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δ-Atracotoxins from Australian funnel-web spiders compete with scorpion α-toxin binding on both rat brain and insect sodium channels

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Abstract δ-Atracotoxins are novel peptide toxins from the venom of Australian funnel-web spiders that slow sodium current inactivation in a similar manner to scorpion α-toxins. To analyse their interaction with known sodium channel neurotoxin receptor sites we determined their effect on scorpion toxin, batrachotoxin and saxitoxin binding. Nanomolar concentrations of δ-atracotoxin-Hv1 and δ-atracotoxin-Ar1 completely inhibited the binding of the scorpion α-toxin AaH II to rat brain synaptosomes as well as the binding of LqhαIT, a scorpion α-toxin highly active on insects, to cockroach neuronal membranes. Moreover, δ-atracotoxin-Hv1 cooperatively enhanced batrachotoxin binding to rat brain synaptosomes in an analogous fashion to scorpion α-toxins. Thus the δ-atracotoxins represent a new class of toxins which bind to both mammalian and insect sodium channels at sites similar to, or partially overlapping with, the receptor binding sites of scorpion α-toxins.

Key words: Funnel-web spider toxin; δ-Atracotoxin; Sodium channel; Scorpion toxin; Rat brain synaptosome; Cockroach neuronal membrane

1. Introduction

Australian funnel-web spiders (Araneae: Hexathelidae: Atracinae) belong to the infraorder Mygalomorphae and are found mainly along the southeastern seaboard of Australia. More than 30 species of funnel-web spiders have so far been identified but it would appear that the male Sydney funnel-web spider *Atrax robustus* Cambridge has been solely responsible for the 14 human fatalities recorded since 1927 [1]. Interestingly, however, non-primates do not appear to exhibit symptoms following envenomation due to the presence of inactivating IgG antibodies in their plasma [2]. A variety of peptide neurotoxins have been isolated from the venom of this, and other, funnel-web spiders including calcium channel blockers which target insects [3] and more recently potassium channel blockers [4]. However, the toxin responsible for the major primate-specific symptoms of envenomation, δ-atracotoxin-Ar1 [5], has been recently shown to target the voltage-gated sodium channel. Both δ-atracotoxin-Ar1 and its homologue δ-atracotoxin-Hv1 [6], from *Hadronyche versuta* (Rainbow), act in rat dorsal root ganglion neurons by slowing tetrodotoxin-sensitive sodium current inactivation [7,8], an action similar to polypeptide scorpion α-toxins and sea anemone toxins [9,10].

δ-Atracotoxin-Ar1 and δ-atracotoxin-Hv1 are peptide neurotoxins consisting of a single chain of 42 amino acid residues; they are highly homologous with only two non-conserved substitutions (Fig. 1E). Both toxins have a high proportion of basic residues and appear to be tightly folded molecules cross-linked by four conserved intramolecular disulfide bonds, including unblocked N- and C-terminal cysteines. These toxins show no significant sequence homology with any presently known neurotoxins, but have a similar cysteine motif to other spider neurotoxins. Recently, the solution structures for δ-atracotoxin-Ar1 [11] and δ-atracotoxin-Hv1 [12] have been determined using NMR spectroscopy. Both display a small triple-stranded antiparallel β-sheet and a disulfide bonding pattern which places them in a class of peptides with an 'inhibitor cystine knot' motif including a number of other animal and plant toxins and protease inhibitors [13]. Interestingly the three-dimensional fold of these toxins is completely different to the previously determined structures for the scorpion α-toxins AaH II [14] and LqhαIT [15] and the sea anemone toxin anthopleurin-B [16], despite similar apparent actions on sodium current inactivation [9,10,17].

A variety of neurotoxins target different receptor sites on the voltage-gated sodium channel, leading to the characterisation of at least seven orphan receptors, referred to as neurotoxin receptor sites 1–7 ([18,19]; for a review see [20,21]). The polypeptide scorpion α-toxins and sea anemone toxins bind to neurotoxin receptor site 3 on the extracellular surface of the sodium channel (reviewed in [21]). This receptor site has been shown to have complex allosteric interactions with neurotoxin receptor site 2 which binds several alkaloid toxins including batrachotoxin (reviewed in [21]). Alkaloid toxins

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Abbreviations: δ-Atracotoxin-Ar1 (formerly robustoxin) from *Atrax robustus*; δ-Atracotoxin-Hv1 (formerly versutoxin) from *Hadronyche versuta*; AaH II, α-toxin from the venom of the scorpion *Androctonus australis hector*; LqhαIT, α-toxin highly active on insects from the venom of the scorpion *Leiurus quinquestriatus hebraeus*; TFA, trifluoroacetic acid; RP-HPLC, reverse phase high performance liquid chromatography; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; HEPES, *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid; Tris, 2-amino-2-(hydroxymethyl) propane-1,3-diol; [^{3}H]STX, [^{3}H]saxitoxin; [^{3}H]BTX, [^{3}H]batrachotoxinin A-20α-benzoate; BSA, bovine serum albumin; AaIT, excitatory insect-selective toxin from the venom of the scorpion *Androctonus australis hector*

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induce persistent activation of sodium channels by shifting the voltage-dependence of activation to very negative membrane potentials and inhibiting channel inactivation [22,23]. Scorpion α-toxins were shown to cooperatively enhance the effect of alkaloid toxins by increasing their binding affinity and activity [24,25].

