

Miniaturization of Scorpion β -Toxins Uncovers a Putative Ancestral Surface of Interaction with Voltage-gated Sodium Channels^{*♦}

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The bioactive surface of scorpion β -toxins that interact with receptor site-4 at voltage-gated sodium channels is constituted of residues of the conserved $\beta\alpha\beta\beta$ core and the C-tail. In an attempt to evaluate the extent by which residues of the toxin core contribute to bioactivity, the anti-insect and anti-mammalian β -toxins Bj-xtrIT and C_{ss}4 were truncated at their N and C termini, resulting in miniature peptides composed essentially of the core secondary structure motives. The truncated β -toxins ($\Delta\Delta$ Bj-xtrIT and $\Delta\Delta$ C_{ss}4) were non-toxic and did not compete with the parental toxins on binding at receptor site-4. Surprisingly, $\Delta\Delta$ Bj-xtrIT and $\Delta\Delta$ C_{ss}4 were capable of modulating in an allosteric manner the binding and effects of site-3 scorpion α -toxins in a way reminiscent of that of brevetoxins, which bind at receptor site-5. While reducing the binding and effect of the scorpion α -toxin Lqh2 at mammalian sodium channels, they enhanced the binding and effect of Lqh α IT at insect sodium channels. Co-application of $\Delta\Delta$ Bj-xtrIT or $\Delta\Delta$ C_{ss}4 with brevetoxin abolished the brevetoxin effect, although they did not compete in binding. These results denote a novel surface at $\Delta\Delta$ Bj-xtrIT and $\Delta\Delta$ C_{ss}4 capable of interaction with sodium channels at a site other than sites 3, 4, or 5, which prior to the truncation was masked by the bioactive surface that interacts with receptor site-4. The disclosure of this hidden surface at both β -toxins may be viewed as an exercise in “reverse evolution,” providing a clue as to their evolution from a smaller ancestor of similar scaffold.

Voltage-gated sodium channels (Na_vs)³ are critical for generation and propagation of action potentials in excitable cells and are targeted by a large variety of ligands that bind at distinct receptor sites on the pore-forming α -subunit (1). Most lipid-

soluble Na_v activators, including pyrethroid insecticides, toxic alkaloids (e.g. veratridine, from the plant family of *Liliaceae*, and batrachotoxin, from the skin of the Colombian frog *Phylllobates aurotaenia*), and marine cyclic polyether toxins (e.g. brevetoxins produced by “red tide” dinoflagellates), are effective in invertebrates as well as vertebrates. In addition, a wide array of proteinaceous Na_v modifiers exists in the venom of scorpions, spiders, cone snails, and sea anemones, which are utilized for prey and defense (1, 2).

Na_v gating modifiers from scorpion venom are divided into two classes, α and β , according to their mode of action and binding properties (3–5). α -Toxins prolong the action potential by inhibiting the fast inactivation of the Na_v upon binding to receptor site-3 (4, 6), which involves extracellular loops in domains 1 and 4 (6). These toxins are divided into three groups (7): (i) anti-mammalian α -toxins (e.g. Lqh2 from *Leiurus quinquestriatus hebraeus*), which are highly toxic in mammalian brain and bind with high affinity to rat brain Na_vs; (ii) anti-insect α -toxins (e.g. Lqh α IT), which are highly toxic to insects and show weak activity in mammalian brain; and (iii) α -like toxins (e.g. Lqh3), which are toxic to insects and mice (by intracerebroventricular and peripheral injections) but bind weakly to rat brain synaptosomes (3). Alterations in the binding of α -toxins to receptor site-3 on rat brain and insect Na_vs are allosterically modulated by a variety of Na_v lipophilic activators such as site-2, site-5, and site-7 toxins (e.g. veratridine, brevetoxin, and pyrethroids, respectively; Refs. 3, 7–10).

β -Toxins shift the voltage dependence of Na_v activation to more negative membrane potentials upon binding to receptor site-4, assigned to extracellular loops in domains 2 and 3 (11–15). This class is divided into: (i) anti-mammalian β -toxins (e.g. C_{ss}4 from *Centruroides suffusus suffusus*; Ref. 4); (ii) β -toxins that affect both insect and mammalian Na_vs (e.g. Ts1 from *Tityus serrulatus*; Ref. 4); (iii) anti-insect selective depressant β -toxins (e.g. LqhIT2 from *L. quinquestriatus hebraeus*) typified by the flaccid paralysis they produce in blowfly larvae and depolarization followed by block of action potentials in an isolated cockroach axon (reviewed in Ref. 5); and (iv) anti-insect selective excitatory toxins (e.g. Bj-xtrIT from *Hotentota judaica*), which induce an immediate contraction paralysis upon injection of blowfly larvae, induce spontaneous repetitive firing in cockroach axon (16), and exhibit a unique structure (17, 18). Binding studies with scorpion α - and β -toxins have

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³ The abbreviation used is: Na_v, voltage-gated sodium channel.

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shown that receptor sites 3 and 4 of insect Na_v s interact allosterically, thus contributing to the synergistic effects in toxicity obtained upon their co-administration (19).

Short chain (30–50-residue-long) and long chain (60–76-residue-long) scorpion toxins that affect potassium and sodium channels, respectively, share a common $\beta\alpha\beta\beta$ scaffold with highly variable loops connecting the main secondary structure elements of the toxin core (see Fig. 1) (reviewed in Ref. 20). The structure of excitatory β -toxins, such as Bj-xtrIT (18), is unique. The core motif is very similar to those of other scorpion toxins (α -helix packed against a three-stranded β -sheet stabilized by three spatially conserved disulfide bonds: in Bj-xtrIT Cys-16–Cys-42, Cys-27–Cys-47, Cys-31–Cys-49; in Css4 Cys-16–Cys-41, Cys-25–Cys-46, Cys-29–Cys-48; see Fig. 1) but varies prominently in its C-terminal region and the spatial arrangement of its fourth, non-conserved disulfide bond (in Bj-xtrIT, Cys-43–Cys-69; in Css4, Cys-12–Cys-65; Refs. 17, 18, 21). Moreover, the C-terminal region of Bj-xtrIT (residues 60–76) contains an additional α -helix, α_2 , which is unique for excitatory toxins (residues 63–69 in Bj-xtrIT; Refs. 18, 20). The differences in the C-tail arrangement, which stabilizes the conformation of the C-terminal region against the molecular core, were suggested to determine the functional divergence of scorpion toxins (17).

