δ-Atracotoxins from the venom of Australian funnel-web spiders compete with scorpion α-toxin binding but differentially modulate alkaloid toxin activation of voltage-gated sodium channels*

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δ-Atracotoxins from the venom of Australian funnel-web spiders are a unique group of peptide toxins that slow sodium current inactivation in a manner similar to scorpion α-toxins. To analyze their interaction with known sodium channel neurotoxin receptor sites, we studied their effect on [3H]batrachotoxin and 125I-Lqh II (where Lqh is α-toxin II from the venom of the scorpion Leiurus quinquestratus hebraeus) binding and on alkaloid toxin-stimulated 22Na+ uptake in rat brain synaptosomes. δ-Atracotoxins significantly increased [3H]batrachotoxin binding yet decreased maximal batrachotoxin-activated 22Na+ uptake by 70–80%, the latter in marked contrast to the effect of scorpion α-toxins. Unlike the inhibition of batrachotoxin-activated 22Na+ uptake, δ-atracotoxins increased veratridine-stimulated 22Na+ uptake by converting veratridine from a partial to a full agonist, analogous to scorpion α-toxins. Hence, δ-atracotoxins may be able to differentiate between the open state of the sodium channel stabilized by batrachotoxin and veratridine and suggest a distinct sub-conductance state stabilized by δ-atracotoxins. Despite these actions, low concentrations of δ-atracotoxins completely inhibited the binding of the scorpion α-toxin, 125I-Lqh II, indicating that they bind to similar, or partially overlapping, receptor sites. The apparent uncoupling between the increase in binding but inhibition of the effect of batrachotoxin induced by δ-atracotoxins suggests that the binding and action of certain alkaloid toxins may represent at least two distinguishable steps. These results further contribute to the understanding of the complex dynamic interactions between neurotoxin receptor site areas related to sodium channel gating.

The venom from Australian funnel-web spiders (Araneae: Hexathelidae: Atracinae) appears to contain a variety of neurotoxins that target various ionic channels in excitable cells, including calcium (1) and potassium channels (2). The toxins so far identified to be responsible for severe envenomation or lethality in humans, however, were shown to be two peptide neurotoxins that target the voltage-gated sodium channel: δ-atracotoxin-Ar1 (formerly robustoxin) from Atrax robustus and δ-atracotoxin-Hv1 (formerly versutoxin) from Hadronyche versuta (3, 4). δ-Atracotoxin-Hv1 and δ-atracotoxin-Ar1 have been shown to exert their neurotoxicity by slowing or removing tetrodotoxin-sensitive sodium current inactivation in rat dorsal root ganglion neurons (5, 6), an action similar to that of polypeptide scorpion α-toxins and sea anemone toxins (7).

Both δ-atracotoxin-Ar1 and δ-atracotoxin-Hv1 consist of 42 amino acids and are highly homologous with only 7 substitutions. These toxins show no significant sequence homology with any presently known neurotoxins. Both spider toxins have a high proportion of basic residues and appear to be tightly folded molecules containing four conserved disulfide bonds, including N- and C-terminal cysteines. Recently, the three-dimensional solution structure for δ-atracotoxin-Hv1 (8) and δ-atracotoxin-Ar1 (9) has been determined. Both display a small three-stranded anti-parallel β-sheet with an “inhibitor cystine knot” motif (10). Interestingly the three-dimensional fold of these toxins is different from the previously determined structures of the scorpion α-toxins AaH II (11) and LqhαIT (12) despite similar actions on sodium current inactivation (13, 14).

Out of the seven identified neurotoxin receptor sites on the voltage-gated sodium channel, determined by direct radiolabeled toxin studies, at least two receptor sites were shown to bind various peptide toxins from different animal venoms that inhibit sodium current inactivation. Scorpion α-toxins and sea anemone toxins such as ATX II bind to the so-called neurotoxin receptor site 3 and δ-conotoxins which bind to receptor site 6 on the α-subunit of the sodium channel (Refs. 15 and 16; for a review see Refs. 7 and 17). Both of these receptor sites have been shown to have complex allosteric interactions with neurotoxin receptor site 2 shown to bind several alkaloid toxins

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†† The abbreviations used are: δ-atracotoxin-Ar1 (formerly robustoxin) from the venom of the spider Atrax robustus; δ-atracotoxin-Hv1 (formerly versutoxin) from the venom of the scorpion Hadronyche versuta; TTX, tetrodotoxin; AaH II, α-toxin II from the venom of the scorpion Androctonus australis hector; ATX II, sea anemone toxin II from Ane- monia sulcata; [3H]BTX, [3H]batrachotoxinin A-20a-benzoate; Lqh II, α-toxin II from the venom of the scorpion Leiurus quinquestratus hebraeus; TEA, tetraethylammonium; HPLC, high performance liquid chromatography; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; BSA, bovine serum albumin.

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such as batrachotoxin and veratridine (17). 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 (18, 19). Scorpion α-toxins were shown to cooperatively enhance the effect of alkaloid toxins by increasing their binding affinity and activity (20, 21).

Based on apparent similarity in the effect of scorpion α-toxins and δ-atracotoxins, the aim of the present study was to determine the likely receptor binding site of funnel-web spider toxins on the sodium channel and their possible interaction with alkaloid toxin binding and action using both 22Na uptake and radiolabeled neurotoxin binding assays. We report that δ-atracotoxins interact with nanomolar affinities with neurotoxin receptor site 3 in rat brain synaptosomes but exhibit unusual allosteric interactions with the site 2 alkaloid toxin receptor.