Given the similarity between the actions of δ-atracotoxins and scorpion α-toxins the aim of the present study was to determine the likely receptor site of δ-atracotoxins and their interactions with other known neurotoxin receptor binding sites on the voltage-gated sodium channel. The study also aimed to determine whether δ-atracotoxins, like scorpion α-toxins, display a differential phyla-specific interaction with insects or mammalian sodium channels [26]. Here we describe that δ-atracotoxins interact with nanomolar affinities with the scorpion α-toxin receptor binding site in both whole rat brain synaptosomes and cockroach neuronal membranes, and display a classical positive allosteric interaction with neurotoxin receptor site 2. As such they represent a new class of toxins interacting with neurotoxin receptor site 3.

2. Materials and methods

2.1. Purification of toxins

Colonies of male *Atrax robustus* and female *Hadronyche versuta* spiders were 'milked' by direct aspiration from the chelicerae of live spiders using glass pipettes. Collected crude venom was flushed from pipettes using 0.1% (v/v) TFA and initial fractionation performed via RP-HPLC using a Vydac semi-preparative column (C_{18}, 10.4×250 mm, 15 μm) on a Waters HPLC system. Elution of venom components was performed using a linear gradient of 0–60% acetonitrile/0.1% TFA over 36 min with a flow rate of 4 ml/min. Fractions containing δ-atracotoxins were lyophilised then resuspended in 25% acetonitrile/0.1% TFA in preparation for further purification on a Vydac analytical column (C_{18}, 4.6×250 mm, 5 μm). δ-Atracotoxins were eluted from the analytical column using a linear gradient of 25–50% acetonitrile/0.1% TFA over 30 min at a flow rate of 1 ml/min. Purity of eluted toxins was confirmed by both SDS-PAGE under reducing and alkylating conditions with a Tris/tricine buffer system and by sequencing on an Applied Biosystems Model 470A Gas Phase Sequencer using automated Edman degradation. Protein content was determined using amino acid composition analysis. Toxins were stored lyophilised at −20°C until required, at which time they were dissolved in 20 mM HEPES-Tris (pH 6.0, 4°C). AaH II was purified according to the methods of Miranda et al. [27]. LqhαIT was a generous gift from Professor Eliahu Zlotkin (The Hebrew University of Jerusalem, Jerusalem, Israel) and was purified as described by Eitan et al. [17].

2.2. Toxicity assays

Mammalian toxicity of δ-atracotoxin-Hv1 was determined by intracerebroventricular (i.c.v.) and subcutaneous (s.c.) injection of increasing amounts of toxin in 0.1–0.2 ml saline into adult male mice (strain C57BL/6, 20±2 g). Lethality tests were conducted with a minimum number of animals and the development of toxicity monitored over 24 h. Insect toxicity was determined by microinjection of δ-atracotoxin-Hv1 using a microapplicator (Burkard, Herts, UK) fitted with a 29-gauge hypodermic needle. Paralysis and lethality were determined by lateroventral thoracic injection into 4th instar crickets (*Gryllus* sp., 70±15 mg body weight) or ventroposterior intersegmental injection into 5- or 6-day-old sheep blowfly larvae (*Lucilia cuprina*, 65±10 mg body weight). δ-Atracotoxin-Hv1 was dissolved in insect saline of the following composition (in mM): NaCl 200, KCl 3.1, $CaCl_2$ 5.4, $MgCl_2$ 5, $NaHCO_3$ 2, NaH_2PO_4 0.1, pH 7.2. Insects, 10 or 12 per group, were injected with 8 concentrations of δ-atracotoxin-Hv1. A positive result was scored when a characteristic paralysis (contraction and immobilisation) was observed. The median paralytic dose (PD_{50}) was defined as the dose that paralyses 50% of insects within 30 min whilst the LD_{50} is the dose that kills 50% of crickets within 72 h of injection.

2.3. Neuronal membrane preparation

Synaptosomes for ^{125}I-AaH II, [^{3}H]STX and [^{3}H]BTX binding assays were prepared from brains of male Wistar rats (4–8 weeks, 250–350 g) using a combination of homogenisation and differential and density gradient centrifugation according to the method described by Tamkun and Catterall [28]. Synaptosomes were suspended in a solution consisting of (in mM): choline chloride 130, KCl 5.4, $MgSO_4$ 0.8, D-glucose 5.5, and HEPES-Tris 50 (pH 7.4, 37°C). The protein concentration of the final synaptosome suspension was determined according to the method of Peterson [29] using BSA as a standard. Insect synaptosomes (P_2L preparation) were prepared from the central nervous system of adult American cockroaches (*Periplaneta americana*) according to the methods described by Gordon et al. [30] and Moskowitz et al. [31]. A combination of protease inhibitors consisting of phenylmethylsulfonyl fluoride (50 μg/ml), pepstatin A (1 μM), iodoacetamide (1 mM), and 1,10-phenanthroline (1 mM) was present in all buffers used for this procedure. Cockroach neuronal membrane protein concentration was determined using a Bio-Rad protein assay with BSA as a standard.