A number of reports have described in detail the bioactive surfaces of a few scorpion β -toxins, the anti-insect excitatory and depressant toxins, Bj-xtrIT and LqhIT2 (22–24), and the anti-mammalian β -toxin, Css4 (25). These studies highlighted a conserved pharmacophore composed of a key negatively charged Glu at the α -helix motif, flanked by hydrophobic aromatic residues that may isolate the point of interaction from bulk solvent. The residues that comprise the pharmacophore of β -toxins belong to the protein core (Fig. 1), which is common to both long chain scorpion α and β -toxins and most short chain scorpion toxins affecting voltage-gated potassium channels (20, 26). An additional hydrophobic cluster of bioactive amino acids at the C-tail of Bj-xtrIT and on the loop connecting the second and the third β -strands of Css4 was suggested to be involved in determining toxin selectivity (5, 25).

To examine the commonality of the pharmacophore region in β -toxins and the extent of its contribution to recognition of receptor site-4, we deleted the N- and C-terminal regions of two β -toxins, Bj-xtrIT and Css4, and analyzed the binding properties of the resulting truncated derivatives ($\Delta\Delta\beta$ -toxins). Unpredictably the $\Delta\Delta\beta$ -toxins were non-toxic and did not bind at receptor site-4; nonetheless they were able to allosterically modulate α -toxin activity on insect and mammalian Na_v s and to antagonize the effects of brevetoxins. These results indicated that the seemingly inactive $\Delta\Delta\beta$ -toxins were able to interact with the Na_v .

EXPERIMENTAL PROCEDURES

Toxins and Mutagenesis—Bj-xtrIT, $\Delta\Delta$ Bj-xtrIT, and Lqh α IT were produced in a recombinant form as was described previously (16, 25), and Css4 and $\Delta\Delta$ Css4 were produced in fusion to a His tag extension at their N termini as described (25). PCR-driven mutagenesis, expression in *Escherichia coli*, *in vitro* folding, and purification of Css4 derivatives have been described in detail (25). Brevetoxins 2 and 3 (PbTx-2; PbTx-3) were pur-

chased from Latoxan (Valance, France). Charybdotoxin was kindly provided by Prof. B. Attali, Tel Aviv University.

Binding Experiments—Locust neuronal membranes were prepared from dissected brains and ventral nerve cords of adult *Locusta migratoria* using an established method (7). Rat brain synaptosomes were prepared from adult (~300 g) albino Wistar strain (laboratory-bred) as was described previously (27). Membrane protein concentration was determined by a Bio-Rad protein assay, using bovine serum albumin as standard. Css4 and Bj-xtrIT were radioiodinated by lactoperoxidase (catalog number L8257; 7 units/60 μl of reaction mix; Sigma) using 10 μg of toxin and 0.5 mCi of carrier-free Na^{125}I (Amersham Biosciences,) and the monoiodotoxin was purified as was described previously (25).

Labeling of PbTx-3—Solution of PbTx-3 (1 mg/ml acetonitrile) was mixed with 0.1 Ci of NaBa^3H_4 (69 Ci/mmol) for 5 min reduction at room temperature. [^3H]PbTx-3 was purified on a C_{18} high pressure liquid chromatography column using an isocratic gradient composed of 77% methanol and 23% water. The specific radioactivity of [^3H]PbTx-3 was 17 Ci/mmol.

The composition of media used in binding assays and termination of the reactions was previously described (27, 28). Nonspecific binding was determined in the presence of 1–10 μM of the unlabeled toxin and typically consisted of 10–30% of total binding (for scorpion toxins). Equilibrium competition binding assays were performed and analyzed as was described previously (25). For [^3H]PbTx-3, filters were dried 1 h at 80 $^\circ\text{C}$, mixed with 5 ml of ULTIMUMA Gold (PerkinElmer Life Sciences), and the radioactivity was monitored. Nonspecific binding was determined in the presence of 2 μM PbTx-2. Each experiment was performed in duplicate and repeated at least three times as indicated (*n*). Data are presented as mean \pm S.E. of the number of independent experiments.

Two-electrode Voltage Clamp Experiments—The genes encoding for the *Drosophila melanogaster* sodium channel α -subunit (Dm Na_v1) and the auxiliary TipE subunit were kindly provided by J. Warmke, Merck, and M. S. Williamson, Division of Plant and Invertebrate Ecology-Rothamsted, UK, respectively. The genes encoding for the rat brain and skeletal muscle sodium channels (r $\text{Na}_v1.2$ and r $\text{Na}_v1.4$) were a gift from Dr. R. G. Kallen, University of Pennsylvania, Philadelphia, PA. These genes and that for the auxiliary subunit h β 1 were transcribed *in vitro* using T7 RNA-polymerase and the mMACHINETM system (Ambion, Austin, TX), and the cRNAs were injected into *Xenopus laevis* oocytes as was described previously (13). Currents were measured 1–2 days after injection using a two-electrode voltage clamp and a Gene Clamp 500 amplifier (Axon Instruments, Union City, CA). Data were sampled at 10 kHz and filtered at 5 kHz. Data acquisition was controlled by a Macintosh PPC 7100/80 computer, equipped with ITC-16 analog/digital converter (Instrutech Corp., Port Washington, NY), utilizing Synapse (Synergistic Systems, Sweden). Capacitance transients and leak currents were removed by subtracting a scaled control trace utilizing a P/6 protocol (29). Bath solution contained (in mM): 96 NaCl, 2 KCl, 1 MgCl_2 , 2 CaCl_2 , and 5 HEPES, pH 7.85. Oocytes were washed with the bath solution using a BPS-8 perfusion system (ALA Scientific Instruments, Westbury, NY) with a positive pressure of 4 p.s.i.

