Scorpion Toxins Affecting Sodium Current Inactivation Bind to Distinct Homologous Receptor Sites on Rat Brain and Insect Sodium Channels*

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Sodium channels possess receptor sites for many neurotoxins, of which several groups were shown to inhibit sodium current inactivation. Receptor sites that bind α- and α-like scorpion toxins are of particular interest since neurotoxin binding at these extracellular regions can affect the inactivation process at intramembranal segments of the channel. We examined, for the first time, the interaction of different scorpion neurotoxins, all affecting sodium current inactivation and toxic to mammals, with α-scorpion toxin receptor sites on both mammalian and insect sodium channels. As specific probes for rat and insect sodium channels, we used the radio-labeled α-scorpion toxins AaH II and Lqhα IT, the most active α-toxins on mammals and insect, respectively. We demonstrate that the different scorpion toxins may be classified to several groups, according to their in vivo and in vitro activity on mammalian and insect sodium channels. Analysis of competitive binding interaction reveal that each group may occupy a distinct receptor site on sodium channels. The α-mammal scorpion toxins and the anti-insect Lqhα IT bind to homologous but not identical receptor sites on both rat brain and insect sodium channels. Sea anemone toxin ATX II, previously considered to share receptor site 3 with α-scorpion toxins, is suggested to bind to a partially overlapping receptor site with both AaH II and Lqhα IT. Competitive binding interactions with other scorpion toxins suggest the presence of a putative additional receptor site on sodium channels, which may bind a unique group of these scorpion toxins (Bom III and IV), active on both mammals and insects. We suggest the presence of a cluster of receptor sites for scorpion toxins that inhibit sodium current inactivation, which is very similar on insect and rat brain sodium channels, in spite of the structural and pharmacological differences between them. The sea anemone toxin ATX II is also suggested to bind within this cluster.

† The abbreviations used are: TTX, tetrodotoxin; AaIT, excitatory α-toxin; AαIT, inhibitory α-toxin; AaH I–III, α-toxins I, II, and III from the venom of the scorpion Androctonus australis Hector, also called AaH IT; AaH I–III, α-toxins I, II, and III from the venom of the scorpion A. australis Hector; ATX II, toxin II from the sea anemone Anemonia sulcata; Bom III and Bom IV, toxin III and IV from the venom of the scorpion Buthus occitanus mardochei from Mexico; BSA, bovine serum albumin; Css II and Css VI, scorpion β-toxins II and VI.
tritiated derivative of batrachotoxin (\(^{3}H\)
batrachotoxin A 20-mono-benzoate) and veratridine (receptor site 2, Soderlund et al., 1988; Dong et al., 1993; Church and Knowles, 1993; Gordon et al., 1989, 1990, 1993), composed of about 2000 amino acids comprising four homologous repeated domains (I–IV), each containing six putative transmembrane \(\alpha\)-helices (for a review, see Gordon and Zlotkin, 1992). Insect sodium channels were shown to resemble their vertebrate counterparts by their primary structure (Loughney et al., 1989), topological organization (Gordon et al., 1992; Moskowitz et al., 1994), and basic biochemical (Gordon et al., 1988, 1990, 1992, 1993; Moskowitz et al., 1991, 1994) and pharmacological (Pelhate and Sattelle, 1988; Pelhate and Zlotkin, 1982; Cestele et al., 1995) properties. On the other hand, a possible uniqueness of the insect sodium channels was suggested by the description of two groups of scorpion toxins that modify sodium conductance exclusively in insect neuronal preparations, the excitatory and depressant insect-selective toxins (Pelhate and Zlotkin, 1982; Zlotkin et al., 1985, 1991). These toxins bind selectively to insect sodium channels at two distinct receptor sites (Gordon et al., 1992; Moskowitz et al., 1994) and therefore indicate the existence of unique features in the structure of insect channels, as compared to their mammalian counterparts (Gordon et al., 1984, 1992, 1993). Thus, a comparative study of mammalian and insect neurotoxin receptor sites on the respective sodium channels may elucidate the structural features involved in the binding and activity of the various neurotoxins and may contribute to the clarification of structure-function relationship in sodium channels.