2.4. Binding assays

Equilibrium competition assays were performed using increasing concentrations of the unlabelled toxin in the presence of a constant low concentration of the radiolabelled toxin. Equilibrium competition experiments with ^{125}I-scorpion toxins were analysed by the iterative computer program LIGAND, using 'Drug' analysis (Elsevier Biosoft, UK). Standard binding medium composition was (in mM): choline chloride 130, $CaCl_2$ 1.8, KCl 5.4, $MgSO_4$ 0.8, D-glucose 5.5, HEPES 50, pH 7.4, BSA 1 mg/ml. Standard wash buffer composition was (in mM): choline chloride 163, $CaCl_2$ 1.8, KCl 5.4, $MgSO_4$ 0.8, HEPES 5, pH 7.4, BSA 1 mg/ml. The binding medium composition for ^{125}I-LqhαIT binding was (in mM): choline chloride 140, $CaCl_2$ 1.8, KCl 5.4, $MgSO_4$ 0.8, D-glucose 10, HEPES 25, pH 7.4, BSA 2 mg/ml. The wash buffer composition for ^{125}I-LqhαIT binding was (in mM): choline chloride 140, $CaCl_2$ 1.8, KCl 5.4, $MgSO_4$ 0.8, HEPES 25, pH 7.4, BSA 5 mg/ml. After incubation the reaction was terminated by dilution with 4 ml of ice-cold wash solution for [^{3}H]STX, [^{3}H]BTX and ^{125}I-AaH II binding experiments or 2 ml of ice-cold wash solution for ^{125}I-LqhαIT binding experiments. Separation of free from bound toxin was achieved by rapid filtration under vacuum using Whatman GF/C filters, except for [^{3}H]STX binding which used Whatman GF/B filters (Crown Scientific, Australia). The filter discs were washed with a further 2×4 ml of wash solution for [^{3}H]STX, [^{3}H]BTX and ^{125}I-AaH II binding or 2×2 ml washes for ^{125}I-LqhαIT binding.

AaH II was radioiodinated by lactoperoxidase as described previously [32] using 1 nmol of toxin and 1 mCi of carrier-free Na^{125}I (Australian Radioisotopes, ANSTO, Lucas Heights, N.S.W., Australia). ^{125}I-AaH II was purified by suspension with AG 1×8 (50–100 mesh) anion exchange resin (Bio-Rad, Hercules, CA, USA) according to the methods of Teitelbaum et al. [33]. Competition binding experiments using ^{125}I-AaH II were performed according to a variation of the method described by Cestèle et al. [34]. Briefly, rat brain synaptosomes (150 μg protein/ml) were suspended in 200 μl of binding buffer containing 0.5 nM ^{125}I-AaH II. After incubation for 30 min at 37°C the reaction was terminated by rapid filtration. Non-specific toxin binding was determined in the presence of 300 nM AaH II and was typically 25–30% of total binding.

LqhαIT was iodinated using IODO-GEN (Pierce) using 0.7 nmol of toxin and 0.5 mCi of carrier-free Na^{125}I as described by Gordon and Zlotkin [35]. The monoiodotoxin was purified via RP-HPLC using a Merck analytical C_8 column and a linear gradient of 5–90% acetonitrile/0.1% TFA at a flow rate of 1 ml/min, as previously described [34]. The concentration of the radiolabeled toxins was determined according to the specific activity of the ^{125}I corresponding to 4200–3450 dpm/fmol monoiodotoxin, depending on the time of use (the age of the iodine). Competition binding experiments using ^{125}I-LqhαIT were performed according to the methods of Gordon et al. [36]. Briefly, insect synaptosomes (P_2L, 3.3 μg protein/ml) were suspended in 0.3 ml of binding buffer containing 30 pM ^{125}I-LqhαIT. After incubation for 60 min at 22°C the reaction was terminated by filtration. Non-specific toxin binding was determined in the presence of 1 μM LqhαIT and was typically 1% of total binding.

[^{3}H]BTX binding experiments were performed according to a modification of the method described by Catterall and colleagues [37]. Rat brain synaptosomes (350 μg protein/ml) were suspended in 0.2 ml

buffer, containing 15 nM [^{3}H]BTX (0.8 μCi; DuPont, Boston, MA, USA). After incubation for 50 min at 37°C, the reaction was terminated by rapid filtration. Non-specific binding was determined in the presence of 300 μM veratridine (Sigma Aldrich, St. Louis, MO, USA) and was typically 5–10% of total binding.

[^{3}H]STX binding assays were performed using the method previously described by Catterall and colleagues [38]. Rat brain synaptosomes (300 μg) and 2 nM [^{3}H]STX (0.2 μCi; Amersham, Little Chalfont, Buckinghamshire, UK) were incubated for 30 min at 37°C followed by filtration. Non-specific binding was determined in the presence of 1 μM tetrodotoxin (Calbiochem, Alexandria, N.S.W., Australia) and was typically 5–15% of total binding.

2.5. Data analysis

The PD_{50} and LD_{50} values for toxicity assays and the IC_{50} values for the inhibition of ^{125}I-scorpion toxin binding were determined by non-linear regression analysis using the Hill equation. The EC_{50} values for the enhancement of [^{3}H]BTX binding were determined by non-linear regression using the Logistic equation. All curve fitting procedures used a non-linear least squares method of regression and splining routines. Competition binding studies with ^{125}I-AaH II and ^{125}I-LqhαIT were analysed by the iterative computer program LIGAND (Biosoft, UK). The IC_{50} values obtained for inhibition of scorpion toxin binding were subsequently converted to K_i values according to the relationship described by Cheng and Prusoff [39]. All data are presented as mean ± standard error of the mean (S.E.). All experiments were performed in duplicate or triplicate.