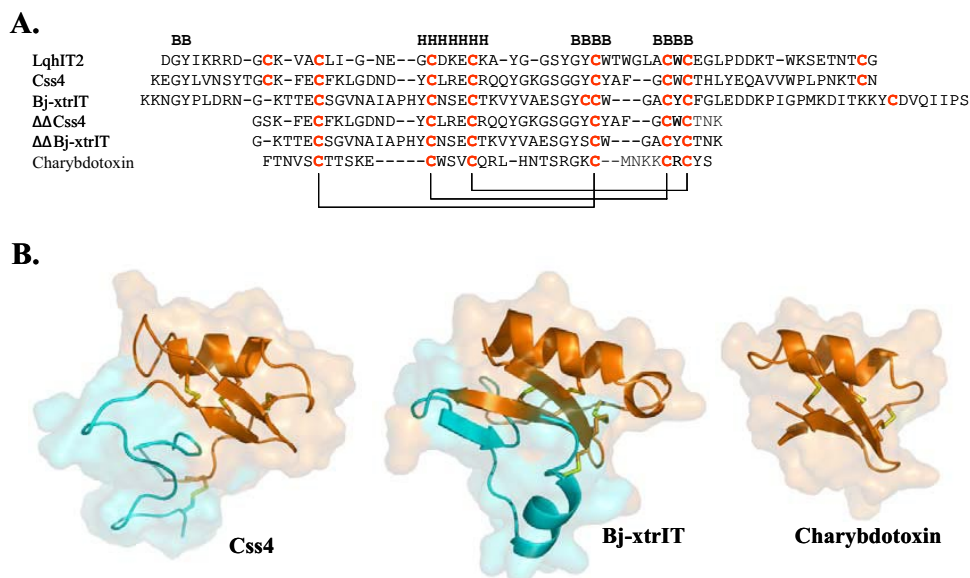


FIGURE 1. Sequence alignment and three-dimensional structure of representative scorpion toxins. *A*, sequences were aligned according to the conserved cysteine residues, and the disulfide bonds formed between cysteine pairs are marked in *solid lines*. *Dashes* indicate gaps. Secondary structure motifs (*B*, β -strand; *H*, α -helix) in Cys4 follow the published structure of Cn2 (Protein Data Bank accession 1Cn2). *B*, schematic diagrams of the $C\alpha$ model structures of Cys4, Bj-xtrIT, and charybdotoxin covered by semitransparent molecular surfaces. The structures of Bj-xtrIT and charybdotoxin are derived from the Protein Data Bank accession numbers 1BCG and 2CRD, respectively. The Cys4 model is based on the NMR structure of Cn2 (25) and is spatially aligned with that of Bj-xtrIT and charybdotoxin. The moieties representing $\Delta\Delta$ Bj-xtrIT and $\Delta\Delta$ Css4 are colored in *orange* on the ribbon structure of the parent toxins, and the *cyan ribbons* represent the deleted N- and C termini. The disulfide bonds are colored in *yellow*. Panel *B* was prepared using PyMOL.

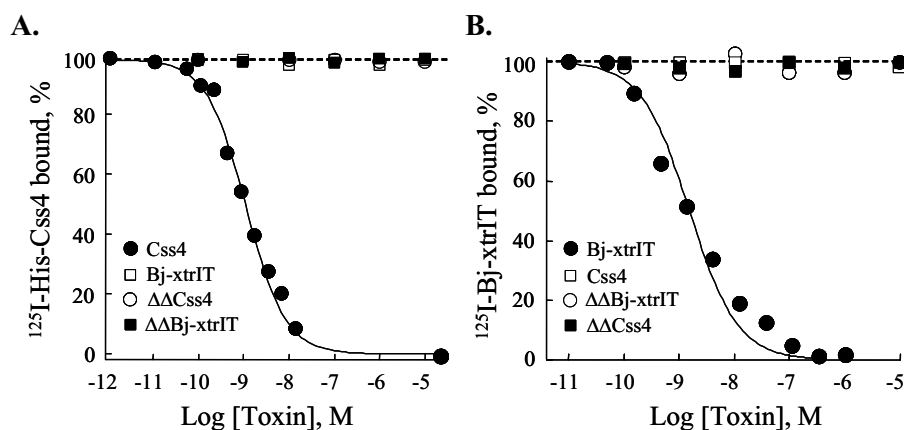


FIGURE 2. The $\Delta\Delta\beta$ -toxins do not compete with site-4 toxins. *A*, rat brain synaptosomes were incubated 60 min at 22 °C with 0.1 nM 125 I-Css4 and increasing concentrations of Cys4, Bj-xtrIT, $\Delta\Delta$ Bj-xtrIT, and $\Delta\Delta$ Css4. Nonspecific binding, determined in the presence of 1 μ M Cys4 (comprising 10% of total binding), was subtracted. The calculated K_i values were: Cys4, 0.98 ± 0.1 nM ($n = 8$); Bj-xtrIT, $\Delta\Delta$ Bj-xtrIT, and $\Delta\Delta$ Css4, $\gg 10,000$ nM. *B*, locust neuronal membranes (160 μ g) were incubated with 0.1 nM 125 I-Bj-xtrIT 45 min at 22 °C. Nonspecific binding, measured in the presence of 1 μ M unlabeled Bj-xtrIT (comprising 30% of total binding), was subtracted. The K_i value of Bj-xtrIT was 1.6 ± 0.3 nM ($n = 3$); Cys4, $\Delta\Delta$ Bj-xtrIT, and $\Delta\Delta$ Css4, $\gg 10,000$ nM.

Toxins were diluted with bath solution and applied directly to the bath to the final desired concentration.

GV Analysis—Mean conductance (G) was calculated from peak current/voltage relationship using the equation $G = I/(V - V_{rev})$, where I is the peak current, V is the membrane potential, and V_{rev} is the reversal potential. Normalized conductance voltage relations were fit with one Boltzman distribution according to the following equation $G/G_{max} = 1 + \exp[(V_{1/2} - V)/k]$, where $V_{1/2}$ is the respective membrane potential for which the mean conductance is half-maximal; k is the respective slope.

To obtain a dose-response curve for α -toxins, currents were elicited by depolarization to -10 mV from a holding potential of -80 mV in the presence of various toxin concentrations. At each toxin concentration, currents were allowed to reach a steady-state level prior to the final measurement, and the steady-state current at -10 mV was normalized relatively to the current in control.

RESULTS

The N and C termini of β -toxins vary in sequence, structure, and disulfide bond organization, but their core is conserved (Fig. 1) (Ref. 17). To examine the contribution of the pharmacophore of scorpion β -toxins (25) to their binding affinity, we deleted (Fig. 1) the first 10 N-terminal residues as well as the C-tail stretch beyond the penultimate Cys of Bj-xtrIT (anti-insect; 27 residues) and Cys4 (anti mammalian; 18 residues). Since the $\Delta\Delta\beta$ -toxins ended with a Cys residue, we added the triplet Thr-Asn-Lys, which appears at the C-tail of various potassium channel blockers (26). The Cys residues that remained unpaired in the two toxins (position 12 in Cys4 and 43 in Bj-xtrIT) were substituted by Ser to prevent incorrect folding or dimerization. $\Delta\Delta$ Css4 and $\Delta\Delta$ Bj-xtrIT were produced recombinantly using the *E. coli* expression system as was described for the unmodified parental toxins.