**EXPERIMENTAL PROCEDURES**

**Purification of Toxins—**Colonies of male *A. robustus* and female *H. versuta* spiders were “milked” by direct aspiration from the chelicerae of live females using glass pipettes. Climbing spider toxins were eluted from the brain from pipettes using 0.1% (v/v) trifluoroacetic acid and initial fractionation performed via reverse-phase high performance liquid chromatography (HPLC) using a Vydac semi-preparative column (C18, 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% trifluoroacetic acid over 36 min with a flow rate of 4 ml/min. The fraction containing δ-atracotoxin-Ar1 eluted with approximately 43% acetonitrile, whereas δ-atracotoxin-Hv1 eluted with 40% acetonitrile. These fractions were lyophilized and then resuspended in 25% acetonitrile, 0.1% trifluoroacetic acid in preparation for further purification on a Vydac analytical column (C18, 4.6 × 250 mm, 5 μm). The funnel-web spider toxins were eluted from the analytical column using a linear gradient of 25–50% acetonitrile, 0.1% trifluoroacetic acid over 30 min at a flow rate of 1 ml/min. Purity of eluted toxins was confirmed by SDS-polyacrylamide gel electrophoresis under reducing and alkylating conditions with a Tris/Tricine buffer system. Protein concentration was determined using amino acid composition analysis. Toxins were stored lyophilized at −20 °C until required, at which time they were dissolved in 20 mM HEPES-Tris (pH 6.0, 4 °C).

**Other Chemicals—**δ-atracotoxin-Ar1 and Lqh II were purified according to the methods of Miranda et al. (22) and Sautter et al. (23) respectively. Batrachotoxin was a generous gift from Dr. John Daly (Laboratory of Bioorganic Chemistry, NIDDK, National Institutes of Health, Bethesda). Lqh II (catalogue number LTX001) was purchased from Latoxan Bioorganic Chemistry, NIDDK, National Institutes of Health, Bethesda. Batrachotoxin was a generous gift from Dr. John Daly (Laboratory of Bioorganic Chemistry, NIDDK, National Institutes of Health, Bethesda).

**Electrophysiological Recordings—**Whole cell patch clamp recordings of tetrodotoxin-sensitive sodium currents were made from both dorsal root ganglion and periaqueductal gray neurons. Acutely dissociated dorsal ganglion neurons were prepared from 4- to 12-day-old Wistar rats according to the methods of Connor and Christie (5, 6). Micropipettes (0.67–2 meghoms) were pulled from borosilicate glass capillary tubing and were filled with (in mM) CsCl 110, NaCl 20, EGTA 10, CaCl2 2, MgATP 2, HEPES 10 with the pH adjusted to 7.3 with 1 x CsOH. The external solution contained (in mM) NaCl 80, TEA chloride 60, MgCl2 5, CsCl 5, n-glucose 10; HEPES 10 with the pH adjusted to 7.4 with 1 x TEA hydroxide. Membrane current recordings were made at room temperature (21–25 °C).

The experiments used in this study were rejected if there were large leak currents or currents showed signs of poor space clamping such as an abrupt activation of currents upon relatively small depolarizing pulses. Stimulation and recording were both controlled by an AxoData or pClamp data acquisition system (Axon Instruments, Foster City, Calif.). Membrane currents were filtered at 10 kHz (low pass Bessel filter), and digital sampling rate was 25–50 kHz. Leakage and capacitative currents were digitally subtracted with P-P4 procedures, and series resistance compensation was >80% for all cells.

**Neuronal Membrane Preparations—**Synaptosomes for 22Na uptake and 125I/HTX binding assays were prepared from brains of male Wistar rats (4–8 weeks, 250–350 g) using a combination of homogenization and differential and density gradient centrifugation according to the method of Gray and Whittaker (26) as described by Tamkun and Catterall (27). Synaptosomes were suspended in a solution consisting of (in mM) choline chloride 130, KCl 5.4, MgSO4 0.8, n-glucose 5.5, and HEPES-Tris 50 (pH 7.4, 37 °C) and were stored in liquid nitrogen until required for 22Na uptake assays, or used within 3 h of preparation for 125I/HTX binding assays. For 125I-Lqh II binding assays, synaptosomes were prepared according to the method of Kanner (28) (using in mM) d-mannitol 300, EDTA 10, HEPES 10 (pH 7.4, 4 °C), and Ficoll (type 400 Sigma) gradients (14). A combination of proteinase inhibitors consisting of phenylmethylsulfonyl fluoride (50 μg/ml), pepstatin A (1 μM), iodoacetamide (1 μM), and 1,10-phenanthroline (1 mM) was present in all buffers used for this procedure. Synaptosomes were frozen at −80 °C in mannitol buffer until use. Membrane protein concentration was determined using a Bio-Rad protein assay with BSA as a standard.

