At present, the best opportunity for determination of the bioactive surface of δ-ACTX most likely arises from the solid-phase peptide synthesis approach recently described for the production of synthetic δ-ACTX-Ar1a (Alewood et al., 2003). This provides for a direct means to identify the pharmacophore of the δ-ACTX by synthesizing analogues with selected residue changes. This would involve substitutions with non-natural amino acids as has been achieved with other toxins such as ShK, a potassium channel blocker from the sea anemone *Stichodactyla helianthus* (Kalman et al., 1998). Future structure-function studies to map the pharmacophores of these toxins using synthetic toxin analogues, combined with binding and electrophysiological approaches, should contribute to a more detailed mapping of site-3. This should provide structure-activity data critical for determining the phyla- and tissue-specific actions of this family of atracotoxins, and site-3

neurotoxins in general. This information may enable the design of insect-selective δ-ACTX even though selectively insecticidal δ-ACTX are unlikely to be found in funnel-web spider venom.

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References

Alewood, D., Birinyi-Strachan, L.C., Pallaghy, P., Norton, R., Nicholson, G.M., Alewood, P.F., 2003. Synthesis and characterisation of δ-atracotoxin-Ar1 the lethal neurotoxin from venom of the Sydney funnel-web spider (*Atrax robustus*). Biochemistry in press.

Benzinger, G.R., Kyle, J.W., Blumenthal, K.M., Hanck, D.A., 1998. A specific interaction between the cardiac sodium channel and site-3 toxin anthopleurin B. J. Biol. Chem. 273, 80-84.

Bernard, C., Corzo, G., Mosbah, A., Nakajima, T., Darbon, H., 2001. Solution structure of Ptu1, a toxin from the assassin bug *Peirates turpis* that blocks the voltage-sensitive calcium channel N-type. Biochemistry 40, 12795-12800.

Bernard, C., Legros, C., Ferrat, G., Bischoff, U., Marquardt, A., Pongs, O., Darbon, H., 2000. Solution structure of hpTX2, a toxin from *Heteropoda venatoria* spider that blocks $K_v4.2$ potassium channel. Protein Sci. 9, 2059-2067.

Brown, M.R., Sheumack, D.D., Tyler, M.I., Howden, M.E.H., 1988. Amino acid sequence of versutoxin, a lethal neurotoxin from the venom of the funnel-web spider *Atrax versutus*. Biochem. J. 250, 401-405.

Calvete, J.J., Wang, Y., Mann, K., Schafer, W., Niewiarowski, S., Stewart, G.J., 1992. The disulfide bridge pattern of snake venom disintegrins, flavoridin and echistatin. FEBS Lett. 309, 316-320.

Catterall, W.A., 1977. Activation of the action potential Na^+ ionophore by neurotoxins. J. Biol. Chem. 252, 8669-8676.

Catterall, W.A., 1992. Cellular and molecular biology of voltage-gated sodium channels. Physiol. Rev. 72, S15-48.

Cestèle, S., Ben Khalifa, R.B., Pelhate, M., Rochat, H., Gordon, D., 1995. Alpha-scorpion toxins binding on rat brain and insect sodium channels reveal divergent allosteric modulations by brevetoxin and veratridine. J. Biol. Chem. 270, 15153-15161.

Cestèle, S., Catterall, W.A., 2000. Molecular mechanisms of neurotoxin action on voltage-gated sodium channels. Biochimie 82, 883-892.

Civera, C., Vazquez, A., Sevilla, J.M., Bruix, M., Gago, F., Garcia, A.G., Sevilla, P., 1999. Solution structure determination by two-dimensional ^{1}H NMR of ω-conotoxin MVIID, a calcium channel blocker peptide. Biochem. Biophys. Res. Commun. 254, 32-35.

Darbon, H., Zlotkin, E., Kopeyan, C., van Rietschoten, J., Rochat, H., 1982. Covalent structure of the insect toxin of the North African scorpion *Androctonus australis* Hector. Int. J. Pept. Protein Res. 20, 320-330.

DeBin, J.A., Maggio, J.E., Strichartz, G.R., 1993. Purification and characterization of chlorotoxin, a chloride channel ligand from the venom of the scorpion. Am. J. Physiol. 264, C361-369.

Dias-Kadambi, B.L., Drum, C.L., Hanck, D.A., Blumenthal, K.M., 1996. Leucine 18, a hydrophobic residue essential for high affinity binding of anthopleurin B to the voltage-sensitive sodium channel. J. Biol. Chem. 271, 9422-9428.

Dieckmann, J., Prebble, J., McDonogh, A., Sara, A., Fisher, M., 1989. Efficacy of funnel-web spider antivenom in human envenomation by *Hadronyche* species. Med. J. Aust. 151, 706-707.

Eitan, M., Fowler, E., Herrmann, R., Duval, A., Pelhate, M., Zlotkin, E., 1990. A scorpion venom neurotoxin paralytic to insects that affects sodium current inactivation - purification, primary structure, and mode of action. Biochemistry 29, 5941–5947.

Escoubas, P., Diochot, S., Corzo, G., 2000. Structure and pharmacology of spider venom neurotoxins. Biochimie 82, 893-907.

Fajloun, Z., Kharrat, R., Chen, L., Lecomte, C., Di Luccio, E., Bichet, D., El Ayeb, M., Rochat, H., Allen, P.D., Pessah, I.N., De Waard, M., Sabatier, J.M., 2000. Chemical synthesis and characterization of maurocalcine, a scorpion toxin that activates Ca^{2+} release channel/ryanodine receptors. FEBS Lett. 469, 179-185.

Farr-Jones, S., Miljanich, G.P., Nadasdi, L., Ramachandran, J., Basus, V.J., 1995. Solution structure of ω-conotoxin MVIIC, a high affinity ligand of P-type calcium channels, using ^{1}H NMR spectroscopy and complete relaxation matrix analysis. J. Mol. Biol. 248, 106-124.

Fletcher, J.I., Chapman, B.E., Mackay, J.P., Howden, M.E.H., King, G.F., 1997a. The structure of versutoxin (δ-atracotoxin-Hv1): implications for binding of site-3 toxins to the voltage-gated sodium channel. Structure 5, 1525-1535.

Fletcher, J.I., Dingley, A.J., Smith, R., Connor, M., Christie, M.J., King, G.F., 1999. High-resolution solution structure of gurmarin, a sweet-taste-suppressing plant polypeptide. Eur. J. Biochem. 264, 525-533.