Functional Analysis of the $\Delta\Delta\beta$ -Toxins— $\Delta\Delta$ Bj-xtrIT and $\Delta\Delta$ Css4 were non-toxic to blowfly larvae and mice by injection even at very high concentrations (>20 nmol/g of animal) and were unable to displace 125 I-Bj-xtrIT and 125 I-Css4 at locust and rat brain neuronal membranes in binding competition assays (up to 20 μ M; Fig. 2). These results indicated that the two $\Delta\Delta\beta$ -toxins lost their ability to bind at receptor site-4 of Na_v s. Moreover, $\Delta\Delta$ Bj-xtrIT and $\Delta\Delta$ Css4 (up to 20 μ M) had no effect on the conductance and gating properties of the mammalian channels rNa_v1.2 (brain) and rNa_v1.4 (skeletal muscle), and the insect channel DmNa_v1, all expressed in *X. laevis* oocytes (Table 1). Thus, at first glance, these results have suggested that the truncated toxins lost any bioactive competence. At this stage, we used another sensitive measure to assess whether the truncated toxins could interact

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TABLE 1

Alterations of the mid-voltage of activation ($V_{1/2}$) derived from conductance-voltage (G-V) curves of DmNa_v1/TipE, rNa_v1.2/h β 1 and rNa_v1.4/h β 1 expressed in *Xenopus* oocytes and induced by PbTx-2 and the $\Delta\Delta\beta$ -toxins

The data represent the mean \pm S.E. of at least six independent experiments. The oocytes were clamped at -100 mV and then 50-ms depolarization pulses from -90 to $+50$ mV were applied at 5-mV increments. The $V_{1/2}$ was calculated as described under "Experimental Procedures."

	DmNa _v 1/TipE		rNa _v 1.2/h β 1		rNa _v 1.4/h β 1	
	μ M	$V_{1/2}$, mV	μ M	$V_{1/2}$, mV	μ M	$V_{1/2}$, mV
Control		-15.9 ± 0.2		-24.9 ± 0.3		-24.9 ± 0.2
PbTx-2	0.5	-27.7 ± 0.3	1	-33.4 ± 0.6	2	-32.6 ± 0.5
$\Delta\Delta$ Bj-xtrIT	20	-17.0 ± 0.8	20	-25.1 ± 0.4	20	-25.0 ± 0.5
$\Delta\Delta$ Css4	20	-16.4 ± 0.2	20	-24.9 ± 0.4	20	-24.7 ± 0.4
$\Delta\Delta$ Bj-xtrIT + PbTx-2 ^a	1 + 2	-16.4 ± 0.3	1 + 3	-25.1 ± 0.3	2 + 5	-25.1 ± 0.2
$\Delta\Delta$ Css4 + PbTx-2 ^a	1 + 2	-16.5 ± 0.3	1 + 3	-25.0 ± 0.3	2 + 5	-25.0 ± 0.2

^a The $\Delta\Delta\beta$ -toxin was applied together with PbTx-2.

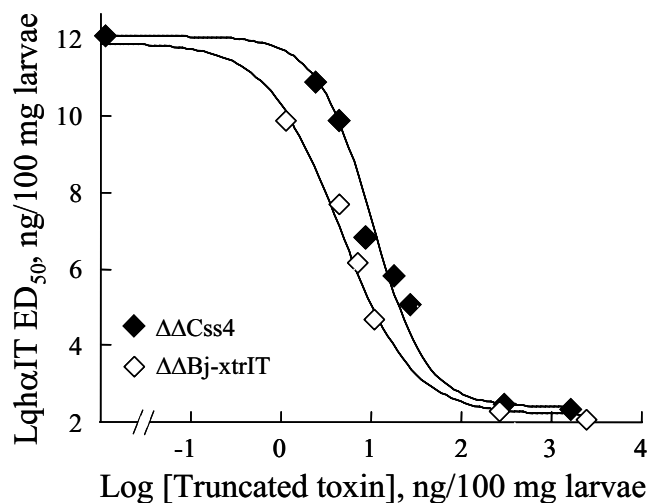


FIGURE 3. The toxicity of Lqh α IT is enhanced by the $\Delta\Delta\beta$ -toxins. Dose-response curves for toxicity of Lqh α IT upon injection to blowfly larvae in the presence of increasing concentrations of $\Delta\Delta$ Bj-xtrIT or $\Delta\Delta$ Css4 (in ng/100 mg of body weight). Forty larvae were used to generate each data point, which represents the ED₅₀ equivalent of Lqh α IT in the presence of the indicated dose of $\Delta\Delta$ Bj-xtrIT or $\Delta\Delta$ Css4. The half-maximal doses of $\Delta\Delta$ Bj-xtrIT or $\Delta\Delta$ Css4 (EC₅₀) that increase the toxicity (decrease in ED₅₀) of Lqh α IT are 5.2 and 9.6 ng/100 mg, respectively. Maximal enhancement in Lqh α IT toxicity (~600%) is achieved in the presence of less than 1 μ g of $\Delta\Delta$ Bj-xtrIT or $\Delta\Delta$ Css4.

with the Na_v. It has been shown that the interaction of scorpion α -toxins with receptor site-3 is modulated by a variety of ligands that bind at other receptor sites on the Na_v, such as veratridine (site-2; Refs. 8,30), scorpion β -toxins (site-4; Ref. 19), brevetoxins (site-5; Refs. 7, 9), and pyrethroids (site-7; Ref. 10). We therefore tested whether $\Delta\Delta$ Bj-xtrIT and $\Delta\Delta$ Css4 were capable of modulating the activity of the scorpion α -toxin Lqh α IT on insects. Surprisingly, $\Delta\Delta$ Bj-xtrIT and $\Delta\Delta$ Css4 increased 6-fold the toxicity of Lqh α IT to blowfly larvae, with half-maximal doses (EC₅₀) of 5.2 and 9.6 ng/100 mg, respectively (Fig. 3). This unexpected synergism prompted us to examine the binding capability of the $\Delta\Delta\beta$ -toxins to Na_vs.

$\Delta\Delta$ Bj-xtrIT and $\Delta\Delta$ Css4 were examined in binding studies using iodinated scorpion α -toxins that interact well with insect and rat brain neuronal membranes. In locust neuronal membranes, $\Delta\Delta$ Bj-xtrIT and $\Delta\Delta$ Css4 increased 1.7-fold ¹²⁵I-Lqh α IT binding (EC₅₀ 2.9 \pm 0.5 and 3.8 \pm 1.4 nM, respectively; Fig. 4A). This positive cooperativity indicated that the $\Delta\Delta\beta$ -toxins interact with the insect Na_v at a site other than receptor site-3. Notably, in rat brain synaptosomes, $\Delta\Delta$ Bj-xtrIT and $\Delta\Delta$ Css4 inhibited ¹²⁵I-Lqh2 binding in a dose-dependent manner with an IC₅₀ of 76 \pm 18 and 105 \pm 29 nM, respectively, whereas the

parental β -toxins had no effect (Fig. 4B). It is noteworthy that ¹²⁵I- $\Delta\Delta$ Bj-xtrIT and ¹²⁵I- $\Delta\Delta$ Css4 did not exhibit significant specific binding to insect or rat brain neuronal preparations, and therefore, direct assessment of their binding interaction could not be performed.