**Measurement of 22Na Uptake—**The effect of δ-atracotoxins on 22Na uptake in rat brain synaptosomes was measured using the method described by Tamkun and Catterall (27). Briefly, rat brain synaptosomes (500 μg of membrane protein) were preincubated for 10 min with toxins in 100 μl of sodium-free preincubation medium at 37 °C. The preincubation 22Na uptake medium contained (in mM) choline chloride 130, KCl 5.4, MgSO4 0.8, glucose 5.5, HEPES-Tris (pH 7.4) 50, and 1 mg/ml BSA. Uptake was initiated by adding 150 μl of assay medium containing (in mM) choline chloride 128, KCl 5.4, MgSO4 0.8, glucose 5.5, HEPES-Tris (pH 7.4) 50, NaCl 2.68, ouabain 5 mM BSA, and 0.9 mg/ml of Δ-atracotoxins. At 5 s at 37 °C, uptake was terminated by the addition of 4 ml of ice-cold wash solution and rapid filtration through 0.45-μm nitrocellulose membrane filters (Millipore, Sydney, Australia). The wash solution consisted of (mM) choline chloride 163, CaCl2 1.8, MgSO4 0.8, HEPES-Tris (pH 7.4) 5, and 1 mg/ml BSA. Maximal 22Na uptake was determined in the presence of 1 μM batrachotoxin, whereas uptake not mediated by the voltage-gated sodium channel was determined in the presence of 1 μM TTX. Nonspecific 22Na uptake was typically less than 35% of total uptake.

**Binding Assays—**Equilibrium competition and saturation assays were performed using increasing concentrations of the unlabeled toxin in the presence of a constant low concentration of the radiolabeled toxin. In order to obtain saturation curves (“cold” saturation), the specific radioactivity and the amount of bound toxin were calculated and determined for each toxin concentration. Equilibrium saturation or competition experiments were analyzed by the iterative computer program LIGAND, using “Cold saturation” and “Drug” analysis, respectively (Elsevier Biosoft, UK). The composition of the binding medium for 125I-Lqh II binding was (in mM) choline Cl 140, CaCl2 1.8, KCl 5.4, MgSO4 0.8, HEPES 25 (pH 7.4); n-glucose 10, BSA 2 mg/ml. Wash buffer composition was (in mM) choline Cl 140, CaCl2 1.8, KCl 5.4, MgSO4 0.8, HEPES 25 (pH 7.4). BSA 5 mg/ml. 125I/HTX binding experiments used the preincubation medium and wash solution as for 22Na uptake experiments (see above). After incubation, reactions were terminated by dilution with 4 ml of ice-cold wash solution for 125I/HTX binding experiments or 2 ml of ice-cold wash solution for 125I-Lqh II binding experiments. Separation of free from bound toxin was achieved using Whatman GF/C filters. The filter discs were washed with a further 2 × 4 ml of wash solution for 125I/HTX binding or 2 × 2 ml washes for 125I-Lqh II binding.

**125I/HTX Binding Experiments—**Experiments were performed according to a modification of the method described by Catterall and colleagues (29). Rat brain synaptosomes (350 μg of protein/ml) were suspended in 0.2 ml of buffer containing 15 mM [125I](HTX) (0.8 μCi). After
incubation for 50 min at 37 °C, the reaction mixture was diluted with 4 ml of ice-cold wash buffer and filtered. Nonspecific binding was determined in the presence of 300 µM veratridine and was typically 5–15% of total binding. Equilibrium saturation experiments were analyzed using EBDA and LIGAND computer programs (Elsevier Biosoft, UK).

\[ \text{IC}_{50} \text{ values obtained for inhibition of } 125\text{I}-\text{Lqh II binding to } K_v \text{ values, according to the relationship described by Cheng and Prusoff (30). Equilibrium saturation of } [3\text{H}]\text{BTX binding data was fitted according to the following single-site hyperbolic equation mathematically equivalent to the Langmuir isotherm.} \]

\[
[3\text{H}]\text{BTX binding (nM)} = \frac{B_{\text{max}} \times x}{K_d + x} \quad (\text{Eq. 1})
\]

where \( B_{\text{max}} \) is the maximal number of moles of [3H]BTX bound, \( K_v \) is the dissociation constant for [3H]BTX, and \( x \) is the total concentration of batrachotoxin activation.

Mathematical curve fitting was accomplished using SigmaPlot version 4.14 for Macintosh. All curve fitting routines used a nonlinear least squares method and splining routines. All data are presented as means ± S.E. All experiments were performed in duplicate or triplicate.

**RESULTS**

**Effect of δ-Atracotoxins on Sodium Currents**—Electrophysiological studies using whole cell patch clamp of dorsal root ganglion neurons have shown that δ-atracotoxins slow sodium current inactivation and shift the voltage dependence of activation in a manner similar to scorpion \( \alpha \)-toxins (5, 6). As shown in Fig. 1, A and B, 30 nM δ-atracotoxin-Hv1 slows TTX-sensitive sodium current inactivation in both dorsal root ganglion neurons and periadqueductal gray neurons from rat brain. In addition, 30 nM δ-atracotoxin-Hv1 shifts the threshold of sodium channel activation by approximately 10 mV to more negative membrane potentials (Fig. 1, C and D). These actions are similar to those reported for scorpion \( \alpha \)-toxins (13, 34). However, δ-atracotoxin-Hv1 caused differential actions on peak sodium current amplitude in these two populations of neurons. In dorsal root ganglion neurons δ-atracotoxin-Hv1 caused a marked reduction in peak sodium current (Fig. 1A), whereas in periadqueductal gray neurons there was no significant change in peak sodium current (Fig. 1B). This is in contrast to scorpion \( \alpha \)-toxins that typically increase peak sodium current (5, 6, 13, 35, 36). Given these similar actions on sodium currents, we further compared the actions of δ-atracotoxins with those of scorpion \( \alpha \)-toxins to produce positive cooperativity with [3H]BTX binding, enhancement of alkaloid toxin-stimulated \(^{22}\text{Na}^+\) uptake, and competition in radioiodinated scorpion toxin binding assays.