3. Results

3.1. HPLC purification of δ-atracotoxins

δ-Atracotoxins were purified from funnel-web spider venom by RP-HPLC (Fig. 1). Samples were initially purified by semi-preparative RP-HPLC column and fractions containing δ-atracotoxin-Ar1 (39% acetonitrile/0.1% TFA) and δ-atracotoxin-Hv1 (37% acetonitrile/0.1% TFA) were pooled and lyophilised. Crude δ-atracotoxins were then further purified by analytical RP-HPLC with δ-atracotoxin-Ar1 and δ-atracotoxin-Hv1 eluting at 46% and 44% acetonitrile/0.1% TFA, respectively.

3.2. Inhibition of ^{125}I-scorpion toxin binding by δ-atracotoxins

Binding of scorpion toxins to neurotoxin receptor site 3 on the sodium channel can be measured directly using ^{125}I-labelled scorpion toxins [40,41]. A range of concentrations of δ-atracotoxins were incubated with 0.5 nM ^{125}I-AaH II and rat brain synaptosomes (Fig. 2). Both δ-atracotoxins were found to compete with ^{125}I-AaH II binding in a concentration-dependent manner and completely inhibit its binding at higher concentrations. The competition curves for ^{125}I-AaH II binding in rat brain synaptosomes reveal that δ-atracotoxins displace ^{125}I-AaH II with IC_{50} values less than one order of magnitude higher than unlabelled AaH II (Fig. 2A). The calculated K_i values were 5.4 ± 1.4 nM and 4.3 ± 1.5 nM for δ-atracotoxin-Hv1 and δ-atracotoxin-Ar1, respectively, as compared with 0.8 ± 0.4 nM for unlabelled AaH II.

To assess if funnel-web spider toxins compete for the scorpion α-toxin site on insect sodium channels a range of concentrations of δ-atracotoxins were incubated with 30 pM ^{125}I-LqhαIT and cockroach neuronal membranes. Surprisingly,