The allosteric effects of the $\Delta\Delta\beta$ -toxins were analyzed on the activity of Lqh α IT and Lqh2 on insect and rat brain Na_vs expressed in *Xenopus* oocytes. Lqh α IT increases the sodium peak current and inhibits the fast inactivation of the insect sodium channel, DmNa_v1, with an EC₅₀ of 2.6 \pm 0.2 nM (Fig. 5A) (Ref. 3). When Lqh α IT was applied together with either $\Delta\Delta$ Bj-xtrIT or $\Delta\Delta$ Css4 in a 1:50 molar ratio, the EC₅₀ of Lqh α IT in the context of the mixture dropped to 0.6 \pm 0.2 and 0.7 \pm 0.2 nM, respectively (Fig. 5A), indicating enhancement in the α -toxin activity at the insect sodium channel. In contrast, joint application of either $\Delta\Delta$ Bj-xtrIT or $\Delta\Delta$ Css4 in a 1:10 molar ratio with Lqh2, which inhibits the fast inactivation of rNa_v1.2 with an EC₅₀ of 13.2 \pm 0.3 nM (3), increased the EC₅₀ of Lqh2 in the context of the mixture to 44.4 \pm 1 and 42 \pm 1.4 nM, respectively (Fig. 5B), indicating decrease in activity at this mammalian sodium channel. Once more, these opposite effects have indicated that the $\Delta\Delta\beta$ -toxins, which by themselves had no effect on the sodium currents (Table 1), modulate the effect of site-3 α -toxins on mammalian and insect Na_vs in a different manner, further supporting their interaction with the sodium channels.

Since the structure of the truncated toxins is composed of the $\beta\alpha\beta\beta$ fold as it appears in most scorpion toxins, we questioned the specificity of their interaction with the Na_v by employing another short chain toxin of similar size and fold, charybdotoxin (from the scorpion *L. quinquestratus hebraeus*; Fig. 1) (Ref. 26). Charybdotoxin, which is a blocker of voltage-gated potassium channels, had no effect on Lqh α IT activity at DmNa_v1 and on Lqh2 activity at rNa_v1.2 (not shown), suggesting that the activity of the $\Delta\Delta\beta$ -toxins is not derived only from their $\beta\alpha\beta\beta$ fold.

The $\Delta\Delta\beta$ -Toxins Inhibit Brevetoxin Action on Na_vs—The $\Delta\Delta$ Bj-xtrIT and $\Delta\Delta$ Css4 opposing modulations of the binding and activity of α -toxins at insect and mammalian Na_vs was reminiscent of that described for brevetoxins, which bind at receptor site-5 (7–9, 31). Therefore, we examined whether $\Delta\Delta$ Bj-xtrIT had any effect on brevetoxin-2 (PbTx-2) toxicity *in vivo*. PbTx-2 is highly toxic to blowfly larvae (contraction and paralysis 60 s after injection; ED₅₀ = 10 ng/100 mg of larvae), but when injected after or together with the $\Delta\Delta\beta$ -toxins at 1:10 molar ratio, it was not toxic. Thus, the $\Delta\Delta\beta$ -toxins act *in vivo* as antagonists of brevetoxin in insects. However, when the $\Delta\Delta\beta$ -

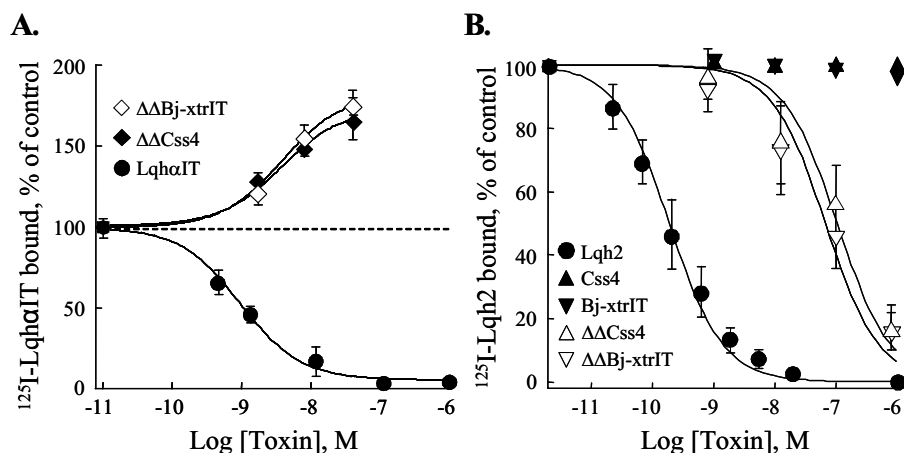


FIGURE 4. Effects of the $\Delta\Delta\beta$ -toxins on the binding of α -toxins. Rat brain and locust neuronal membranes were incubated with 0.1 nM ^{125}I -toxin at room temperature for 20 or 45 min, respectively, in the presence of increasing concentrations of the indicated toxin derivatives. Nonspecific binding, measured in the presence of 1 μM unlabeled toxin, was subtracted. Data points represent mean \pm S.E. of three independent experiments. **A**, enhancement of ^{125}I -Lqh α IT binding to locust neuronal membranes (160 μg of protein). The dashed line indicates maximal specific binding of ^{125}I -Lqh α IT under control conditions. The K_i value for Lqh α IT was 1.1 ± 0.2 nM. Maximal increase in ^{125}I -Lqh α IT binding in the presence of $\Delta\Delta\text{Bj-xtrIT}$ or $\Delta\Delta\text{Ccss4}$ was 172 ± 11 and $163 \pm 14\%$ of the control with an EC_{50} of 2.9 ± 0.5 and 3.8 ± 1.4 nM, respectively. **B**, inhibition of ^{125}I -Lqh2 binding to rat brain synaptosomes (41 μg of protein). Lqh2 displaces ^{125}I -Lqh2 with a K_i of 0.2 ± 0.02 nM. $\Delta\Delta\text{Bj-xtrIT}$ and $\Delta\Delta\text{Ccss4}$ inhibit the binding of ^{125}I -Lqh2 with an apparent K_i of 76 ± 18 and 105 ± 29 nM, respectively. Bj-xtrIT and Ccss4 (up to 20 μM) did not inhibit the binding of ^{125}I -Lqh2.