**Enhancement of [3H]BTX Binding by δ-Atracotoxin-Hv1**—Scorpion \( \alpha \)-toxins have been shown to act in a positive allosteric fashion to increase the binding of site 2 alkaloid toxins such as batrachotoxin (27, 29). We therefore assessed the ability of δ-atracotoxins to enhance [3H]BTX binding to rat brain synaptosomes. δ-Atracotoxin-Hv1 was found to significantly enhance the binding of [3H]BTX approximately 12-fold with a concentration required for half-maximal enhancement (EC_{50}) of 25.2 ± 4.5 nM (n = 4, Fig. 2A). In comparison, AaH II-enhanced [3H]BTX binding under the same conditions gave a 5.6-fold increase in the maximal response (E_{max}) with an EC_{50} of 14.2 ± 2.3 nM (Fig. 2A, inset). In order to show that the enhancement of [3H]BTX binding represents an increase in binding to neurotoxin receptor site 2, δ-atracotoxin-Hv1-enhanced [3H]BTX binding was measured in the presence of increasing concentrations of unlabeled batrachotoxins or veratridine. Both alkaloid toxins were able to completely inhibit [3H]BTX binding enhanced by 3 \( \mu \)M δ-atracotoxin-Hv1 in a concentration-dependent manner indicating the enhancement is specifically mediated through receptor site 2 (Fig. 2B).

Analysis of batrachotoxin binding indicated that in the presence of 100 nM AaH II, 20 nM δ-atracotoxin-Hv1, or 3 \( \mu \)M δ-atracotoxin-Hv1, [3H]BTX binds to a single class of high affinity binding sites (Table I) in agreement with earlier studies (29). In the absence of scorpion \( \alpha \)-toxins or sea anemone toxins, the estimated K_d for [3H]BTX was reported to be 700 nM (29). Under the present conditions, the K_d of [3H]BTX in the absence of enhancement was 862 ± 310 nM. The addition of either AaH II or δ-atracotoxin-Hv1 reduced the K_d approximately 3.2-fold while not significantly altering the total num-

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**FIG. 1. Actions of δ-atracotoxin-Hv1 on whole cell tetrodotoxin-sensitive sodium currents in rat dorsal root ganglion and periadqueductal gray neurons. Upper panels show the slowing of sodium current inactivation induced by δ-atracotoxin-Hv1 in dorsal root ganglion neurons (A) and periadqueductal gray neurons from rat brain (B); a represents the control trace, and b shows the current recorded in the presence of 30 nM δ-atracotoxin-Hv1. A, currents were activated by depolarizing test pulses from −80 mV to −10 mV for 50 ms every 10 s. B, currents were activated by depolarizing test pulses from −90 mV to 0 mV for 6 ms every 10 s. C and D show the I/V relationships recorded before (○) and after (○) 30 nM δ-atracotoxin-Hv1 from the same neurons as in A and B, respectively. Note the negative shift in the voltage dependence of activation in the presence of δ-atracotoxin-Hv1.**
FIG. 2. Enhancement of [3H]BTX binding by \( \delta \)-atracotoxin-Hv1 in rat brain synaptosomes. A, enhancement of [3H]BTX binding by increasing concentrations of \( \delta \)-atracotoxin-Hv1. Rat brain synaptosomes (350 \( \mu \)g of protein) were preincubated with 15 nM [3H]BTX for 50 min at 37 °C in the presence of increasing concentrations of \( \delta \)-atracotoxin-Hv1 (main panel) or AaH II (inset). The data points represent the mean ± S.E. of four experiments (see “Experimental Procedures” for details). B, inhibition of \( \delta \)-atracotoxin-Hv1 enhanced [3H]BTX binding by veratridine and batrachotoxin. Rat brain synaptosomes were incubated with 15 nM [3H]BTX and 3 \( \mu \)M \( \delta \)-atracotoxin-Hv1 in the presence of a range of concentrations of batrachotoxin (●) and veratridine (○) under the same conditions as in A. Data are presented as a percentage of maximal [3H]BTX binding in the presence of 3 \( \mu \)M \( \delta \)-atracotoxin-Hv1. Values represent the mean ± S.E. of three experiments for both veratridine and batrachotoxin. B, inset, lack of enhancement of \( \delta \)-atracotoxin-Hv1-enhanced [3H]BTX binding by AaH II. Increasing concentrations of AaH II were incubated with 15 nM [3H]BTX in the absence (gray columns) and presence (black columns) of a saturating concentration of \( \delta \)-atracotoxin-Hv1 (10 \( \mu \)M) under the same conditions as in A. Values represent the mean ± S.E. of three experiments.