Fletcher, J.I., Smith, R., O'Donoghue, S.I., Nilges, M., Connor, M., Howden, M.E.H., Christie, M.J., King, G.F., 1997b. The structure of a novel insecticidal neurotoxin, ω-atracotoxin-HV1, from the venom of an Australian funnel web spider. Nature Struct. Biol. 4, 559-566.

Fontecilla-Camps, J.C., Habersetzer-Rochat, C., Rochat, H., 1988. Orthorhombic crystals and three-dimensional structure of the potent toxin II from the scorpion *Androctonus australis* Hector. Proc. Natl. Acad. Sci. USA 85, 7443-7447.

Fontecilla–Camps, J.C., Almassy, R.J., Suddath, F.L., Bugg, C.E., 1982. The three-dimensional structure of scorpion neurotoxins. Toxicon 20, 1–7.

Gilles, N., Harrison, G., Karbat, I., Gurevitz, M., Nicholson, G.M., Gordon, D., 2002. Variations in receptor site-3 on rat brain and insect sodium channels highlighted by binding of a funnel-web spider δ-atracotoxin. Eur. J. Biochem. 269, 1500-1510.

Gilles, N., Krimm, I., Bouet, F., Froy, O., Gurevitz, M., Lancelin, J.M., Gordon, D., 2000. Structural implications on the interaction of scorpion alpha-like toxins with the sodium channel receptor site inferred from toxin iodination and pH-dependent binding. J. Neurochem. 75, 1735-1745.

Gilles, N., Leipold, E., Chen, H., Heinemann, S.H., Gordon, D., 2001. Effect of depolarization on binding kinetics of scorpion alpha-toxin highlights conformational changes of rat brain sodium channels. Biochemistry 40, 14576-14584.

Gordon, D., 1997a. A new approach to insect-pest control-combination of neurotoxins interacting with voltage sensitive sodium channels to increase selectivity and specificity. Invert. Neurosci. 3, 103-116.

Gordon, D., 1997b. In: Lazarowici, P., Gutman, Y. (Eds.), Sodium channels as targets for neurotoxins: mode of action and interaction of neurotoxins with receptor sites on sodium channels, Toxins and Signal Transduction, Harwood Press, Amsterdam, pp. 119-149.

Gordon, D., Martin-Eauclaire, M.-F., Cestèle, S., Kopeyan, C., Carlier, E., Khalifa, R.B., Pelhate, M., Rochat, H., 1996. Scorpion toxins affecting sodium channel current inactivation bind to distinct homologous receptor sites on rat brain and insect sodium channels. J. Biol. Chem. 271, 8034-8045.

Gordon, D., Savarin, P., Gurevitz, M., Zinn-Justin, S., 1998. Functional anatomy of scorpion toxins affecting sodium channels. J. Toxicol.-Toxin Rev. 17, 131-159.

Gordon, D., Zlotkin, E., 1993. Binding of an α-scorpion toxin to insect sodium channels is not dependent on membrane potential. FEBS Lett. 315, 125–128.

Graudins, A., Wilson, D., Alewood, P.F., Broady, K.W., Nicholson, G.M., 2002. Cross-reactivity of Sydney funnel-web spider antivenom: Neutralization of the *in vitro* toxicity of other Australian funnel-web (*Atrax* and *Hadronyche*) spider venoms. Toxicon 40, 259-266.

Gray, M.R., 1988. In: Austin, A. D., Heather, N. W. (Eds.), Aspects of the systematics of the Australian funnel web spiders (Araneae:Hexathelidae:Atracinae) based upon morphological and electrophoretic data, Australian Arachnology, The Australian Entomological Society, Brisbane, pp. 113–125.

Gregson, R.P., Spence, I., 1983. Isolation and characterization of a protein neurotoxin from the venom glands of the funnel-web spider (*Atrax robustus*). Comp. Biochem. Physiol. C 74, 125-132.

Grolleau, F., Stankiewicz, M., Birinyi-Strachan, L.C., Wang, X.-H., Nicholson, G.M., Pelhate, M., Lapied, B., 2001. Electrophysiological analysis of the neurotoxic action of a funnel-web spider toxin, δ-atracotoxin-Hv1a, on insect voltage-gated Na^+ channels. J. Exp. Biol. 204, 711-721.

Gurevitz, M., Gordon, D., Ben-Natan, S., Turkov, M., Froy, O., 2001. Diversification of neurotoxins by C-tail 'wiggling': a scorpion recipe for survival. FASEB J. 15, 1201-1205.

Hanck, D.A., Sheets, M.F., 1995. Modification of inactivation in cardiac sodium channels: ionic current studies with anthopleurin-A toxin. J. Gen. Physiol. 106, 601-616.

Harrington, A.P., Raven, R.J., Bowe, P.C., Hawdon, G.M., Winkel, K.D., 1999. Funnel-web spider (*Hadronyche infensa*) envenomations in coastal south-east Queensland. Med. J. Aust. 171, 651-653.

Hill, J.M., Alewood, P.F., Craik, D.J., 1997. Solution structure of the sodium channel antagonist conotoxin GS: a new molecular caliper for probing sodium channel geometry. Structure 5, 571-583.

Kalman, K., Pennington, M.W., Lanigan, M.D., Nguyen, A., Rauer, H., Mahnir, V., Paschetto, K., Kem, W.R., Grissmer, S., Gutman, G.A., Christian, E.P., Cahalan, M.D., Norton, R.S., Chandy, K.G., 1998. ShK-Dap22, a potent K_v1.3-specific immunosuppressive polypeptide. J. Biol. Chem. 273, 32697-32707.

Khera, P.K., Blumenthal, K.M., 1996. Importance of highly conserved anionic residues and electrostatic interactions in the activity and structure of the cardiotonic polypeptide anthopleurin B. Biochemistry 35, 3503-3507.

Kim, J.I., Konishi, S., Iwai, H., Kohno, T., Gouda, H., Shimada, I., Sato, K., Arata, Y., 1995. Three-dimensional solution structure of the calcium channel antagonist ω-agatoxin IVA; consensus molecular folding of calcium channel blockers. J. Mol. Biol. 250, 659–671.

King, G.F., Tedford, H.W., Maggio, F., 2002. Structure and function of insecticidal neurotoxins from Australian funnel-web spiders. J. Toxicol.-Toxin Rev. 21, 359-389.