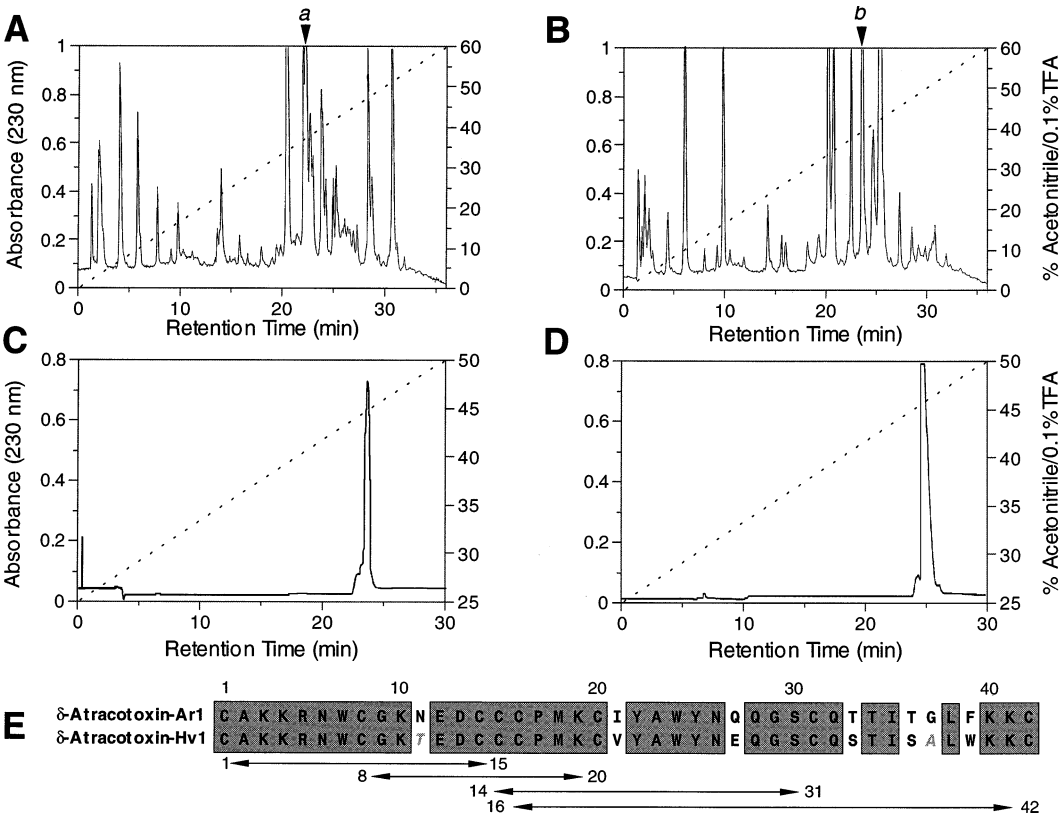

Fig. 1. Isolation of δ-atracotoxins from funnel-web spider venom. A and B: Semi-preparative RP-HPLC traces of whole *H. versuta* (A) and *A. robustus* venom (B). The arrows mark the lethal fractions containing δ-atracotoxin-Hv1 (a) and δ-atracotoxin-Ar1 (b). C and D: Analytical RP-HPLC chromatograms of the lethal fraction from A and B. E: Comparison of the primary structures of δ-atracotoxin-Ar1 and δ-atracotoxin-Hv1. Sequences were obtained from Brown et al. [5] and Sheumack et al. [6], respectively. Identities are boxed and residues in grey at positions 11 and 37 indicate non-conserved substitutions. The disulfide bonding pattern for both toxins, shown below the sequences, was taken from Pallaghy et al. [11] and Fletcher et al. [12]. The first three disulfide bonds form the inhibitory cystine knot motif (see Section 1).

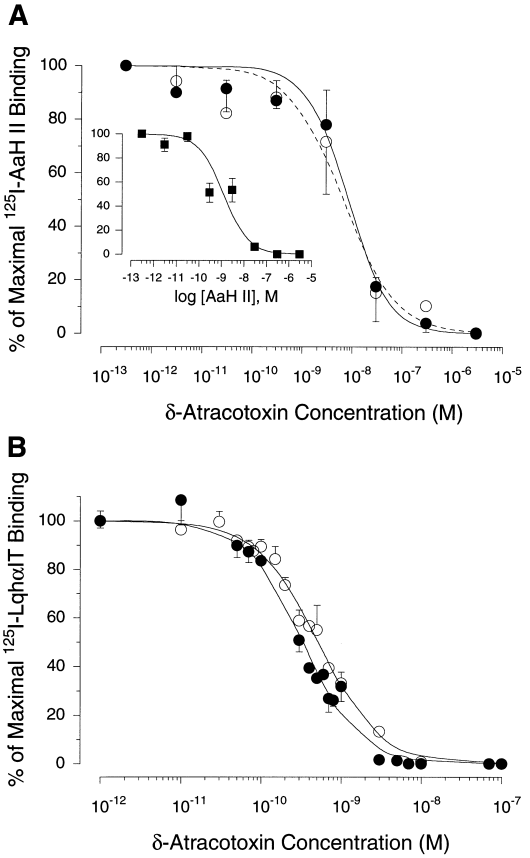

Fig. 2. Inhibition of scorpion toxin binding by δ-atracotoxins. A: Inhibition of ^{125}I-AaH II binding δ-atracotoxins in rat brain synaptosomes. A range of concentrations of δ-atracotoxin-Hv1 (●), δ-atracotoxin-Ar1 (○) and AaH II (inset) were incubated with 150 μg rat synaptosomes and 0.5 nM ^{125}I-AaH II for 30 min at 37°C. Data have been presented as a percentage of maximal ^{125}I-AaH II following correction for non-specific binding determined in the presence of 300 nM AaH II. Values represent the mean ± S.E. of 3 experiments. The curves were fitted using the Hill equation with regression coefficients (R^2) higher than 0.92. The calculated IC_{50} values and Hill coefficients (n_H) for inhibition of ^{125}I-AaH II, respectively, were: δ-atracotoxin-Hv1, 8.2 ± 2.1 nM, 1.07 ± 0.23; δ-atracotoxin-Ar1, 6.5 ± 2.3 nM, 0.82 ± 0.21; AaH II, 1.2 ± 0.6 nM, 0.79 ± 0.26. B: Inhibition of ^{125}I-LqhαIT binding by δ-atracotoxins in cockroach neuronal membranes. A range of concentrations of δ-atracotoxin-Hv1 (●) and δ-atracotoxin-Ar1 (○) were incubated with 1 μg *P. americana* neuronal membranes and 0.03 nM ^{125}I-LqhαIT for 60 min at 22°C. Data have been presented as a percentage of maximal ^{125}I-LqhαIT binding following correction for non-specific binding determined in the presence of 1 μM LqhαIT. Values represent the mean ± S.E. of 3 experiments. The curves were fitted using the Hill equation with regression coefficients (R^2) higher than 0.987. The calculated IC_{50} values and Hill coefficients (n_H) for inhibition of ^{125}I-LqhαIT, respectively, were: δ-atracotoxin-Hv1, 333 ± 21 pM, 1.22 ± 0.10; δ-atracotoxin-Ar1, 514 ± 21 pM, 1.13 ± 0.05.

δ-atracotoxin-Hv1 and δ-atracotoxin-Ar1 completely displaced ^{125}I-LqhαIT binding at picomolar concentrations (Fig. 2B). The calculated K_i values for inhibition of ^{125}I-LqhαIT binding were 111 ± 7 pM for δ-atracotoxin-Hv1 and 171 ± 7 nM for δ-atracotoxin-Ar1; under the same conditions LqhαIT was reported to bind with a K_d of 30 pM [36].