Rat brain synaptosomes were incubated with 15 nM [3H]BTX and increasing concentrations of unlabeled batrachotoxin in the presence of the indicated concentrations of AaH II or \( \delta \)-atracotoxin-Hv1 as described under “Experimental Procedures.” Data have been presented as the means ± S.E. (\( n = 3 \)) as determined from nonlinear regression (see Equation 1 under “Experimental Procedures”). Statistical analysis using a one-way analysis of variance revealed no significant differences between any of the data.

### TABLE I

| Toxin | Concentration | BTX \( K_d \) | BTX \( B_{max} \) |
|-------|---------------|--------------|-----------------|
| AaH II | 10 \( \times 10^{-6} \) | 267 ± 53 | 4.4 ± 0.7 |
| \( \delta \)-Atracotoxin-Hv1 | 2 \( \times 10^{-6} \) | 241 ± 17 | 4.3 ± 0.5 |
| \( \delta \)-Atracotoxin-Hv1 | 3 \( \times 10^{-6} \) | 196 ± 37 | 6.1 ± 0.5 |

Effects of \( \delta \)-Atracotoxins on Voltage-gated Sodium Channels

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**Activation of \( 22 \text{Na}^+ \) Uptake by \( \delta \)-Atracotoxins**—To determine whether \( \delta \)-atracotoxins can directly activate voltage-gated sodium channels, their ability to increase \( 22 \text{Na}^+ \) uptake by rat brain synaptosomes was examined. Both \( \delta \)-atracotoxins were able to activate \( 22 \text{Na}^+ \) uptake in a concentration-dependent manner to 30 and 28% of maximum, with concentrations for half-maximal activation \((\text{EC}_{50})\) of 25 ± 8 and 44 ± 18 nM for \( \delta \)-atracotoxin-Hv1 and \( \delta \)-atracotoxin-Ar1, respectively (Fig. 3). Maximal \( 22 \text{Na}^+ \) uptake was determined in the presence of 1 \( \mu \)M BTX, and the direct stimulation of \( 22 \text{Na}^+ \) uptake by \( \delta \)-atracotoxins was expressed as a percentage of maximal flux. Moreover, the \( \delta \)-atracotoxin-activated \( 22 \text{Na}^+ \) uptake was TTX-sensitive (Fig. 3B, inset) confirming that it was mediated via the voltage-gated sodium channel. Similarly, the scorpion \( \alpha \)-toxin AaH II was also able to directly activate \( 22 \text{Na}^+ \) uptake to 14 ± 3% at a concentration of 30 nM (Fig. 3A, inset).

Scorpion \( \alpha \)-toxins that bind to neurotoxin receptor site 3 have been shown to enhance activation of \( 22 \text{Na}^+ \) uptake by site 2 alkaloid toxins via an increase in binding affinity and efficacy (22). This is believed to occur via a positive allosteric interaction between sites 2 and 3 (for review see Ref. 7). To determine if AaH II and \( \delta \)-atracotoxin-Ar1 have a cooperative action on \( 22 \text{Na}^+ \) uptake, increasing concentrations of \( \delta \)-atracotoxin-Ar1 were incubated with 30 nM AaH II. This concentration of AaH II was able to directly activate \( 22 \text{Na}^+ \) uptake (Fig. 3A, inset). No additivity or cooperativity was observed as \( 22 \text{Na}^+ \) uptake activated by the highest concentrations of both toxins (Fig. 3C) was identical to flux produced by \( \delta \)-atracotoxin-Ar1 alone (approximately 30% of maximal). Similar results were obtained with \( \delta \)-atracotoxin-Hv1 (data not shown). These results are in accordance with the effect of concurrent administration of AaH II and \( \delta \)-atracotoxins on [3H]BTX binding (Fig. 2B, inset).

Low Concentrations of \( \delta \)-Atracotoxins Cooperatively Enhance Batrachotoxin- and Veratridine-activated \( 22 \text{Na}^+ \) Uptake—Since \( \delta \)-atracotoxins enhance [3H]BTX binding, we assessed the effect of 6 nM \( \delta \)-atracotoxin on batrachotoxin-activated \( 22 \text{Na}^+ \) uptake (Fig. 4). This concentration of \( \delta \)-atracotoxin-Hv1 gives minimal direct activation of \( 22 \text{Na}^+ \) uptake (Fig. 3); nevertheless, it is able to significantly shift the EC\(_{50}\) of batrachotoxin to activate \( 22 \text{Na}^+ \) uptake from 155 ± 36 to 15 ± 10 nM (Fig. 4A). Unexpectedly, this low concentration of \( \delta \)-atracotoxin-Hv1 also produced a 30 ± 7% inhibition of the maximum \( 22 \text{Na}^+ \) uptake, activated by 1 \( \mu \)M BTX (Fig. 4A).