Kobayashi, K., Sasaki, T., Sato, K., Kohno, T., 2000. Three-dimensional solution structure of ω-conotoxin TxVII, an L-type calcium channel blocker. Biochemistry 39, 14761-14767.

Kohno, T., Sasaki, T., Kobayashi, K., Fainzilber, M., Sato, K., 2002. Three-dimensional solution structure of the sodium channel agonist/antagonist δ-conotoxin TxVIA. J. Biol. Chem. 277, 36387-36391.

Little, M.J., Wilson, H., Zappia, C., Cestèle, S., Tyler, M.I., Martin-Eauclaire, M.-F., Gordon, D., Nicholson, G.M., 1998a. δ-Atracotoxins from Australian funnel-web spiders compete with scorpion α-toxin binding on both rat brain and insect sodium channels. FEBS Lett. 439, 246–252.

Little, M.J., Zappia, C., Gilles, N., Connor, M., Tyler, M.I., Martin-Eauclaire, M.-F., Gordon, D., Nicholson, G.M., 1998b. δ-Atracotoxins from Australian funnel-web spiders compete with

scorpion α-toxin binding but differentially modulate alkaloid toxin activation of voltage-gated sodium channels. J. Biol. Chem. 273, 27076-27083.

Martin-Eauclaire, M.-F., Courand, F., 1995. In: Chang, L. W., Dyer, R. S. (Eds.), Scorpion neurotoxins: effects and mechanisms, Handbook of Neurotoxicology, Marcel Dekker, New York, pp. 683-716.

Miller, M.K., Whyte, I.M., White, J., Keir, P.M., 2000. Clinical features and management of *Hadronyche* envenomation in man. Toxicon 38, 409-427.

Monks, S.A., Pallaghy, P.K., Scanlon, M.J., Norton, R.S., 1995. Solution structure of the cardiostimulant polypeptide anthopleurin-B and comparison with anthopleurin-A. Structure 3, 791–803.

Mylecharane, E.J., Spence, I., Sheumack, D.D., Claassens, R., Howden, M.E.H., 1989. Actions of robustoxin, a neurotoxic polypeptide from the venom of the male funnel-web spider (*Atrax robustus*), in anaesthetized monkeys. Toxicon 27, 481-492.

Naranjo, D., 2002. Inhibition of single *Shaker* K channels by κ-conotoxin-PVIIA. Biophys. J. 82, 3003-3011.

Nicholson, G.M., Walsh, R., Little, M.J., Tyler, M.I., 1998. Characterisation of the effects of robustoxin, the lethal neurotoxin from the Sydney funnel-web spider *Atrax robustus*, on sodium channel activation and inactivation. Pflügers Archiv. (Eur. J. Physiol.) 436, 117-126.

Nicholson, G.M., Willow, M., Howden, M.E.H., Narahashi, T., 1994. Modification of sodium channel gating and kinetics by versutoxin from the Australian funnel-web spider *Hadronyche versuta*. Pflügers Archiv. (Eur. J. Physiol.) 428, 400-409.

Nielsen, K.J., Thomas, L., Lewis, R.J., Alewood, P.F., Craik, D.J., 1996. A consensus structure for ω-conotoxins with different selectivities for voltage-sensitive calcium channel subtypes: comparison of MVIIA, SVIB and SNX-202. J. Mol. Biol. 263, 297-310.

Norton, R.S., Pallaghy, P.K., 1998. The cystine knot structure of ion channel toxins and related polypeptides. Toxicon 36, 1573–1583.

Omecinsky, D.O., Holub, K.E., Adams, M.E., Reily, M.D., 1996. Three-dimensional structure analysis of μ-agatoxins: further evidence for common motifs among neurotoxins with diverse ion channel specificities. Biochemistry 35, 2836-2844.

Oswald, R.E., Suchyna, T.M., McFeeters, R., Gottlieb, P., Sachs, F., 2002. Solution structure of peptide toxins that block mechanosensitive ion channels. J. Biol. Chem. 277, 34443-34450.

Pallaghy, P.K., Alewood, D., Alewood, P.F., Norton, R.S., 1997. Solution structure of robustoxin, the lethal neurotoxin from the funnelweb spider *Atrax robustus*. FEBS Lett. 419, 191-196.

Pallaghy, P.K., Neilsen, K.J., Craik, D.J., Norton, R.S., 1994. A common structural motif incorporating a cystine knot and a triple-stranded β-sheet in toxic and inhibitory polypeptides. Protein Sci. 3, 1833-1839.

Pallaghy, P.K., Norton, R.S., 1999. Refined solution structure of ω-conotoxin GVIA: implications for calcium channel binding. J. Pept. Res. 53, 343-351.

Peng, K., Shu, Q., Liu, Z., Liang, S., 2002. Function and solution structure of huwentoxin-IV, a potent neuronal tetrodotoxin (TTX)-sensitive sodium channel antagonist from Chinese bird spider *Selenocosmia huwena*. J. Biol. Chem. 277, 47564-47571.

Phillips, C.A., Spence, I., Mylecharane, E.J., Brown, M.R., Sheumack, D.D., Claassens, R., Howden, M.E.H., 1987. The effects of the venom from the Blue Mountains funnel-web spider (*Atrax versutus*) in anaesthetized monkeys. Clin. Exp. Pharmacol. Physiol. Suppl 10, 14–15.

Qu, Y., Liang, S., Ding, J., Liu, X., Zhang, R., Gu, X., 1997. Proton nuclear magnetic resonance studies on huwentoxin-I from the venom of the spider *Selenocosmia huwena*: 2. Three-dimensional structure in solution. J. Protein Chem. 16, 565-574.

Rash, L.D., Hodgson, W.C., 2002. Pharmacology and biochemistry of spider venoms. Toxicon 40, 225-254.

Ray, R., Morrow, C.S., Catterall, W.A., 1978. Binding of scorpion toxin to receptor sites associated with voltage-sensitive sodium channels in synaptic nerve ending particles. J. Biol. Chem. 253, 7307-7313.

Reily, M.D., Thanabal, V., Adams, M.E., 1995. The solution structure of ω-Aga-IVB, a P-type calcium channel antagonist from venom of the funnel web spider, *Agelenopsis aperta*. J. Biomol. NMR 5, 122-132.

Rogers, J.C., Qu, Y., Tanada, T.N., Scheuer, T., Catterall, W.A., 1996. Molecular determinants of high affinity binding of α-scorpion toxin and sea anemone toxin in the S3-S4 extracellular loop in domain IV of the Na^+ channel α subunit. J. Biol. Chem. 271, 15950–15962.