Receptor sites for peptide neurotoxins that inhibit sodium current inactivation in neurons (the classical effect induced by \(\alpha\)-scorpion and sea anemone toxins; see Table I) are of particular interest for the study of the dynamics of channel gating, since neurotoxin binding at these extracellular regions can affect the inactivation process at intramembranous segments of the channel (Catterall, 1992). The most studied neurotoxins that induce inhibition of sodium current inactivation are the \(\alpha\)-scorpion toxins and sea anemone toxins, which are believed to share receptor site 3 on sodium channels (Couraud et al., 1978; Catterall and Beress, 1978; Catterall, 1980). Several \(\alpha\)-scorpion toxins have been identified by their high toxicity to mammals and by a high homology in their amino acid sequence (reviewed by Martin-Eauclaire and Couraud, 1995).

In the present study we have used AaH II, the \(\alpha\)-scorpion toxin that reveals the highest affinity to rat brain synaptosomes (I over et al., 1978), and Lqh\(\text{III}\)T, the \(\alpha\)-scorpion toxin that reveals significantly higher activity to insects as compared to vertebrates (Eitan et al., 1990; Gordon and Zlotkin, 1993) as specific probes for receptor site 3 in rat brain and insect sodium channels, respectively. Lqh\(\text{III}\)T binding characteristics to locust neuronal membranes have been shown to be similar to those described for the \(\alpha\)-scorpion toxins Lqq V and AAh II (I over et al., 1978) on rat brain sodium channels, except that its binding is not dependent on membrane potential (Gordon and Zlotkin, 1993). Thus, the receptor site for Lqh\(\text{III}\)T on insect sodium channels has been considered to be homologous to receptor site 3 in vertebrate sodium channels (Eitan et al., 1990; Gordon and Zlotkin, 1993; Zlotkin et al., 1994).

We have compared the toxic activity and binding interactions of various scorpion toxins on mammals and insects. Three different neuronal sodium channel preparations have been chosen: rat brain synaptosomes, which are the most studied; and two different insect central nervous system membranes, locust and cockroach neuronal membranes, which served for neurotoxin binding studies in insects. Cockroach axons have been used as the main preparation for physiological effects of neurotoxins in insects. We have tested binding interactions of several different scorpion toxins, which reveal peculiarity in their toxic and pharmacological behavior, to get some insight into their possible receptor sites on sodium channels.

The results of our comparative study suggest that scorpion toxins affecting inactivation of sodium current may be divided into several different groups according to their mammal versus insect activities, each possessing its distinct receptor site on sodium channels. The \(\alpha\)-toxin receptor site on sodium channels is suggested to be a macrosite, which includes the Lqh\(\text{III}\)T/Lqq \(\text{III}\) receptor site that partially overlaps with both ATX II and AAh II receptor sites. The other groups of \(\alpha\)-like scorpion toxins are suggested to bind to distinct receptor sites on both rat brain and insect sodium channels, which interact with receptor site 3. A cluster of receptor sites that preferentially bind scorpion toxins affecting current inactivation is suggested to be present on both rat brain and insect sodium channels. ATX II receptor site is suggested to be included in this cluster.

| Site | Toxin | Effect |
|------|-------|--------|
| 1    | Tetrodotoxin Saxitoxin \(\mu\)-Conotoxin | Inhibition of ion conductance |
| 2    | Veratridine Batrachotoxin Acobamine Grayanotoxin | Persistent activation |
| 3    | Scorpion \(\alpha\)-toxins Sea anemone toxin | Inhibit inactivation; enhance persistent activation |
| 4    | Scorpion \(\beta\)-toxins | Shift voltage dependence of activation |
| 5    | Brevetoxins Ciguatoxins | Repetitive firing; shift voltage dependence of activation |
| 6    | \(\delta\)-Conotoxins \(\delta\)TxVI | Inhibit inactivation |

| Unidentified sites | Goniopora coral toxin Conus striatus toxin | Inhibit inactivation |

Toxins bound by neurotoxin receptor sites 1–6 and additional unidentified sites on vertebrate sodium channels

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1. D. Gordon and M. Fainzilber, unpublished results.

2. D. Gordon and M. Fainzilber, unpublished results.
Neuronal Membrane Preparations

Rat brain synaptosomes were prepared from adult albino Wistar rats (about 300 g, laboratory-bred), according to the procedure of Dodd et al. (1987). Insect synaptosomes (P. lusoria) were prepared from the central nervous system of adult locusts (Locusta migratoria) and cockroach (Periplaneta americana) according to established methods (Gordon et al., 1990, 1992; Moskowitz et al., 1994). All buffers contained a mixture of proteinase inhibitors composed of: phenylmethylsulfonyl fluoride (50 μg/ml), pepstatin A (1 μM), iodoacetamide (1 mM), and 1,10-phenanthroline. Membrane protein concentration was determined using a Bio-Rad protein assay, with BSA as standard.