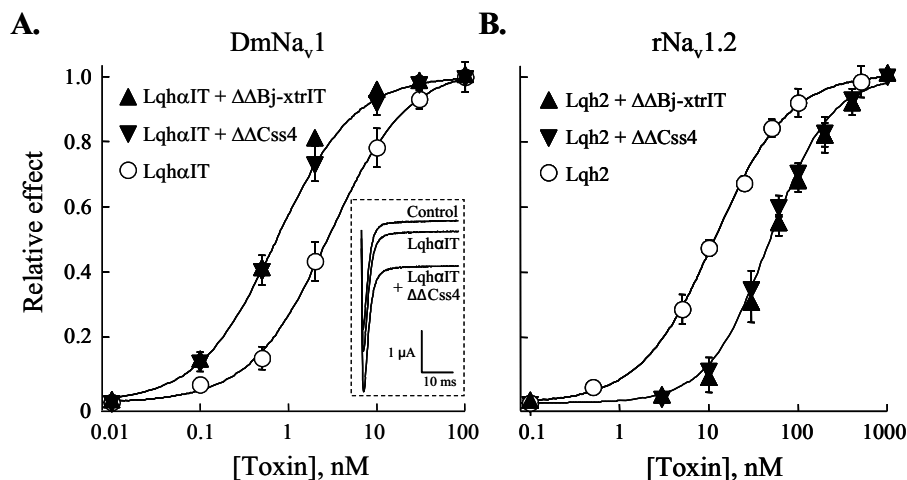


FIGURE 5. Effects of the $\Delta\Delta\beta$ -toxins on the activity of α -toxins. Oocytes expressing DmNa_v1/TipE and rNa_v1.2/h β 1 were clamped at -80 mV, and currents were elicited by step depolarization to -10 mV. Increasing concentrations of the toxins and toxin mixtures were applied, and their relative effect on the current inactivation (sustained current) at -10 mV was determined after 50 ms. **A**, activity of Lqh α IT ($\text{EC}_{50} = 2.6 \pm 0.2$ nM) and Lqh α IT in a mixture with $\Delta\Delta\text{Bj-xtrIT}$ or $\Delta\Delta\text{Ccss4}$ (1:50 molar ratio) on DmNa_v1 provide the following EC_{50} values: 0.6 ± 0.2 and 0.7 ± 0.2 nM, respectively. **Inset**, representative current traces demonstrating the effect of 25 nM $\Delta\Delta\text{Ccss4}$ on the level of the sustained current induced by 0.5 nM Lqh α IT. **B**, activity of Lqh2 and Lqh2 in a mixture with $\Delta\Delta\text{Bj-xtrIT}$ and $\Delta\Delta\text{Ccss4}$ (1:10 molar ratio) on rNa_v1.2 provide the following EC_{50} values: 13.2 ± 0.3 , 44.4 ± 1 , and 42 ± 1.4 nM, respectively. The maximal inhibition of inactivation by Lqh α IT or Lqh2 in the absence or presence of the $\Delta\Delta\beta$ -toxins was identical.

toxins were administered to blowfly larvae subsequent to PbTx-2 injection and after a paralytic effect already developed, even at a 1:100 molar ratio, the larvae were not resuscitated, indicating that the $\Delta\Delta\beta$ -toxins were unable to abolish the brevetoxin effect. To further clarify this point, we examined the effects of PbTx-2 in the presence of the truncated toxins on rNa_v1.2, rNa_v1.4, and DmNa_v1 expressed in *Xenopus* oocytes. Although PbTx-2 in concentrations of 0.5–2 μM shifted the voltage dependence of activation of the three channels to more hyperpolarizing membrane potentials, pre- or co-application of

$\Delta\Delta\text{Bj-xtrIT}$ or $\Delta\Delta\text{Ccss4}$ with PbTx-2 in a 1:2–3 molar ratio abolished these effects (Fig. 6 and Table 1). Once more, when the $\Delta\Delta\beta$ -toxins were applied subsequent to PbTx-2, when its effect had already developed, they had no inhibitory effect (not shown).

We further tested the effects of the $\Delta\Delta\beta$ -toxins on the binding of α -toxins in the presence of PbTx-2. 1 μM PbTx-2 increased the binding of ^{125}I -Lqh α IT to locust neuronal membranes by 30% (19). 100 nM $\Delta\Delta\text{Bj-xtrIT}$ increased the binding of ^{125}I -Lqh α IT to the same membranes by 70%. However, co-application of PbTx-2 and $\Delta\Delta\text{Bj-xtrIT}$ had no additive effect on Lqh α IT binding (Fig. 7A). These data suggest that the binding sites of brevetoxin and the $\Delta\Delta\beta$ -toxins are not identical.

To examine whether the $\Delta\Delta\beta$ -toxins were directly or indirectly involved with brevetoxin binding, we analyzed their effect on the binding of [^3H]PbTx-3, a specific marker of receptor site-5 at rat brain synaptosomes (32). High concentrations (1–3 μM) of either $\Delta\Delta\text{Bj-xtrIT}$ or $\Delta\Delta\text{Ccss4}$ had no effect on [^3H]PbTx-3 binding, not even when incubated for 1 h with the membranes prior to the addition of [^3H]PbTx-3 (Fig. 7B). These results have indicated that the $\Delta\Delta\beta$ -toxins do not compete for the brevetoxin binding site at rat brain Na_vs.

Residues Important for $\Delta\Delta\text{Ccss4}$ Effect—The inability of $\Delta\Delta\text{Bj-xtrIT}$ and $\Delta\Delta\text{Ccss4}$ to compete on binding at receptor sites 3 (Figs. 4 and 5), 4 (Fig. 2), and 5 (Fig. 7B) have suggested that they recognize another receptor site on the sodium channel. Since the two $\Delta\Delta\beta$ -toxins exhibited similar activities, we focused on $\Delta\Delta\text{Ccss4}$ and substituted 16 putative solvent-exposed residues to Ala with

the objective to identify those residues important for interaction with the channel. The $\Delta\Delta\text{Ccss4}$ mutants were tested for their ability to influence Lqh α IT toxicity by mixing each mutant in a 5:1 (w/w) ratio with Lqh α IT, and the ED_{50} of the mixtures was determined on blowfly larvae (Fig. 8). A number of substitutions, which in the context of Ccss4 had no effect on the binding affinity (K13A, D21A, D23A, Q31A, and Q32A; Ref. 25), decreased the positive cooperative effect of $\Delta\Delta\text{Ccss4}$ on Lqh α IT, with the utmost effect achieved with K13A. In contrast, substitutions, which in the context of Ccss4 decreased substantially the binding affinity for rat brain synapto-