Rosengren, K.J., Wilson, D., Daly, N.L., Alewood, P.F., Craik, D.J., 2002. Solution structures of the cis- and trans-Pro30 isomers of a novel 38-residue toxin from the venom of *Hadronyche infensa* sp. that contains a cystine-knot motif within its four disulfide bonds. Biochemistry 41, 3294-3301.

Sautière, P., Cestèle, S., Kopeyan, C., Martinage, A., Drobecq, H., Doljansky, Y., Gordon, D., 1998. New toxins acting on sodium channels from the scorpion *Leiurus quinquestriatus hebraeus* suggest a clue to mammalian vs insect selectivity. Toxicon 36, 1141-1154.

Sheumack, D.D., Baldo, B.A., Carroll, P.R., Hampson, F., Howden, M.E., Skorulis, A., 1984. A comparative study of properties and toxic constituents of funnel web spider (*Atrax*) venoms. Comp. Biochem. Physiol. C 78, 55-68.

Sheumack, D.D., Claassens, R., Whiteley, N.M., Howden, M.E.H., 1985. Complete amino acid sequence of a new type of lethal neurotoxin from the venom of the funnel-web spider *Atrax robustus*. FEBS Lett. 181, 154-156.

Sheumack, D.D., Comis, A., Claassens, R., Mylecharane, E.J., Spence, I., Howden, M.E.H., 1991. An endogenous antitoxin to the lethal venom of the funnel web spider, *Atrax robustus*, in rabbit sera. Comp. Biochem. Physiol. 99C, 157-161.

Strichartz, G.R., Wang, G.K., 1986. Rapid voltage-dependent dissociation of scorpion α-toxins coupled to Na channel inactivation in amphibian myelinated nerves. J. Gen. Physiol. 88, 413-435.

Sutherland, S.K., 1980. Antivenom to the venom of the male Sydney funnel-web spider *Atrax robustus*. Preliminary report. Med. J. Aust. 2, 437-441.

Sutherland, S.K., Tibballs, J., 2001. In: The genera *Atrax* and *Hadronyche*, Funnel-web spiders, Australian Animal Toxins: The Creatures, Their Toxins and Care of the Poisoned Patient, Oxford University Press, Melbourne, pp. 402-464.

Szeto, T.H., Birinyi-Strachan, L.C., Wang, X.-H., Smith, R., Connor, M., Christie, M.J., King, G.F., Nicholson, G.M., 2000. Isolation and pharmacological characterisation of δ-atracotoxin-Hv1b, a vertebrate-selective sodium channel toxin. FEBS Lett. 470, 293-299.

Takahashi, H., Kim, J.I., Min, H.J., Sato, K., Swartz, K.J., Shimada, I., 2000. Solution structure of hanatoxin1, a gating modifier of voltage-dependent K^+ channels: common surface features of gating modifier toxins. J. Mol. Biol. 297, 771-780.

Takeuchi, K., Park, E., Lee, C., Kim, J., Takahashi, H., Swartz, K., Shimada, I., 2002. Solution structure of ω-grammotoxin SIA, a gating modifier of P/Q and N-type Ca^{2+} channel. J. Mol. Biol. 321, 517.

Torda, T.A., Loong, E., Greaves, I., 1980. Severe lung oedema and fatal consumption coagulopathy after funnel-web bite. Med. J. Aust. 2, 442-444.

Tugarinov, V., Kustanovich, I., Zilberberg, N., Gurevitz, M., Anglister, J., 1997. Solution structures of a highly insecticidal recombinant scorpion alpha-toxin and a mutant with increased activity. Biochemistry 36, 2414–2424.

Wang, X.-H., Connor, M., Smith, R., Maciejewski, M.W., Howden, M.E.H., Nicholson, G.M., Christie, M.J., King, G.F., 2000. Discovery and characterization of a family of insecticidal neurotoxins with a rare vicinal disulfide bridge. Nature Struct. Biol. 7, 505-513.

Wang, X.-H., Connor, M., Wilson, D., Wilson, H., Nicholson, G.M., Smith, R., Shaw, D., Mackay, J.P., Alewood, P.F., Christie, M.J., King, G.F., 2001. Biopesticide panning: discovery of a potent and highly specific peptide antagonist of insect calcium channels. J. Biol. Chem. 276, 40306-40312.

Wang, X.-H., Smith, R., Fletcher, J.I., Wilson, H., Wood, C.J., Howden, M.E.H., King, G.F., 1999. Structure-function studies of omega-atracotoxin, a potent antagonist of insect voltage-gated calcium channels. Eur. J. Biochem. 264, 488-494.

White, J., Carduso, J.L., Fan, H.W., 1995. In: Meier, J., White, J. (Eds.), Clinical toxicology of spider bites, Handbook of Clinical Toxicology of Animal Venoms and Poisons, CRC Press, New York, pp. 259-329.

Zilberberg, N., Froy, O., Loret, E., Cestèle, S., Arad, D., Gordon, D., Gurevitz, M., 1997. Identification of structural elements of a scorpion α-neurotoxin important for receptor site recognition. J. Biol. Chem. 272, 14810-14816.

Zlotkin, E., Fishman, Y., Elazar, M., 2000. AaIT: from neurotoxin to insecticide. Biochimie 82, 869-881.

Table 1. Receptor sites 1-7 identified by neurotoxins and pyrethroids on mammalian voltage-gated sodium channels.

Neurotoxin receptor site	Toxin	Effect on ion permeation or gating	Allosteric coupling[a]
Site-1	Tetrodotoxin (TTX) Saxitoxin (STX) μ-Conotoxin	Block Na^+ conductance	+3, +5, –2
Site-2	Batrachotoxin (BTX) Veratridine Aconitine Grayanotoxin	Persistent activation Hyperpolarising shift in voltage dependence of activation and inhibit inactivation	+3, –6
Site-3	Scorpion α-toxins Sea anemone toxins δ-Atracotoxins	Inhibit inactivation, minor hyperpolarising shift in voltage dependence of activation	+2, –5
Site-4	Scorpion β-toxins	Hyperpolarising shift in voltage dependence of activation	
Site-5	Brevetoxins Ciguatoxins	Hyperpolarising shift in voltage dependence of activation and inhibit inactivation	+2, +4, –3
Site-6	δ-Conotoxins (δ-TxVIA)	Inhibit inactivation	–2
Site-7	Pyrethroids	Inhibit inactivation, shift voltage dependence of activation and slow activation and deactivation kinetics	+2, +3, +5

[a]Allosteric coupling refers to the alteration in affinity of toxins binding at the neurotoxin receptor site (first column) by neurotoxin occupancy at the indicated site (final column). Positive cooperativity (+) indicates enhancement of binding of the toxin and/or stimulation of Na^+ influx while negative cooperativity (–) refers to a decrease in toxin binding (Gordon et al., 1998). Adapted from (Cestèle and Catterall, 2000; Zlotkin et al., 2000).