Neuromuscular Junctions

Neuromuscular junctions were prepared from adult male cockroaches (P. americana) and locusts (Gordon et al., 1983). As a result, higher concentrations of toxins are required to detect their activity. Normal physiological saline had the following composition (in mM): NaCl 200, KCl 3.1, CaCl2 1.0, MgCl2 5.0, HEPES buffer 1, pH 7.2. Experiments were performed at 19–21 °C. When necessary, potassium current was suppressed by 0.5 mM 3,4-diaminopyridine.

Electrophysiological Experiment

Rat neuronal cells—cultured rat cerebellar granule neurons in 45-mm dishes (Costar) were used at day 7-14 of culture for electrophysiological experiments, which were performed at room temperature (20-22 °C) with the single-electrode whole-cell voltage clamp technique, using suction pipettes ranging from 2 to 4 megohms. The Na+ gradient was reversed to eliminate variability in space clamp, allowing recordings of highly reproducible peak currents (Numann et al., 1991; Dargant et al., 1994). The external solution contained 90 mM choline chloride, 15 mM tetraethylammonium chloride, 1 mM MgCl2, 1.5 mM CaCl2, 5 mM glucose, 30 mM HEPES (pH adjusted to 7.3 with NaOH), 1 mM BSA. The internal solution contained 100 mM NaF, 30 mM NaCl, 20 mM CsF, 0.2 mM CdCl2 and 5 mM HEPES (pH adjusted to 7.3 with CsOH). Currents induced by a 50-ms depolarizing test pulse were recorded using Axon Instrument Axopatch 200A patch-clamp amplifier, low-pass-filtered at 2 kHz with an 8-pole Bessel filter, and sampled at 20 kHz using a 12-bit ADC (Labmaster TM 40, Scientific Solution, Foster City, CA). Capacitance and leak currents were subtracted from active currents using a P4 protocol (Bazanelli and Armstrong, 1977). Data acquisition and analysis were controlled by pCLAMP software (Axon Instrument).

In Vivo Animal Bioassays

Fifty percent lethal doses (LD50) were established according to Behrens and Karber (1935). The anti-mammal activity was evaluated in mice by subcutaneous or intracerebroventricular injections into C57 BL/6 mice (20 ± 2 g). Anti-insect activity was evaluated in cockroaches (Blatella germanica, 50 ± 2 mg) using an automatic microsyringe from the Burker Manufacturing Co. (Rickmansworth, United Kingdom).

RESULTS

Correlation between Toxicity and Binding of Scorpion Toxins—Table II reveals a perfect correlation between their toxicity and binding of AaH II on rat brain sodium channels. Receptors related to these toxins (belonging to structural groups III and IV) were shown to have much weaker toxic effects on mice, as compared to the AaH II (Table II). Out of these less active toxins on mice, only Lqq IV and Lqq III have been shown to satisfy the main criterion used for α-scorpion toxin definition (Coureaud et al., 1982), namely competition for AaH II binding in rat brain synaptosomes, although at higher concentration (Table II and Fig. 2, upper inset).

Examination of the correlation between toxicity to mice and the toxins' potency in competing for binding of AaH II on rat brain sodium channels reveals a certain peculiarity (Table II). As a result, higher concentrations of toxins are required to detect their activity. Normal physiological saline had the following composition (in mM): NaCl 200, KCl 3.1, CaCl2 1.0, MgCl2 5.0, HEPES buffer 1, pH 7.2. Experiments were performed at 19–21 °C. When necessary, potassium current was suppressed by 0.5 mM 3,4-diaminopyridine.