Since batrachotoxin and veratridine have been shown to compete for binding to neurotoxin receptor site 2 (see Ref. 29 and Fig. 2B), we compared the effects of \( \delta \)-atracotoxin-Hv1 on veratridine-stimulated \( 22 \text{Na}^+ \) uptake. Similar to the enhancement of batrachotoxin-activated \( 22 \text{Na}^+ \) uptake, \( \delta \)-atracotoxin-Hv1 (6 nM) lowered the EC\(_{50}\) of veratridine 10-fold from 4.0 ± 1.0 to 0.4 ± 0.1 \( \mu \)M (Fig. 4B). In contrast to the inhibition observed in the presence of batrachotoxin, the addition of \( \delta \)-atracotoxin-Hv1 increased the maximal effect of veratridine to that observed in the presence of 1 \( \mu \)M batrachotoxin, thus converting veratridine from a partial agonist to a full agonist. This effect is similar to that of scorpion \( \alpha \)-toxins on veratridine-stimulated \( 22 \text{Na}^+ \) uptake as described previously by Tamkun and Catterall (27).
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**Fig. 3. Activation of ^22^Na\(^+\) uptake by funnel-web spider toxins in rat brain synaptosomes.** Synaptosomes (500 μg) were incubated with a range of concentrations of δ-atracotoxin-Hv1 (A) and δ-atracotoxin-Ar1 (B and C) for 30 min at 37 °C, and ^22^Na\(^+\) uptake was measured after a 5-s period. Nonspecific ^22^Na\(^+\) uptake in the presence of 1 μM batrachotoxin was subtracted, and data were presented as a percentage of maximal uptake as determined in the presence of 1 μM batrachotoxin. A, inset, activation of ^22^Na\(^+\) uptake by AaH II. Synaptosomes were incubated with a range of concentrations of AaH II and ^22^Na\(^+\) uptake measured under the same conditions as above (n = 3). B, inset, inhibition of δ-atracotoxin-activated ^22^Na\(^+\) uptake by TTX. Black columns show ^22^Na\(^+\) uptake recorded in the presence of 10 μM δ-atracotoxin-Hv1, and the gray columns show data recorded in the presence of 1 μM δ-atracotoxin-Ar1. It was found that 1 μM TTX reduced ^22^Na\(^+\) uptake activated by δ-atracotoxin-Hv1 and δ-atracotoxin-Ar1 to 5 ± 2% (n = 3) and 1 ± 1% (n = 3), respectively. C, effect of AaH II on ^22^Na\(^+\) uptake activated by δ-atracotoxin-Ar1. Synaptosomes were incubated with a range of concentrations of δ-atracotoxin-Ar1 in the absence (●) and presence (○) of 30 nM AaH II. All values represent the mean ± S.E. (n = 3–13 for A, n = 8–6 for B, and n = 3 for C).

**Fig. 4. Enhancement of batrachotoxin- and veratridine-stimulated ^22^Na\(^+\) uptake by δ-atracotoxins.** A, increasing concentrations of batrachotoxin were incubated with rat brain synaptosomes in the absence (●) and presence of 6 nM (○) δ-atracotoxin-Hv1 (n = 3). The EC\(_{50}\) of batrachotoxin was reduced from 154 ± 21 to 9.0 ± 4.4 nM in the presence of δ-atracotoxin-Hv1. Note the 32% inhibition in batrachotoxin-inactivated ^22^Na\(^+\) uptake. B, enhancement of veratridine-activated ^22^Na\(^+\) uptake by δ-atracotoxin-Hv1. A range of concentrations of veratridine was incubated with rat brain synaptosomes in the absence (●) and presence of 6 nM δ-atracotoxin-Hv1 (○) (n = 3). The EC\(_{50}\) of veratridine was reduced from 4.0 ± 0.3 to 0.6 ± 0.4 μM in the presence of δ-atracotoxin-Hv1, and the maximal ^22^Na\(^+\) uptake was increased. For conditions of incubation see “Experimental Procedures.” All data are presented as a percentage of maximal uptake as determined in the presence of 1 μM batrachotoxin.

**Inhibition of Scorpion α-Toxin Binding by δ-Atracotoxins—** AaH II and Lqh II, which differ by only 2 amino acid residues, were shown to have similar LD\(_{50}\) values in mice and reveal differences were not observed with scorpion α-toxin modulation of alkaloid toxin action (27). Accordingly, we examined the ability of δ-atracotoxins to compete directly with the binding of scorpion α-toxins to neurotoxin receptor site 3.

δ-atracotoxin-activated ^22^Na\(^+\) uptake—To analyze further the interactions of δ-atracotoxins with alkaloid toxin-stimulated ^22^Na\(^+\) uptake, we examined the effect of the funnel-web spider toxins on maximal ^22^Na\(^+\) uptake stimulated either by batrachotoxin or veratridine. As previously noted in Fig. 4A, increasing concentrations of δ-atracotoxins strongly inhibited the maximal ^22^Na\(^+\) uptake stimulated by 1 μM batrachotoxin (Fig. 5A) with IC\(_{50}\) values of 10 ± 2 and 14 ± 2 nM for δ-atracotoxin-Hv1 and δ-atracotoxin-Ar1, respectively. Notable was the inhibition of batrachotoxin-activated ^22^Na\(^+\) uptake to a level comparable with that which the δ-atracotoxins activate ^22^Na\(^+\) uptake in the absence of batrachotoxin (see Fig. 3, A and B). As with flux activated directly by δ-atracotoxins, the remaining ^22^Na\(^+\) uptake in the presence of both 1 μM batrachotoxin and saturating concentrations of δ-atracotoxins could be inhibited by 1 μM TTX (data not shown).