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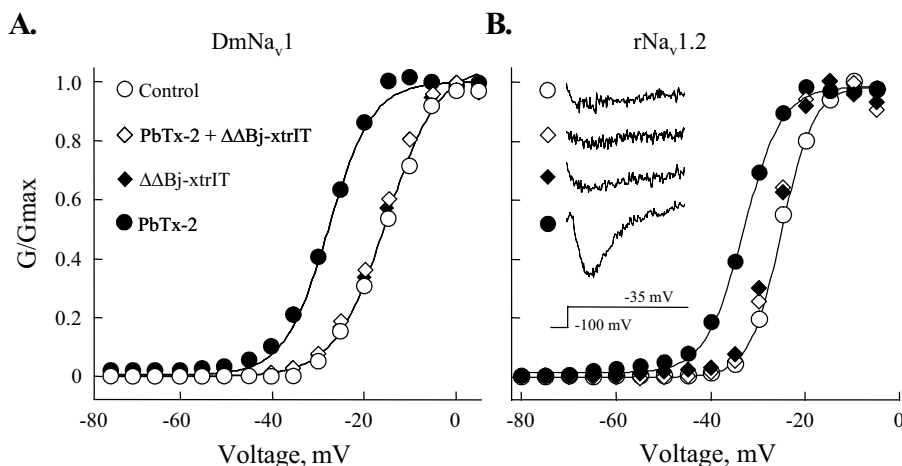


FIGURE 6. **Inhibition of PbTx-2 effect by $\Delta\Delta$ Bj-xtrIT.** *A* and *B*, alteration in conductance-voltage relations of $DmNa_v1$ /TipE (*A*) and $rNa_v1.2/h\beta1$ (*B*) by PbTx-2 in the presence or absence of $\Delta\Delta$ Bj-xtrIT. Toxin concentrations and the activation parameters ($V_{1/2}$) are as described in Table 1. *Inset*, $rNa_v1.2/h\beta1$ currents induced by the indicated toxin in a test pulse to -35 mV. Similar results were obtained with $\Delta\Delta$ Css4 (Table 1).

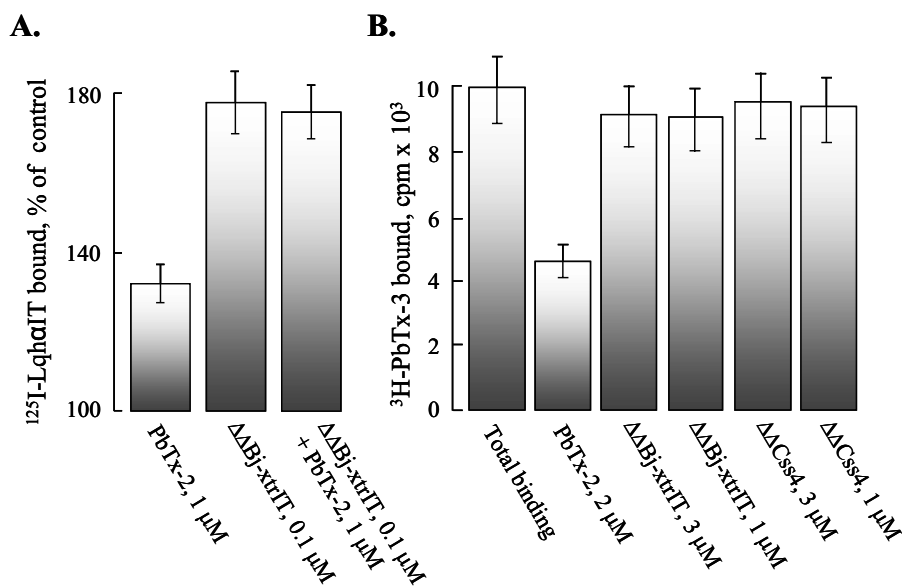


FIGURE 7. **The $\Delta\Delta\beta$ -toxins do not compete with brevetoxin on binding to site-5.** *A*, locust neuronal membranes were incubated with 0.1 nM ^{125}I -Lqh α IT as in Fig. 4. Saturating concentration of PbTx-2 (1 μ M; Ref. 19) increased ^{125}I -Lqh α IT binding by $30 \pm 8\%$. 0.1 μ M $\Delta\Delta$ Bj-xtrIT increased ^{125}I -Lqh α IT binding (maximal enhancement, Fig. 4) by $172 \pm 11\%$, and in the presence of a mixture of both (1 μ M PbTx-2 + 0.1 μ M $\Delta\Delta$ Bj-xtrIT), the increase in ^{125}I -Lqh α IT binding was comparable ($168 \pm 14\%$). *B*, [3H]PbTx-3 binding in the presence of the $\Delta\Delta\beta$ -toxins. [3H]PbTx-3 (10 nM) was incubated with rat brain synaptosomes (70 μ g) for 3 h at room temperature in the absence (*Total binding*) or presence of 2 μ M PbTx-2 (nonspecific binding) or the indicated concentrations of $\Delta\Delta\beta$ -toxins. Identical results were obtained when the $\Delta\Delta\beta$ -toxins were preincubated with the membranes for 1 h prior to the addition of [3H]PbTx-3.

somes, had no effect (L19A, R27A, and Y42A) or only a minor effect (F17A and N22A) on $\Delta\Delta$ Css4 ability to enhance Lqh α IT toxicity (Fig. 8). Since C α ss4 and $\Delta\Delta$ C α ss4 do not bind at the same receptor site on the sodium channel, the difference of bioactive residues was not surprising. The only substitutions that affected both C α ss4 and $\Delta\Delta$ C α ss4 activity were E28A (hot spot) and F14A (numbering follows C α ss4).

DISCUSSION

"Uncoating" the Exterior of Scorpion β -Toxins Exposes a Hidden Bioactive Surface—The C-tail, loops, turns, and unstructured stretches that connect the main secondary structure ele-

ments in long-chain scorpion toxins constitute a large portion of their exteriors and bear residues that participate in bioactivity (reviewed in Refs. 3 and 5). Here we show that removal of about 50% of the amino acid residues of Bj-xtrIT and C α ss4, which changed markedly their exteriors (Fig. 1), abolished any detectable activity and ability to bind at receptor site-4 on the Na_v . However, the ability of $\Delta\Delta$ Bj-xtrIT and $\Delta\Delta$ C α ss4 to reduce the activity of Lqh2 at the brain channel $rNa_v1.2$ and increase Lqh α IT activity at receptor site-3 of insect channels (binding, effect on fast inactivation, and toxicity to blowfly larvae) indicated that they bound to the Na_v . The bioactive residues of $\Delta\Delta$ C α ss4 are different from those of C α ss4 except for Glu-28, which was considered a hot spot in C α ss4 surface of interaction with the $rNa_v1.2$ (25). It is likely that in the context of the new functional surface of $\Delta\Delta$ C α ss4, Glu-28 is unable to interact with the site-4 residue as it did in the context of C α ss4.