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Examination of the correlation between toxicity to mice and the toxins' potency in competing for binding of AaH II on rat brain sodium channels reveals a certain peculiarity (Table II and Fig. 2). The graphic presentation of this correlation (Fig. 2) suggests that toxins related to α-scorpion toxins comprise at least four groups: 1) the "classical" α-toxins, such as AaH I–III and Lqq V (belonging to structural groups I and II), which reveal a perfect correlation between their toxicity and binding inhibition properties in rat brain (Fig. 2, lower inset); 2) Lqq IV, which exhibits a lower toxicity (54-fold less toxic than AaH II) and inhibits AaH II at significantly higher concentrations than other α-toxins (Table II) (this toxin holds an intermediate position on the correlation curve; Fig. 2); 3) Lqq III, which is...
2200-fold less toxic to mice than AaH II, and inhibits the binding of AaH II at very high concentration (Fig. 2 and Table II) (Lqq II is highly homologous to the anti-insect α-toxin LqhαIT(see Fig. 1) and holds a unique place in this correlation curve); 4) toxins belonging to structural group III, represented by Bom III and Bom IV, which are toxic to mice but do not compete for AaH II binding and consequently do not reveal any correlation between these parameters (Table II). The peculiarity of these toxins prompt us to re-examine their toxicity and pharmacology by a comparative approach, using sodium channels from rat and insect central nervous system.

Electrophysiological Activity of α-Like Scorpion Toxins—The toxins presented in Table II intoxicate mice (by intracerebroventricular injection) in a similar manner, leading to paralysis and death at different doses (see Table II). To examine whether the two peculiar scorpion toxins, Bom III and Bom IV, belong to the same category of neurotoxins as the α-scorpion toxin group, namely are able to induce inhibition of sodium current inactivation, we tested their physiological effects on cultured neuronal cells from rat brain (Fig. 3) and on an isolated axon from

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**Table II**

| Structural group | Toxin   | Binding inhibition (IC₅₀) | Toxicity i.c.v. (LD₅₀) | IC₅₀/IC₅₀ AaH II | LD₅₀ i.c.v./LD₅₀ AaH II |
|------------------|---------|--------------------------|------------------------|------------------|------------------------|
| I                | AaH I   | 4.5² | 10 | 22.5 | 20 |
| I                | AaH III | 3.0² | 7.0 | 15 | 14 |
| I                | AaH II  | 0.2² | 0.5 | 1 | 1 |
| II               | Lqq V   | 1.0² | 2.5 | 5 | 5 |
| III              | Lqq III | 700² | 1100² | 3500 | 2200 |
| III              | Bom III | >1000² | 23.0 | — | 43 |
| III              | Bom IV  | >1000² | 23.0 | — | 43 |
| IV               | Lqq IV  | 49 ± 21² | 27.0 | 243 | 54 |

² Competition for ¹²⁵I-AaH II binding in rat brain synaptosomes.

See Martin-Eauclaire et al. (1992) for references. i.c.v., intracerebroventricular.

Kopeyan et al. (1985).

Kopeyan et al. (1993).

No apparent competition (at 1 µM) for ¹²⁵I-AaH II binding.

Fig. 2 (upper inset).

No IC₅₀ available (see footnote e).

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**Fig. 1.** Comparison of scorpion toxin amino acid sequences classified according to their structural homology. A, the structural group is marked on the left (I–IV). The sequences were aligned for maximum similarity by eye inspection. B, a table presenting the percentage of identical and conserved (in brackets) residues calculated for maximum homology between each pair of protein sequences.
cockroach central nervous system (Fig. 4).

In cerebellar granule cells under voltage-clamp conditions, extracellular addition of 0.5 nM AaH II induced a classical α-scorpion toxin effect, namely a slight, progressive decrease of the Na⁺ peak current accompanied by an evident slowing of inactivation time course (Fig. 3A). In the same experimental conditions, the main effect induced by Bom III and Bom IV was slowing down the decline of Na⁺ currents (Fig. 3, B–E), similarly to the one observed with AaH II (Fig. 3A), but Bom IV affects the sodium conductance at higher concentration (Fig. 3, D and E). The higher concentration of Bom III and IV needed for maximal effects are in concert with the lower activity of these toxins on mice (see Table I). Steady-state inactivation curves obtained before and after addition of 0.5 nM AaH II or 25 nM Bom IV showed a notable shift to the left, to more hyperpolarized potentials for both AaH II and Bom IV (Fig. 3, F and G). However, examination of the current changes induced by AaH II compared to Bom toxins reveals that the latter affect the Na⁺ conductance in an additional manner, namely slowing the activation kinetics. Although we did not quantitatively analyzed the activation kinetics of the sodium currents, they appear to be slowed by both Bom toxins (Fig. 3, C and E) but not by AaH II (Fig. 3A), as indicated by the rising phase and time-to-peak current. Unlike AaH II, Bom IV reduced the slope of the activation curve (Fig. 3G). These discrepancies between the two groups of toxins could indicate that Bom IV may modify additional properties of the channel. Since plural mechanisms may account for slowing the decline of sodium current, including reopening of channels that are closed along the inactivation pathway as well as those with slowed or modified activation, further experimentation would be necessary to determine the exact nature of the mechanism involved. Thus, both Bom III and IV induce an apparent phenomenologically similar effect to that of the α-scorpion toxin AaH II on the slowed decline of sodium currents in mammalian neurons, but reveal difference on the activation kinetics. The latter may suggest that the Bom toxins exert their effects by binding to distinct receptor site on the sodium channels.