3.3. Insect and mice bioassay

Non-primates rapidly develop resistance post partum to the actions of δ-atracotoxins, due to the production of IgG antibodies [2]. Previous studies to determine the mammalian toxicity of δ-atracotoxins were therefore performed by s.c. injection into newborn (1-day-old) mice with LD_{50} values of 0.22 mg/kg (4.4 μg/20 g) for δ-atracotoxin-Hv1 and 0.16 mg/kg (3.2 μg/20 g) for δ-atracotoxin-Ar1 [5,42]. However, this may not accurately reflect the toxicity of δ-atracotoxin to mammals since there may already be appreciable expression of this plasma inactivating factor in neonatal animals. Accordingly, intracerebroventricular and subcutaneous injections were performed in adult mice and the LD_{50} of δ-atracotoxin-Hv1 determined to be 1.2 ng/20 g as compared with 4 μg/20 g via subcutaneous injection. This extremely low mammalian LD_{50}, via the intracerebroventricular route, is comparable to that of AaH II (see Table 1). However, the LD_{50} via the subcutaneous route is markedly higher for δ-atracotoxin-Hv1 than for AaH II reflecting inactivation of the toxin by plasma IgG antibodies [2], which probably do not cross the blood-brain barrier.

As predicted from the inhibition of binding of the scorpion α-toxin highly active on insects, ^{125}I-LqhαIT, δ-atracotoxin-Hv1 also showed neurotoxicity in insects observed as a delayed contractile paralysis of blowfly larvae and crickets. The PD_{50} for δ-atracotoxin-Hv1, determined after 30 min, was 200 ± 36 pmol/g in crickets and 54 ± 14 pmol/g in blowfly larvae ($n = 3$). Curiously, however, the crickets tended to recover from this paralysis with the rate of onset and duration of the paralytic contraction being dose-dependent. At the PD_{50}, contractile paralysis commenced within 30 min but crickets mostly recovered over the next 24 h. At higher concentrations contractile paralysis was immediate eventually leading to death 2–4 days later. The LD_{50} of δ-atracotoxin-Hv1 in crickets after 72 h was 770 ± 87 pmol/g ($n = 3$). The nearly four-fold higher LD_{50} as compared to the PD_{50} reflects this recovery from the contractile paralysis. These symptoms are in contrast to excitatory insect toxins from scorpion venom, such as AaIT [43], which produce an immediate and transient contractile paralysis of blowfly larvae, but are similar to those reported for LqhαIT [17].

3.4. Enhancement of [^{3}H]BTX binding by δ-atracotoxin-Hv1

Since scorpion α-toxins act in a cooperative manner to allosterically enhance the binding of site 2 alkaloid toxins [35,46] we examined whether δ-atracotoxin-Hv1 could enhance [^{3}H]BTX binding to rat brain synaptosomes. δ-Atracotoxin-Hv1 was found to produce a 12-fold increase in [^{3}H]BTX binding, with an EC_{50} of 25.2 ± 4.5 nM ($n = 4$). In comparison, AaH II-enhanced [^{3}H]BTX binding under the same conditions gave a 5.6-fold enhancement with an EC_{50} of 14.5 ± 2.3 nM (Fig. 3). In order to test whether the enhancement of [^{3}H]BTX binding represents an increase in binding to neurotoxin receptor site 2, δ-atracotoxin-Hv1-enhanced [^{3}H]BTX binding was measured in the presence of increasing concentrations of unlabelled batrachotoxin and veratridine. Both alkaloid toxins were able to completely inhibit [^{3}H]BTX binding enhanced by 3 μM δ-atracotoxin-Hv1 in a concentration-dependent manner (data not shown).

3.5. Effect of δ-atracotoxin-Hv1 on [^{3}H]STX binding

Tetrodotoxin, saxitoxin and μ-conotoxins can inhibit ion movement through the pore of the voltage-gated sodium channel by binding to neurotoxin receptor site 1 at the mouth of the channel [47–49]. δ-Atracotoxins have been reported to

Table 1
Toxicity of δ-atracotoxin and scorpion toxins to mammals and insects

Toxin	Toxin class	Toxicity (LD_{50})			Toxicity ratio	
		To mice (ng/20 g) i.c.v.	To mice (μg/20 g) s.c.	To insect (pmol/g)	Mice LD_{50} s.c./LD_{50} i.c.v.	Relative toxicity ratio[a] (mammal/insect)
δ-Atracotoxin-Hv1	δ-Atracotoxin	1.2	4	770	3420	0.004
AaH I	α-Toxins	10[b]	0.35[b]	276[e]	35	0.181
AaH II		0.5[b]	0.24[b]	897[e]	480	0.003
AaH III		7[b]	0.5[b]	1490[e]	71	0.024
Lqq IV		27[b]	1.4[b]	1230[e]	52	0.110
Lqq V		2.5[b]	0.5[b]	2317[e]	200	0.005
LqhαIT	α-Toxins highly active on insects	1100[c]	1.2[b]	2.5[e]	1.1	2200
Lqq III		1100[d]	1.1[d]	8.3[d]	1.0	662.7
Bom III	α-Like toxins	23[b]	3.0[b]	52.6[e]	130	2.186
Bom IV		23[e]	5.5[b]	19.7[e]	239	5.838

[a]Relative toxicity ratio = (LD_{50} mice (i.c.v.)/LD_{50} AaH II (i.c.v.))/(LD_{50} insect/LD_{50} LqhαIT).
[b]Martin-Eauclaire et al. [50].
[c]Kopeyan and Gordon, unpublished.
[d]Kopeyan et al. [55].
[e]Gordon et al. [36].

reduce TTX-sensitive sodium currents in rat dorsal root ganglion neurons in a dose-dependent manner [7,8]. In order to determine whether δ-atracotoxin-Hv1 inhibits tetrodotoxin-sensitive sodium currents through an interaction with receptor site 1 of the channel, a range of concentrations of δ-atracotoxin-Hv1 were incubated with rat brain synaptosomes in the presence of 2 nM [^{3}H]STX. δ-Atracotoxin-Hv1 was unable to significantly decrease [^{3}H]STX binding, even at concentrations as high as 3 μM (Fig. 3, inset). This indicates that the reduction in peak sodium current in dorsal root ganglion neurons produced by δ-atracotoxins is not due to an interaction with receptor site 1.