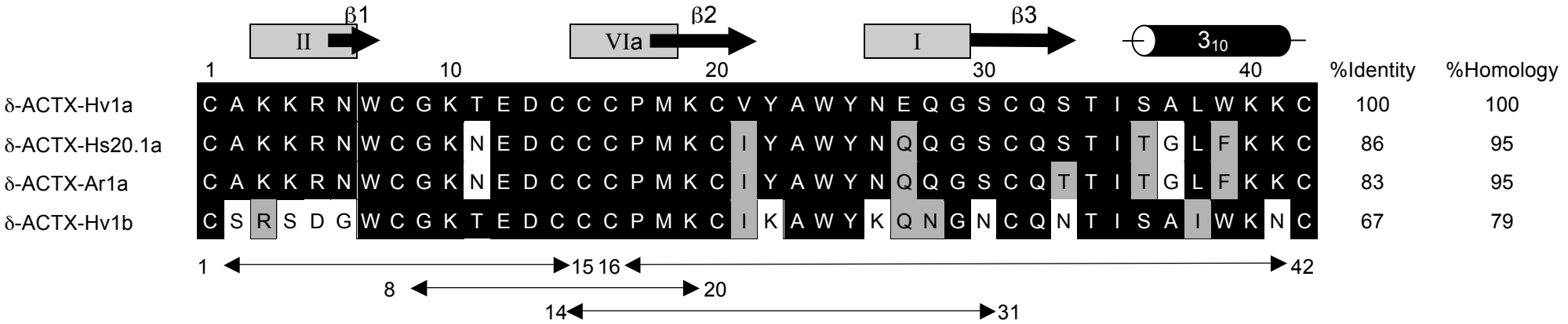

Fig. 1. Comparison of the amino acid sequences of all currently available members of the δ-ACTX family. Identical residues are shaded in black and conservatively substituted residues, relative to δ-ACTX-Hv1a, are shaded in grey. Toxins are ordered by homology to δ-ACTX-Hv1a. The disulfide bonding pattern for the strictly conserved cysteine residues determined for δ-ACTX-Ar1a (Pallaghy et al., 1997) and δ-ACTX-Hv1a (Fletcher et al., 1997a) is indicated below the sequences; it is assumed that δ-ACTX-Hs20.1a and δ-ACTX-Hv1b have the same disulfide bonding pattern. Secondary structure for δ-ACTX-Hv1a is shown at the top of the figure where shaded squares represent β-turns (the type is indicated in the squares), black arrows represent β-strands, and the black cylinder represents a 3_{10} helix (Fletcher et al., 1997a). The percentage identity and homology with δ-ACTX-Hv1a is shown to the right of the sequences. Additional sequence data is taken from δ-ACTX-Hv1b (Szeto et al., 2000), and δ-ACTX-Hs20.1a (Wilson D and Alewood PF, unpublished data).

Source		I	II	III	IV	V	VI	Amino Acids	Target
Spiders	δ-ACTX-Ar1a	- - - - C A K K R N W - C	G K N E D - - C C	C P M K - - C	I Y A W Y N Q Q G S - - - C	Q T T I T G L F K K C		42	Na^+
	δ-ACTX-Hv1a	- - - - C A K K R N W - C	G K T E D - - C C	C P M K - - C	V Y A W Y N E Q G S - - - C	Q S T I S A L W K K C		42	Na^+
	μ-Aga IV	- - - A C V G E N Q Q - C	A D W A G P H C C	D G Y Y - - C	T C R Y F P K - - - - C I C	R N N N		37	Na^+
	μ-Aga I	- - - E C V P E N G H - C	R D W Y D E - C C	E G F Y - - C	S C R Q P P K - - - - C I C	R N N N		36	Na^+
	HWTX-IV	E C L E I F K A - C	N P S N D Q - C C	K S S K L V C	S R K T R W - - - - - - - C	K Y Q I		35	Na^+
	ω-Aga IVB	- E D N C I A E D Y G K C	T W G G T K - C C	R G R P - - C	R C S M I G T N - - - C E C	T P R L I M E G L S F A		48	Ca^{2+} / P,Q
	ω-Aga IVA	- K K K C I A K D Y G R C	K W G G T P - C C	R G R G - - C	I C S I M G T N - - - C E C	K P R L I M E G L G L A		48	Ca^{2+} / P,Q
	ω-ACTX-Hv2a	- L L A C L F G N G R - C	S S N R D - - C C	E L T P V - C	K R G S - - - - - - - - - C	V S S G P G L V G G I L G G I L		45	Ca^{2+} / insect
	ω-ACTX-Hv1a	- S P T C I P S G Q P - C	P Y N E N - - C C	S Q S - - - C	T F K E N E N G N T V K R C	D		37	Ca^{2+} / insect
	ω-GrTx SIA	- - - D C V R F W G K - C	S Q T S D - - C C	P H L A - - C	K S K W P R N I - - - - - C	V W D G S V		36	Ca^{2+} / P,Q,N
	HWTX-I	- - - A C K G V F D A - C	T P G K N E - C C	P N R V - - C	S D K H K W - - - - - - - C	K W K L		33	Ca^{2+} / N
	J-ACTX-Hv1c	- - A I C T G A D R P - C	A A C C P - - C C	P G T S - - C	K A E S N G V S Y - - - - C	R K D E P		37	K^+ / insect
	HaTx1	- - - E C R Y L F G G - C	K T T S D - - C C	K H L G - - C	K F R D K Y - - - - - - - C	A W D F T F S		35	K^+ / 2.1, 4.2
	HpTx2	- - D D C G K L F S G - C	D T N A D - - C C	E G Y V - - C	R L W - - - - - - - - - - C	K L D W		30	K^+ / 4.2
	GsMTx-4	- - - G C L E F W W K - C	N P N D D K - C C	R P K L K - C	S K L F K L - - - - - - - C	N F S S G		35	MSC
Scorpion	MCa	- - G D C L P H L K L - C	K E N K D - - C C	S K K - - - C	K R R G T N I E K R - - - C	R		33	RyR1
Insect	Ptu1	A E K D C I A P G A P - C	F G T D K P - C C	N P R A W - C	S S Y A N K - - - - - - - C	L		34	Ca^{2+} / N
Marine molluscs	Conotoxin GS	- - - A C S G R G S R - C	O O Q - - - - C C	M G L R - - C	G R G N P Q K - - - - - - C	I G A H X D V		34	Na^+
	δ-CTX TxVIA	- - - W C K Q S G E M - C	N L L D Q N - C C	D G Y - - - C	I V L V - - - - - - - - - C	T		27	Na^+
	ω-CTX GVIA	- - - - C K S O G S S - C	S O T S Y N - C C	R S - - - - C	N O Y T K R - - - - - - - C	Y		27	Ca^{2+} / N
	ω-CTX TxVII	- - - - C K Q A D E P - C	D V F S L D - C C	T G I - - - C	L G V - - - - - - - - - - C	M W		26	Ca^{2+} / L
	ω-CTX MVIIC	- - - - C K G K G A P - C	R K T M Y D - C C	S G S - - - C	G R R G K - - - - - - - - C			26	Ca^{2+} / P,Q
	ω-CTX MVIID	- - - - C Q G R G A S - C	R K T M Y N - C C	S G S - - - C	N R G R - - - - - - - - - C			25	Ca^{2+} / P,Q
	ω-CTX MVIIA	- - - - C K G K G A K - C	S R L M Y D - C C	T G S - - - C	R S G K - - - - - - - - - C			25	Ca^{2+} / N
	κ-CTX PVIIA	- - - - C R I O N Q K - C	F Q H L D D - C C	S R K - - - C	N R F N K - - - - - - - - C	V		28	K^+ / Shaker