The similarity in macroscopic effects on the decline of sodium currents has been further exemplified on cockroach axonal preparation (Fig. 4). AaH II and LqhIT were demonstrated to induce prolongation of action potentials in an isolated giant axon of the cockroach due to inhibition of the sodium current turning off (Pelhate and Zlotkin, 1982; Eitan et al., 1990). Bom III affects the cockroach axonal membrane in a similar way (Fig. 4A) at concentrations similar to those needed for insect-selective toxins in this preparation (Eitan et al., 1990; Pelhate and Zlotkin, 1982). In voltage clamp conditions, 10-fold lower concentration of Bom III (62.5 nM) inhibits the inactivation of the sodium current, with no effect on the potassium conductance (Fig. 4B), similar to the effect of α-scorpion toxins in vertebrate and insect preparation (Duval et al., 1989; Wang and Strichartz, 1983; Eitan et al., 1990; Pelhate and Zlotkin, 1982).

Thus, the scorpion toxins listed in Table III reveal some similar electrophysiological phenomenon on sodium conductance (inhibition of sodium current inactivation) in both mammal and insect excitable membranes, as described previously for ATX II and other polypeptide neurotoxins derived from Conus snail and coral venom (Catterall and Beress, 1978; Goni et al., 1986, 1987; Hasson et al., 1993; Fainzilber et al., 1995). Such effects may be a result of many different kinetic modifications produced by different specific action, following binding of the chemically different toxins to distinct receptor sites on sodium channels (see Goni et al., 1986, 1987) and Fainzilber et al. (1994, 1995). Moreover, the Bom toxins have been shown to alter, in addition, the activation kinetics (Fig. 3, C and E). Accordingly, Bom III and IV do not interact with receptor site 3 on vertebrate sodium channels, as indicated by their inability to inhibit the binding of AaH II in rat brain synaptosomes (Table II and Fig. 2, upper inset). For the convenience of discussion and to be consistent with previous classification (Vargas et al., 1987; Martin-Eauclaire et al., 1992), we suggest to term them as α-like scorpion toxins. α-Like toxins include neurotoxins that are toxic to vertebrates, and induce inhibition of sodium current inactivation by occupying a different receptor site from that of α-scorpion toxins.

Competitive Inhibition of LqhIT Binding on Cockroach and Locust Sodium Channels—The activity of the α-like scorpion toxins on cockroach axon indicates that they might be toxic to insects. Using the cockroach (B. germanica) bioassay, Bom III and IV reveal 10-fold and about 4-fold lower toxicity than LqhIT, respectively (200, 75, and 18 ng/g body weight, respectively; see Table III). The toxicity of Bom III and IV to mice and insects is very similar, as compared to the insect/mammal toxicity of LqhIT (3.3-fold more toxic to insects; Table III).

The activity of these α-like toxins on both mammals and insects allowed the examination of their interaction with LqhIT binding on insect sodium channels. LqhIT shares 53–77% identity with other α-scorpion toxins affecting mammals (Fig. 1B), but it reveals high toxicity to insects (Eitan et al., 1990; Table III).