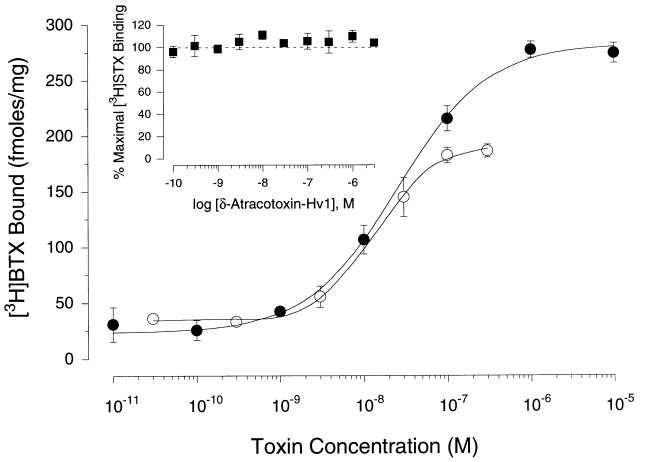

Fig. 3. Effect of δ-atracotoxin-Hv1 on [^{3}H]BTX and [^{3}H]STX binding. Main panel: Enhancement of [^{3}H]BTX by δ-atracotoxin-Hv1 and AaH II. A range of concentrations of AaH II (○) and δ-atracotoxin-Hv1 (●) were incubated with 350 μg protein and 15 nM [^{3}H]BTX at 37°C for 50 min. Data are presented as fmol of [^{3}H]BTX bound per mg of synaptosomal protein following correction for non-specific binding determined in the presence of 300 μM veratridine. Values represent the mean ± S.E. of 4 experiments. Inset: No alteration in [^{3}H]STX binding by δ-atracotoxin-Hv1. A range of concentrations of δ-atracotoxin-Hv1 were incubated with rat brain synaptosomes in the presence of 2 nM [^{3}H]STX at 37°C for 30 min. Data are presented as the percentage of maximal [^{3}H]STX binding following correction for non-specific binding determined in the presence of 1 μM TTX. The dashed line represents [^{3}H]STX binding in the absence of δ-atracotoxin-Hv1. Values represent the mean ± S.E. of 5 experiments.

4. Discussion

Similar to scorpion α-toxins, δ-atracotoxins are polypeptide neurotoxins that slow sodium current inactivation [7–9]. Given these comparable actions on sodium channel gating kinetics, the present study investigated the interaction of δ-atracotoxins with scorpion toxin binding. We have shown that δ-atracotoxins compete at nanomolar concentrations for the binding of the 'classical' scorpion α-toxin AaH II to neurotoxin receptor site 3 on rat brain sodium channels. δ-Atracotoxins also enhanced the binding of the site 2 alkaloid toxin [^{3}H]BTX in a positive allosteric manner (Fig. 3), an action analogous to scorpion α-toxins [20]. An unexpected finding of the present study, however, was that δ-atracotoxins also inhibited the binding of the scorpion α-insect toxin LqhαIT to cockroach neuronal membranes. This finding was supported by toxicity tests in insects which revealed that δ-atracotoxin-Hv1 produced a delayed contractile paralysis but the LD_{50} was relatively higher in comparison with other insect-selective scorpion toxins despite a potent inhibition of ^{125}I-LqhαIT binding. Thus δ-atracotoxins appear to represent a new class of toxins which interact with neurotoxin receptor site 3, but unlike scorpion α-toxins, are able to potently interact with insect sodium channels.

Despite all slowing sodium channel inactivation, scorpion α-toxins are a heterogenous class comprising several different groups determined by their phyla-specific activity on mammals and insects as well as their binding properties. The first group comprises the 'classical' α-toxins, such as AaH II and Lqq V; highly active on mammals but weakly toxic to insects. The second group are the α-toxins highly active on insects (here defined as α-insect toxins) such as LqhαIT and Lqq III; highly toxic to insects but weakly active on mice. The third group are the 'α-like' that are active on mammals but do not compete with classical α-toxins for binding to rat brain synaptosomes (see Fig. 4). Those that have been characterised,