From a structural viewpoint, the ability of $\Delta\Delta$ Bj-xtrIT and $\Delta\Delta$ C α ss4 to bind to the Na_v , as manifested in the modulation of receptor site-3, suggests that a masked functional surface was exposed by miniaturization of the two β -toxins. The probability that this hidden surface has been formed by coincidence is very low as it appears in two pharmacologically distinct toxins, C α ss4 and Bj-xtrIT. One way to rationalize such a finding is by assuming that scorpion toxins affecting sodium channels evolved from smaller peptides bearing the conserved $\beta\alpha\beta\beta$ core that were able to interact with sodium

channels (26, 33). It is conceivable that along evolution, these peptides gained higher affinity and specificity for distinct Na_v targets, whereas increasing in volume. Along this process, the bioactive surface of the ancestral toxin has been masked by new functional exteriors, such as those resolved nowadays by mutagenesis in various β -toxins (22, 23, 25). This hypothesis is in concert with the idea that diversification of long-chain scorpion toxins occurred mainly by mutations and slight structural alterations at the loops, turns, and C-tail rather than at the $\beta\alpha\beta\beta$ core (17). Thus, the truncation of these toxins may be considered an exercise in "reverse evolution" since it was opposite to the precedent natural path of evolution of these molecules.

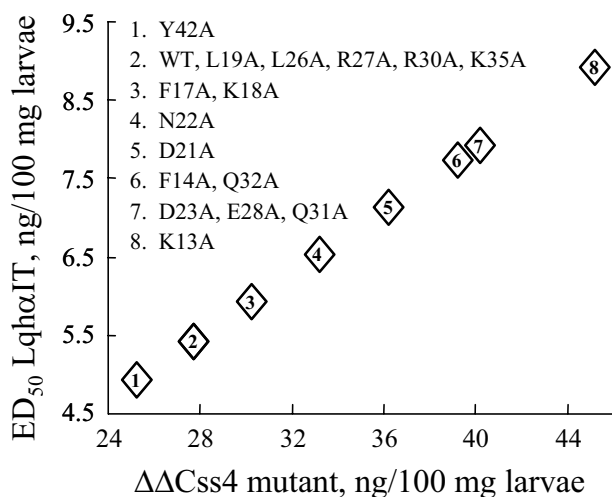


FIGURE 8. **Analysis of $\Delta\Delta$ Css4 bioactive surface.** $\Delta\Delta$ Css4 mutants mixed with Lqh α IT in 5:1 (w/w) ratio were injected to blowfly larvae, and the ED₅₀ of the mixture was determined. The y axis indicates the ED₅₀ of Lqh α IT in the presence of the indicated doses of $\Delta\Delta$ Css4 mutants (x axis). WT, unmodified $\Delta\Delta$ Css4. ED₅₀ of Lqh α IT is 12 ng/100 mg of larvae (see Fig. 3).

The $\Delta\Delta\beta$ -Toxins as Antagonists of Brevetoxins—The similar allosteric effects of the $\Delta\Delta\beta$ -toxins and brevetoxins on the activity of α -toxins (Figs. 4–7) (Refs. 7, 9, 31) have raised the possibility that they both interact with receptor site-5. However, because simultaneous application of $\Delta\Delta$ Bj-xtrIT and PbTx-2 had no additive effect on Lqh α IT binding at insect Na_vs and because $\Delta\Delta$ Bj-xtrIT and $\Delta\Delta$ Css4 did not compete with brevetoxin on binding at receptor site-5 (Fig. 7), it was apparent that the truncated β -toxins bind at a different site. Interestingly, this binding induced allosterically a similar conformational alteration at site-3 to that induced by brevetoxins binding. This conclusion raises the question as to why the $\Delta\Delta\beta$ -toxins are capable of inhibiting PbTx-2 effects on rNa_v1.2, rNa_v1.4, and DmNa_v1 activation only when pre- or co-applied (Fig. 6), whereas having no antagonistic effect when applied after PbTx-2. The 30-Å-long brevetoxin has been suggested to orient its “head” (Lacton ring A) down across the membrane, parallel to the transmembrane hydrophobic α helices S5 and S6 between domains 1 and 4 of the Na_v, whereas its “tail” (“rigid region”; rings H-K) points outward (32, 34). Chemical modifications at the side chain of the K-ring of PbTx-3 (rigid region) hardly affected its binding but markedly influenced its activity (35, 36). In addition, brevetoxins that vary in their rigid region structure exhibit different effects on the binding of α -toxins at receptor site-3 (37). It is therefore likely that the rigid region of brevetoxins interacts with an external channel site whose conformational alteration allosterically affects the activity of α -toxins at receptor site-3. If the $\Delta\Delta\beta$ -toxins interact at least partially with this external channel site, it may suggest that their surface of interaction with the channel, which is responsible for the allosteric effects, is involved in the inhibition of brevetoxin activity. This assumption is supported by previous observations that subtle modifications induced by tetrodotoxin binding to the Na_v external pore are sufficient to prevent the allosteric inhibition caused by brevetoxin on the binding of a scorpion α -toxin to rat brain Na_vs (31, 37).

Therefore, preapplication of the $\Delta\Delta\beta$ -toxins may prevent brevetoxin activity without affecting its binding. As brevetoxins are responsible for massive fish kills and marine mammal mortalities, as well as illness in humans who ingest filter-feeding shellfish or inhale toxic aerosols (34), the $\Delta\Delta\beta$ -toxins may serve as leads for development of brevetoxin antagonists.

General Implications—The continuous search for novel pharmacologically valuable compounds in arthropod venoms requires tedious fractionation and purification followed by biological assays to validate that a desired activity still persists at each purification step. Inactive fractions or substances are usually disregarded as they are not expected to yield a valuable end product. These basic considerations have led to the present situation where of 100–300 distinct polypeptides that can be monitored by mass spectroscopy in the venom of a single scorpion or a spider, the biological activity of only a small portion has been characterized (4, 38, 39). However, there are numerous reasons to believe that compounds that at first glance seem inactive should not be ignored. We have recently shown that scorpion β -toxins that bind to receptor site-4 at the extracellular region of Na_vs may influence allosterically the effects produced by α -toxins that bind at receptor site-3 (19). Moreover, even mere binding of a β -toxin, whose activity was abolished by mutagenesis, was shown to induce this allosteric effect at site-3 (19). We have also shown that the alleged anti-insect selective scorpion depressant toxins affect the mammalian skeletal muscle Na_v provided that the channel was allosterically modulated by the binding of an α -toxin at receptor site-3 (40).

Here we demonstrate the use of the sensitive measure of allosteric interactions to evaluate the ability of substances that *a priori* were considered “biologically inactive” to bind and alter the Na_v conformation. It is therefore rational to believe that when scorpion toxins that affect Na_vs degrade either in the venom glands (39) or in the stung animal (hemolymph or blood), their partially degraded derivatives may “boost” the impact of the entire venom. This phenomenon also provides a conceptual basis to future attempts to decrease doses of drugs by harmless, allosterically acting compounds.

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