In contrast to the inhibition of batrachotoxin-activated ^22^Na\(^+\) uptake, δ-atracotoxin-Hv1 was able to increase uptake activated by saturating (300 μM) concentrations of veratridine (Fig. 5B). No inhibition of veratridine-activated ^22^Na\(^+\) uptake by δ-atracotoxin-Hv1 was observed, even at concentrations up to 3 μM. This marked difference in the modulation of batrachotoxin- and veratridine-stimulated ^22^Na\(^+\) uptake by δ-atracotoxins strongly suggests a differential interaction of the spider toxin receptor site with the alkaloid toxin binding region. Such differences were not observed with scorpion α-toxin modulation of alkaloid toxin action (27). Accordingly, we examined the ability of δ-atracotoxins to compete directly with the binding of scorpion α-toxins to neurotoxin receptor site 3.
FIG. 5. Differential effect of δ-atracotoxins on batrachotoxin- and veratridine-stimulated 22Na uptake. A, inhibition of batrachotoxin-activated 22Na uptake by δ-atracotoxins. Rat brain synaptosomes were incubated for 10 min at 37°C with 1 μM batrachotoxin and a range of concentrations of either δ-atracotoxin-Hv1 (●, n = 3–8) or δ-atracotoxin-Ar1 (○, n = 3–5). B, enhancement of veratridine-activated 22Na uptake by increasing concentrations of δ-atracotoxin-Hv1. Synaptosomes were incubated with the indicated concentrations of δ-atracotoxin-Hv1 in the absence (black columns) and presence (gray columns) of 300 μM veratridine. The left-hand (striped) column shows the activation of 22Na uptake by 300 μM veratridine alone. All data are presented as a percentage of maximal 22Na uptake as determined in the presence of 1 μM batrachotoxin (n = 3).

identical IC50 values in competition binding studies with 125I-Lqh II binding to rat brain synaptosomes (22). Similarly, AaH II and Lqh II compete with identical Ki values for the binding of 125I-Lqh II (Fig. 6). Likewise δ-atracotoxin-Hv1 and δ-atracotoxin-Ar1 are able to completely inhibit the binding of 125I-Lqh II to rat brain sodium channels with Ki values of 0.85 ± 0.03 and 1.07 ± 0.2 nM, respectively (Fig. 6). These data indicate that δ-atracotoxins may share a common binding site with scorpion α-toxins.

DISCUSSION

δ-Atracotoxins Affect Sodium Channels in a Unique Manner—The present study shows that δ-atracotoxins slow sodium current inactivation and shift the voltage dependence of activation in peripheral and central neurons in a similar manner to scorpion α-toxins. Unlike scorpion α-toxins, which typically cause a slight increase or do not alter peak sodium currents (13), δ-atracotoxins were also shown to decrease peak sodium currents in rat dorsal root ganglion but not periaqueductal gray neurons (5, 6). This suggests that the actions of δ-atracotoxins may be, at least in part, subtype-specific as the sodium channel subtypes in sensory and rat brain neurons have been shown to be distinct (37). This assumption deserves further study, but clearly the most prominent effect to slow sodium current inactivation is similar in the two preparations (Fig. 1). One of the most intriguing results of the present study, however, was the observation that δ-atracotoxins markedly increase [3H]BTX binding but decrease batrachotoxin-activated 22Na uptake (Figs. 2A and 5A). The latter is in marked contrast to the effect of scorpion α-toxins on batrachotoxin-activated 22Na uptake in rat brain synaptosomes (27, 34).

The other unexpected result of our study was the differential modulation of batrachotoxin and veratridine action by δ-atracotoxins. Unlike the inhibition of batrachotoxin-activated 22Na uptake (Fig. 5A), the δ-atracotoxins increase veratridine-activated 22Na flux by decreasing the EC80 and converting veratridine from a partial to a full agonist (Fig. 4B). This effect on the activity of veratridine is similar to that observed with scorpion α-toxins (27, 34). Hence δ-atracotoxins, unlike scorpion α-toxins, are able to differentiate between the open state of the sodium channel stabilized by batrachotoxin and veratridine (18, 38). Despite these differences in the effect of δ-atracotoxins and scorpion α-toxins on activation of sodium channels in electrophysiological and 22Na uptake studies, δ-atracotoxins were shown to compete at low concentration with the scorpion α-toxin Lqh II on rat brain sodium channels (Fig. 6). This suggests that δ-atracotoxins bind to similar receptor sites as scorpion α-toxins, despite their differential interaction with batrachotoxin and veratridine.

δ-Atracotoxins May Induce Sub-conductance States—Alkaloid toxins, which bind to receptor site 2, are considered to be persistent activators of sodium channels (7). Their mode of action, however, is very complex; they induce a strong shift in the voltage dependence of activation to very negative membrane potentials, inhibit or remove inactivation, alter ion selectivity, and reduce single channel conductance (18, 19, 36, 39). Thus despite the persistent activation, sodium conductance through the permanently open sodium channels is reduced to around 50% or 15–25% of normal in the presence of batrachotoxin or veratridine, respectively, indicating that these two alkaloids stabilize two discrete sub-conductance states (19, 39, 40). It is conceivable that the decrease in sodium conductance observed with δ-atracotoxins in dorsal root ganglia neurons may be due to stabilization or induction of a distinct sub-conductance state, in addition to actions on sodium...
current inactivation (5, 6). The presence of sub-conductance states is suggested to occur also in central neurons in the presence of batrachotoxin, as indicated by the inhibition of batrachotoxin-activated $^{22}\text{Na}^+$ uptake induced by $\delta$-atracotoxins (Fig. 5A). $\delta$-Atracotoxins can induce 30% of maximal $^{22}\text{Na}^+$ uptake and inhibit the maximal uptake stimulated by batrachotoxin to 20–30% (Figs. 3 and 5A). This may indicate that the sub-conductance state induced by $\delta$-atracotoxins in the presence of batrachotoxin is about 30% that induced by batrachotoxin alone. Moreover, this sub-conductance state should dominate the open state of the channel even in the simultaneous presence of both batrachotoxin and $\delta$-atracotoxins. Our present data, however, cannot provide evidence for this assumption. Clarification of the mechanistic basis for the inhibition of sodium current awaits single channel analysis of $\delta$-atracotoxin-modified currents.