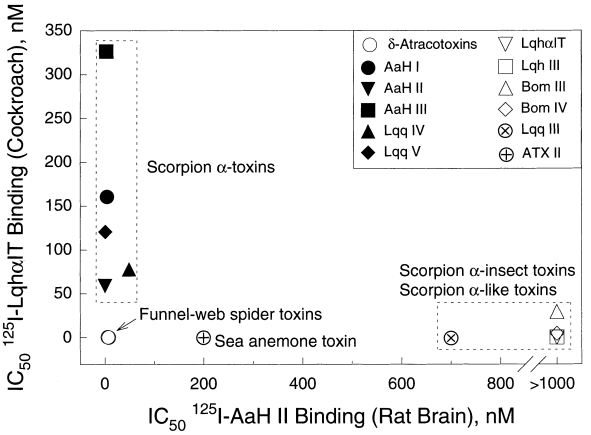

Fig. 4. Correlation between the potency to inhibit the binding of ^{125}I-AaH and ^{125}I-LqhαIT by δ-atracotoxins and scorpion toxins. Abscissa: IC_{50} values of each toxin to inhibit binding of ^{125}I-LqhαIT in cockroach neuronal membranes. Ordinate: IC_{50} values of each toxin to inhibit binding of ^{125}I-AaH II in rat brain synaptosomes. The term scorpion α-insect toxin is defined as an α-toxin highly active on insects. Data are taken from [36] and [51].

such as Bom III, Bom IV and Lqh III are also active on insects ([36,51]; for review see [52]). In the present study competition binding experiments revealed that δ-atracotoxins were able to completely displace both AaH II and LqhαIT binding with less than a 50-fold difference in K_i values. The ability to potently inhibit both scorpion α-toxin and α-insect toxin binding with such high affinities on both mammals and insects (see Fig. 4) clearly sets δ-atracotoxins apart from other scorpion and sea anemone toxins. Thus, δ-atracotoxins represent a novel group of neurotoxins which compete for binding with neurotoxin receptor site 3 on voltage-gated sodium channels.

Earlier studies using whole venom revealed strong insecticidal activity [44], which was generally thought to reside in fractions such as the ω-atracotoxin class of toxins [3]. These toxins produce a slow paralysis in cockroaches leading to death in 1–2 days (J. Fletcher, personal communication). Given the high content of δ-atracotoxins in the venoms on funnel-web spiders (up to 10% with δ-atracotoxin-Ar1) [45] this may also contribute significantly to the rapid paralysis of target insects.

Despite competitive interactions with neurotoxin receptor site 3 (Figs. 2 and 3), and positive allosteric interactions with neurotoxin receptor site 2 (Fig. 3), the actual binding interactions with the channel may differ, at least partially, between δ-atracotoxins and scorpion α-toxins. The main difference is that δ-atracotoxins reduce peak sodium currents in rat dorsal root ganglion neurons whilst scorpion α-toxins increase or fail to alter sodium currents [7–9]. This decrease in peak current was not the result of an interaction with neurotoxin receptor site 1 since δ-atracotoxins failed to alter [^{3}H]STX binding to rat brain synaptosomes (Fig. 3, inset). This may therefore be a result of a reduction in single channel conductance in addition to a slowing of inactivation or a block of the channel distant from receptor site 1. Determination of the mechanism of this reduction in sodium channel conductance will have to await single channel analysis of δ-atracotoxin-modified sodium currents.

δ-Atracotoxins and scorpion α-toxins therefore appear to bind to partially overlapping sites on the sodium channel localised within the area of receptor site 3. In support of this contention, the δ-atracotoxin receptor site, like site 3, is on the extracellular side of the channel since intracellular application of funnel-web spider toxins does not alter sodium channel gating or kinetics [7,8]. In addition, the 'α-like' toxins, that also inhibit sodium current inactivation, were suggested to bind to a partially overlapping receptor site with scorpion α-toxins and sea anemone toxins [36].

Recently the mutagenesis study of Rogers et al. [53] has identified a number of residues in the S3–S4 short extracellular loop of Domain IV of the sodium channel α-subunit critical for the binding of scorpion α-toxins and sea anemone toxins. These residues were found to include cationic and anionic residues suggested to be important for the binding of scorpion α-toxin and sea anemone toxins. Fletcher et al. [12] have investigated whether δ-atracotoxin-Hv1, and other site 3 neurotoxins, share topologically related charged residues that might interact with complementary charged residues in the S3–S4 loop. They found that three cationic and two anionic residues in δ-atracotoxin-Hv1 can be superimposed on the structures of anthopleurin-B and AaH II including those suggested to be important for binding, despite their lack of sequence homology and totally different three-dimensional folds. Since δ-atracotoxins compete for LqhαIT binding we also assessed if δ-atracotoxins share topologically related amino acids with those critical for the binding of LqhαIT [54]. Despite their completely different three-dimensional folds the structure of δ-atracotoxin-Hv1 (pdb accession code 1vtx) and LqhαIT (pdb accession code 1lqi) can be overlaid in such a way that residues Lys^4, Arg^5 and Lys^{19} in δ-atracotoxin-Hv1 superimpose very closely on residues Arg^{64}, Lys^{62} and Lys^8 in LqhαIT. Interestingly, these residues are different to those which superimpose on the structures of AaH II and anthopleurin-B [12].

In summary, the δ-atracotoxins bind to a similar cluster of receptor sites as scorpion α-toxins. Despite the slowing of sodium current inactivation and the competition for scorpion α-toxin binding differences amongst these receptor sites are indicated by the inhibition of peak sodium current induced by δ-atracotoxins in contrast to the usual increase obtained with scorpion α-toxins. Given that δ-atracotoxins target both mammalian and insect sodium channels there is considerable potential as a tool to aid in the investigation of structural requirements for anti-insect versus anti-mammal activity.

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