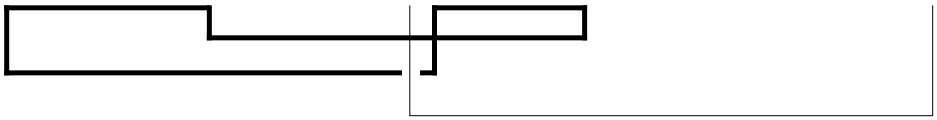

Fig. 2. Alignment of δ-ACTX and other toxic inhibitor cystine-knot (ICK) peptides. Only toxins with experimentally determined cysteine connectivities are shown. Alignment is based on cysteine residues, which are highlighted and numbered I-VI. The ICK motif connectivity (I-IV, II-V, III-IV) is shown below the table. The additional fourth disulfide bond (Cys^{16}-Cys^{42}) in the δ-ACTX-1 family has been omitted for clarity. The sequences are ordered based on source, target and length. Data are from: δ-ACTX-Ar1a (Pallaghy et al., 1997), δ-ACTX-Hv1a (Fletcher et al., 1997a), μ-Aga IV (Omecinsky et al., 1996), μ-Aga I (Omecinsky et al., 1996), HWTX-IV (Peng et al., 2002), ω-Aga IVB (Reily et al., 1995), ω-Aga IVA (Kim et al., 1995), ω-ACTX-Hv2a (Wang et al., 2001), ω-ACTX-Hv1a (Fletcher et al., 1997b), ω-GrTx SIA (Takeuchi et al., 2002), HWTX-1 (Qu et al., 1997), J-ACTX-

Hv1c (Wang et al., 2000), HaTx1 (Takahashi et al., 2000), HpTx2 (Bernard et al., 2000), GsMTx-4 (Oswald et al., 2002), MCa (Fajloun et al., 2000), Ptu1 (Bernard et al., 2001), conotoxin GS (Hill et al., 1997), δ-CTX TxVIA (Kohno et al., 2002), ω-CTX GVIA (Pallaghy and Norton, 1999), ω-CTX TxVII (Kobayashi et al., 2000), ω-CTX MVIIC (Farr-Jones et al., 1995), ω-CTX MVIID (Civera et al., 1999), ω-CTX MVIIA (Nielsen et al., 1996), κ-CTX PVIIA (Naranjo, 2002). MSC, mechanosensitive channel; RyR1, type 1 ryanodine receptor.