$\delta$-Atracotoxins Differentially Interact with Batrachotoxin and Veratridine Receptor Sites—An interesting finding of the present study was that $[^3\text{H}]\text{BTX}$ binding was increased despite a decrease in batrachotoxin-activated $^{22}\text{Na}^+$ uptake. This suggests an uncoupling between the binding of batrachotoxin and its action to activate sodium channels. In support of this hypothesis is the recent study of Wang and Wang (41) that used site-directed mutagenesis in the S6 transmembrane segment of domain I of sodium channels to study the receptor-binding site of batrachotoxin. They revealed that certain mutants could separate the inhibition of channel inactivation from the induction of a sub-conductance state by batrachotoxin. Their results indicate a possible uncoupling between the binding and some of the effects of batrachotoxin on the sodium channel as noted in the present study.

In contrast to the inhibition in batrachotoxin-activated $^{22}\text{Na}^+$ uptake, $\delta$-atracotoxins increased the effect of veratridine-stimulated $^{22}\text{Na}^+$ uptake at all concentrations. This may indicate that $\delta$-atracotoxins interact differentially with the open sub-conducting state induced by veratridine than with that stabilized by batrachotoxin. This is supported by the increase in veratridine-stimulated $^{22}\text{Na}^+$ uptake to the maximal level even at saturating concentrations of $\delta$-atracotoxins (3 $\mu\text{m}$; Fig. 5B), a concentration that induced 70–80% inhibition of batrachotoxin-stimulated uptake. Independent support for this differential interaction hypothesis is the different sub-conductance state reported in the literature for batrachotoxin and veratridine (19, 39, 40).

Implications for the $\delta$-Atracotoxin Receptor Site—Competition binding experiments revealed that both $\delta$-atracotoxins were able to completely displace the $^{125}\text{I}$-scorpion $\alpha$-toxin Lqh II in a concentration-dependent manner. This suggests that $\delta$-atracotoxins bind to at least a partially overlapping receptor site with that of scorpion $\alpha$-toxins. The above considerations suggest, however, that despite competitive interactions in binding studies (Fig. 6), the actual binding interactions may differ, at least partially, between $\delta$-atracotoxins and scorpion $\alpha$-toxins. This is supported by the differential modulation by the $\delta$-atracotoxin receptor site, shown to be on the extracellular side of the channel (5, 6), of the receptor site for batrachotoxin and that for veratridine. Hence, $\delta$-atracotoxins and scorpion $\alpha$-toxins may bind to partially overlapping sites on the sodium channel localized within the area of receptor site 3. In support, another group of scorpion toxins, the “a-like” toxins, which also inhibit sodium current inactivation, were suggested to bind to a partially overlapping receptor site with scorpion $\alpha$-toxins and sea anemone toxins (31). Despite the initial insight into the receptor site 3 area (42, 43), the regions of the sodium channel involved in binding of these peptide toxins still await to be discovered.

In summary, the $\delta$-atracotoxins bind to a similar cluster of receptor sites as scorpion $\alpha$-toxins. Despite the slowing of sodium current inactivation and the competition for scorpion $\alpha$-toxin binding differences among these receptor sites are indicated by the inhibition of peak sodium current in dorsal root ganglion neurons induced by $\delta$-atracotoxins in contrast to the increase obtained with scorpion $\alpha$-toxins. Moreover, $\delta$-atracotoxins also differentially interact with batrachotoxin and veratridine receptor sites to modulate $^{22}\text{Na}^+$ uptake which has not been reported with the scorpion $\alpha$-toxins. The interaction with alkaloid toxin binding is, however, suggested to be very similar for both scorpion $\alpha$-toxins and $\delta$-atracotoxins as they can markedly increase $[^3\text{H}]\text{BTX}$ binding and reduce the EC$_{50}$ values for both batrachotoxin and veratridine. Since $\delta$-atracotoxins enhance batrachotoxin binding yet significantly reduce batrachotoxin-activated $^{22}\text{Na}^+$ uptake, the binding and action of certain alkaloid toxins may represent at least two distinguishable steps. Our study further emphasizes the complexity of peptide toxin interaction with the receptor site 3 area on the extracellular surface of sodium channels and its allosteric interaction with the receptor site 2 area. This suggests that closely related structural elements within both receptor site 2 and 3 areas may be responsible for the differences in the modulation of gating revealed by the combined presence of sodium channel modulators such as $\delta$-atracotoxins, scorpion $\alpha$-toxins, and alkaloid toxins. The present study further highlights the need for more structural data on the neurotoxin receptor sites in combination with electrophysiological and biochemical studies in order to understand the dynamic gating processes of the voltage-gated sodium channel.

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