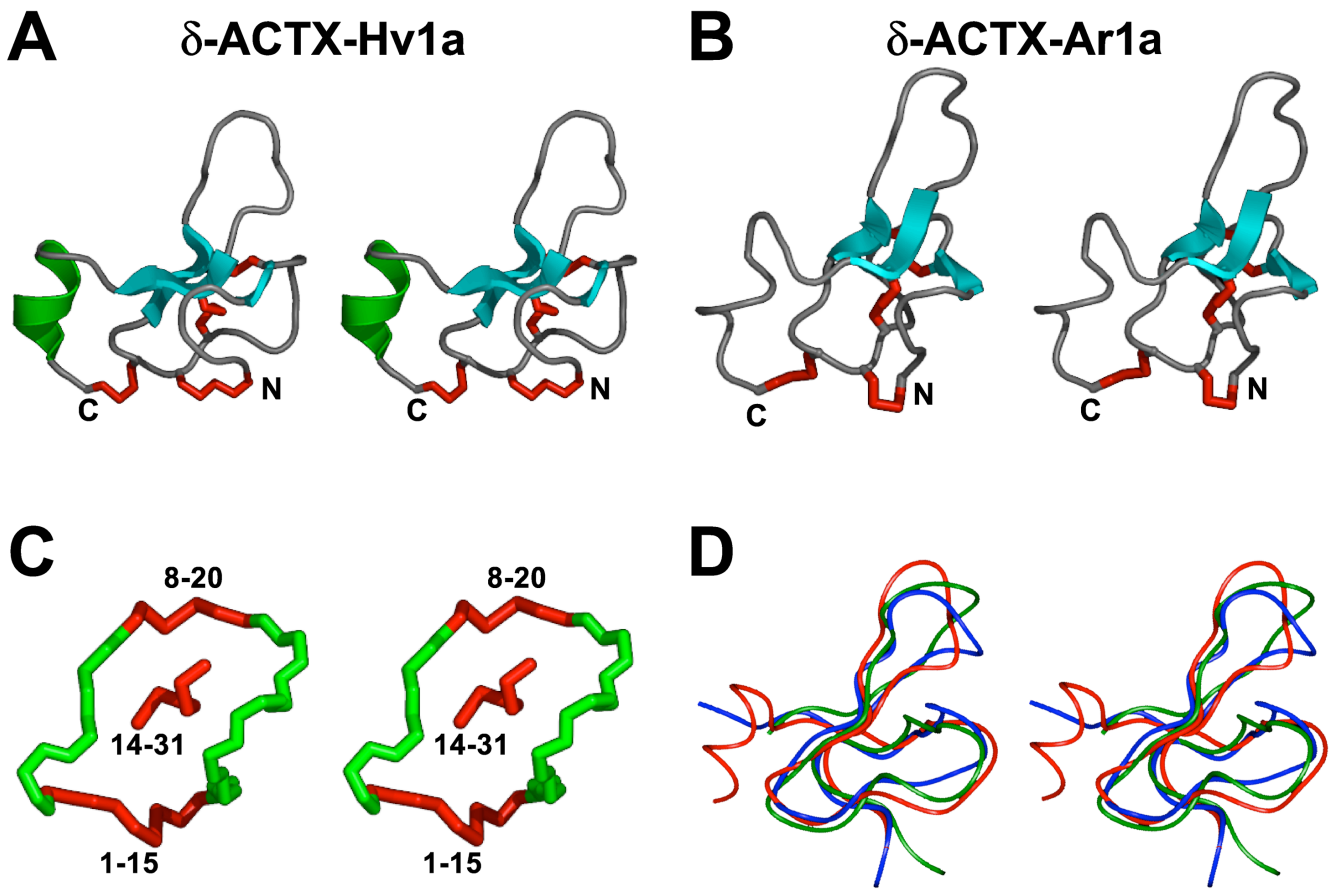

Fig. 3. Structure of δ-ACTX. (A-B) Stereo schematic view of the structures of δ-ACTX-Ar1a (A; PDB file 1VTX) and δ-ACTX-Hv1a (B; PDB file 1QDP) showing the location of β-strands (cyan arrows) and C-terminal 3_{10}-helix (green). The N- and C- termini are labeled. These disulfides bridges form an inhibitor cystine-knot (ICK) motif and are shown as red tubes. (C) Stereo view of the ICK motif of δ-ACTX-Hv1a. The Cys^{14}-Cys^{31} disulfide pierces a 14-residue loop formed by the Cys^{1}-Cys^{15} and Cys^{8}-Cys^{20} disulfides (red) and the intervening sections of the polypeptide backbone (green) to create a pseudo-knot. (D) Structural comparison of δ-ACTX-Hv1a, gurmarin (PDB file 1C4E), and μ-agatoxin I (PDB file 1EIT). Stereo view of an overlay of the backbone of gurmarin (green), δ-ACTX-Hv1a (red), and μ-agatoxin I (blue). Note that δ-ACTX-Hv1a has a C-terminal 3_{10}-helical extension relative to the other two structures. Toxin models were prepared using PyMOL.

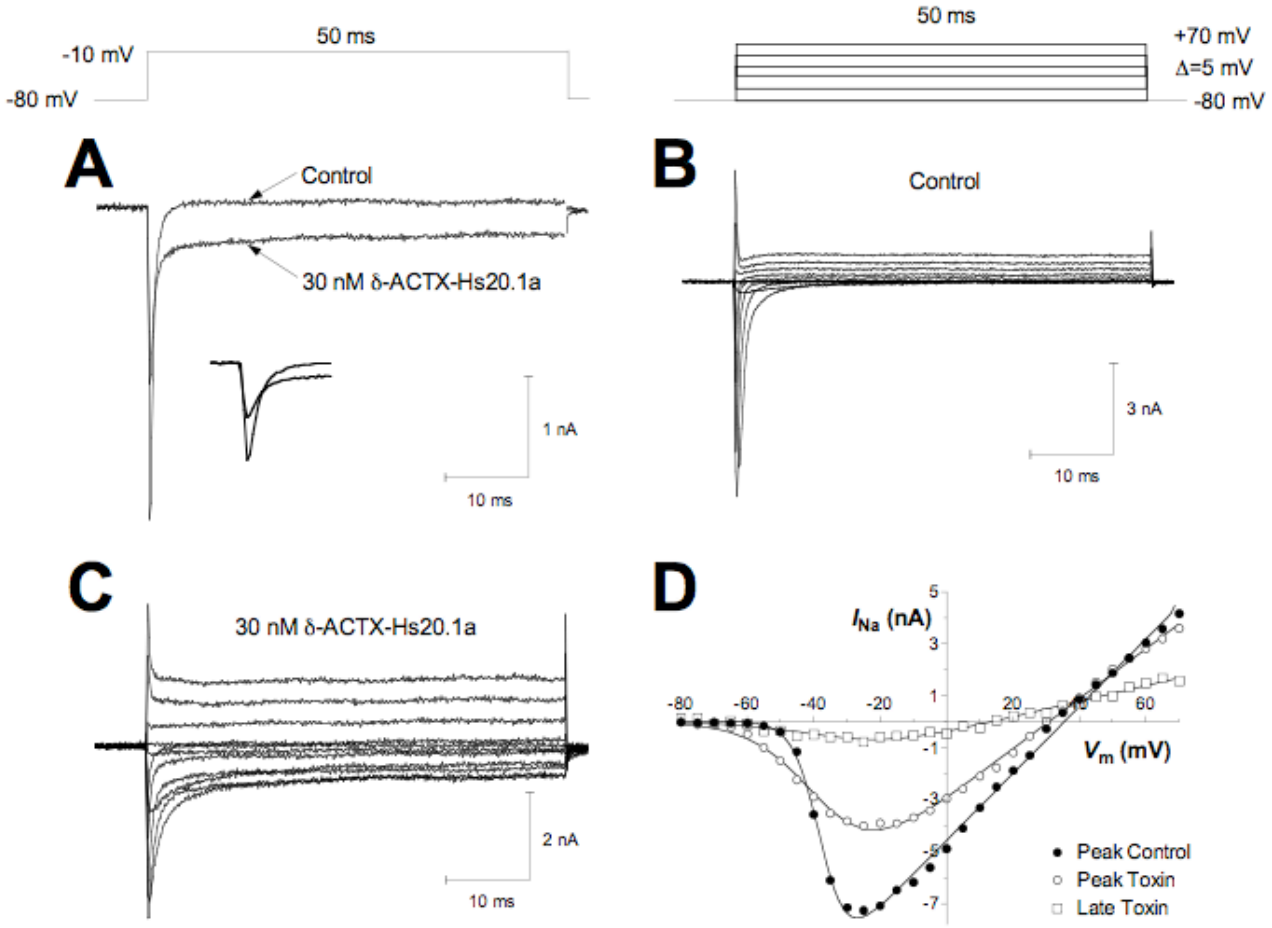

Fig. 4. Effects of δ-ACTX-Hs20.1a on TTX-sensitive sodium currents in rat dorsal root ganglion neurons. δ-ACTX-Hs20.1a was purified from the venom of *Hadronyche* sp. 20 collected from Gerringong, New South Wales, according to the methods of Alewood et al. (2003). Whole-cell patch clamp recording and analysis of isolated sodium currents from immature rat dorsal root ganglion neurons were carried out according to the methods of Szeto et al. (2000). Potassium and calcium currents were blocked as detailed in Szeto et al. (2000). (A) Superimposed TTX-sensitive sodium current traces recorded following a depolarization to –10 mV from a holding potential of –80 mV before, and 10 min after, the addition of 30 nM δ-ACTX-Hs20.1a. Note the slowed sodium current inactivation kinetics; *inset* shows the magnified peak TTX-sensitive sodium currents. (B-C) Typical effects of δ-ACTX-Hs20.1a on the current voltage (*I*/*V*) relationship. Families of sodium currents were evoked by a series of 50-ms depolarizations from –80 to +70 mV in 5-mV steps applied every 10 sec from a holding potential of –80 mV. Families of sodium currents before (B) and after (C) application of

30 nM δ-ACTX-Hs20.1a. For clarity only sodium currents recorded in 15-mV steps are presented. Note that the inactivation kinetics of both inward and outward currents are slowed and incomplete in (C) only. (D) Peak (circles) and late (squares) *I*/*V* relationships are shown before (filled symbols), and following (empty symbols), a 10-min perfusion with 30 nM δ-ACTX-Hs20.1a. Late currents were measured at the end of each 50 ms depolarizing test pulse.

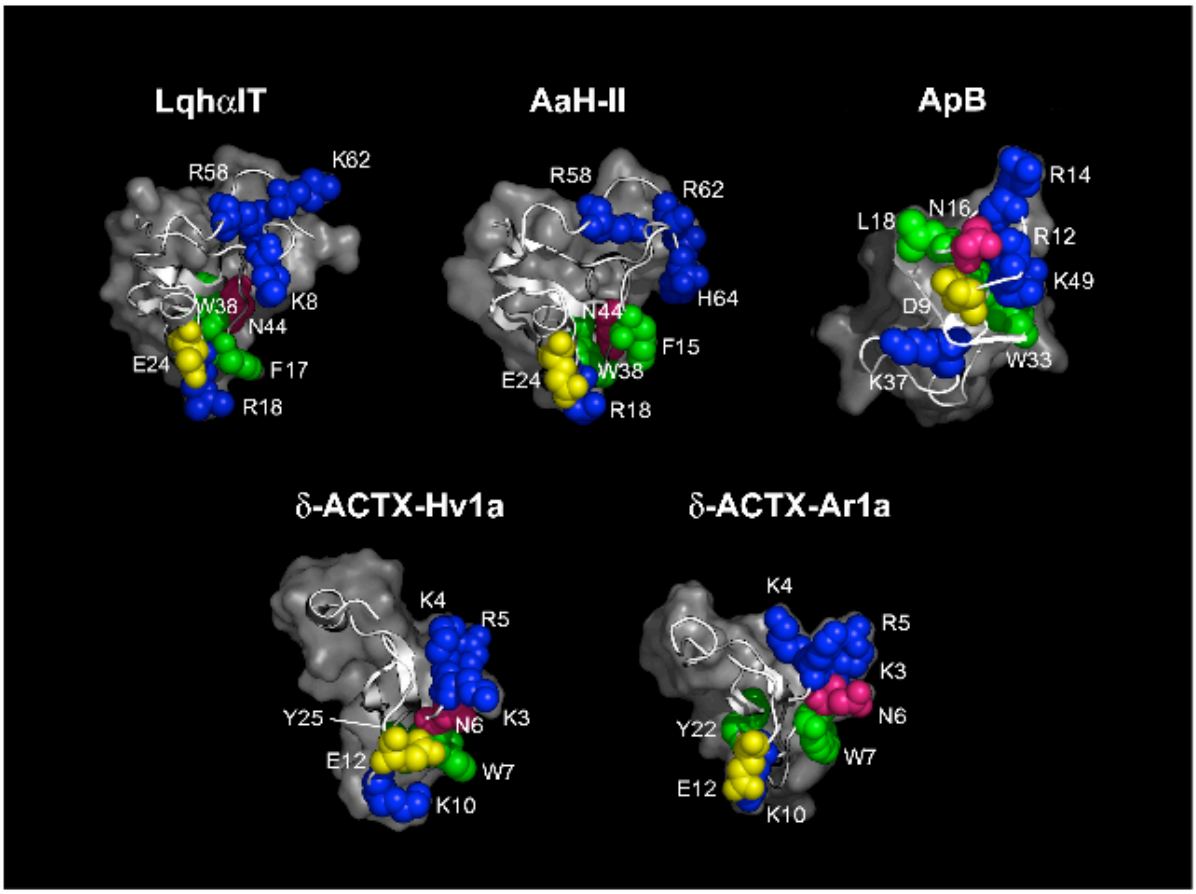

Fig. 5. Structural comparison of site-3 neurotoxins. The structures for AaH-II (Fontecilla-Camps et al., 1988; PDB accession code 1PTX), LqhαIT (Tugarinov et al., 1997; 1LQH), anthopleurin-B (ApB) (Monks et al., 1995; 1APF), δ-ACTX-Hv1a (Fletcher et al., 1997a; 1VTX) and δ-ACTX-Ar1a (Pallaghy et al., 1997; 1QDP) are presented with a similar orientation of their putative bioactive surfaces. Residues determined to form the pharmacophore of LqhαIT (Zilberberg et al., 1997; Gurevitz et al., 2001) and ApB (Dias-Kadambi et al., 1996; Khera and Blumenthal, 1996) together with topologically related residues in AaH-II, δ-ACTX-Hv1a and δ-ACTX-Ar1a are highlighted and colour coded: blue, positively charged; yellow, negatively charged; green, aromatic/hydrophobic; magenta, Asn. Highlighted residues and intervening peptide backbone are superimposed on the toxin molecular surface. Modified from Gilles et al. (2002). Toxin models were prepared using PyMOL.