Comparative binding study of LqhIT in the two insect neuronal membrane preparations, from locust and cockroach central nervous system (Fig. 5) revealed that the affinity of 125I-

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**Fig. 2.** Correlation between the toxicity to mice (intracerebroventricular) of different scorpion toxins and the concentration required to inhibit the binding of 125I-AaH II to rat brain synaptosomes (IC₅₀), relative to the toxicity and IC₅₀ of AaH II. The data are from Table II. Abscissa, LD₅₀ values of each toxin divided by the LD₅₀ of AaH II; ordinate, IC₅₀ values of each toxin divided by IC₅₀ of AaH II. Upper inset, competitive inhibition curves of several toxins for 125I-AaH II binding to rat brain synaptosomes. The results are presented as percent of AaH II maximal specific binding with no competitor. Nonspecific binding, measured in the presence of 200 nM AaH II, was subtracted from all data points. Lower inset, enlargement of the correlation curve (main panel, lower left corner), presenting the correlation between toxicity and binding inhibition of some classical α-scorpion toxins.
Lqh\textsubscript{1}IT to cockroach synaptosomes is about 10–15-fold higher than its binding affinity to locust neuronal membranes ($K_d = 0.03 \pm 0.01 \text{ nM}$ in cockroach and $0.46 \pm 0.14 \text{ nM}$ in locust; Fig. 5, panels A and B (insets) and panel D). This is the highest affinity described so far for an $\alpha$-scorpion toxin to any sodium channel preparation (see Table III). Lqq\textsubscript{1}IT, which possesses only three amino acid substitutions as compared to Lqh\textsubscript{1}IT, reveals similar $I_{C_{50}}$ to that of Lqh\textsubscript{1}IT on cockroach sodium channels (Fig. 5C and Table III). Thus, these two homologous toxins are suggested to share the same receptor site on insect sodium channels. Depolarization of the membrane by osmotic lysis does not affect Lqh\textsubscript{1}IT binding to cockroach (data not shown), confirming the independence of the binding on membrane polarization, as described previously in locust (Gordon and Zlotkin, 1993).

The $\alpha$-toxins highly active on mammals (see Table I) are able to inhibit Lqh\textsubscript{1}IT binding on both cockroach and locust membranes, but at concentrations higher by about 3–4 orders of magnitude than Lqh\textsubscript{1}IT (Fig. 5, A and B, and Table III). In accordance, the toxicity of the classical $\alpha$-toxins to insects is very low (Table III). The inhibitory potency of the classical $\alpha$-toxins in each insect neuronal preparation is comparable ($I_{C_{50}}$ around 1 \text{ mM} in locust and in the range of $60–325 \text{ nM}$ in cockroach; Fig. 5, A and B, and Table III), supporting the notion that the $\alpha$-mammalian toxins bind to a homologous, perhaps overlapping receptor site on insect sodium channels, but with a much weaker affinity, as compared to Lqh\textsubscript{1}IT.

The toxins that reveal no inhibition on AaH\textsubscript{1}IT binding in rat brain sodium channels, BomIII and Bom IV, but were shown to be active on mice (Bom III and Bom IV are 12.5 and 3.5 times less active on mice than AaH\textsubscript{1}IT by subcutaneous injection, respectively; Table III), are able to compete for Lqh\textsubscript{1}IT binding at nanomolar concentrations (Fig. 5, A and B, and Table III). The relative higher toxicity of Bom IV as compared to Bom III in insects is accompanied by lower $I_{C_{50}}$ values in both cockroach and locust (Fig. 5E and Table III).

The intermediate position of Lqq\textsubscript{4}IT, suggested by the correlation of toxicity and binding in mammals (Fig. 2 and Table II), is supported by its very low toxicity to insect (LD$_{50}$ in the range of the classical $\alpha$-mammalian scorpion toxins; see Table III). However, Lqq\textsubscript{4}IT competently inhibits the binding of Lqh\textsubscript{1}IT in both locust and cockroach at moderate concentrations (Fig. 5D). Unlike the increase in $I_{C_{50}}$, detected between cockroach and locust for Lqh\textsubscript{1}IT and ATX\textsubscript{2}IT inhibition (Fig. 5, C and D), the $I_{C_{50}}$ of Lqq\textsubscript{4}IT is lower in locust (Fig. 5D and Table III), in contrast to all the other toxins (Table III), suggesting that this toxin binds to a different receptor site than Lqh\textsubscript{1}IT.

ATX\textsubscript{2}IT has been shown to compete for $\alpha$-scorpion toxins on binding to both rat brain (Couraud et al., 1978; Catterall and Beress, 1978) and locust (Gordon and Zlotkin, 1993) sodium channels. Accordingly, ATX\textsubscript{2}IT inhibits at low concentration ($I_{C_{50}} = 0.53 \pm 0.03 \text{ nM}$) the binding of Lqh\textsubscript{1}IT to cockroach sodium channels (Fig. 5C), suggesting similarity between their receptor sites.

Allosteric Modulation of Lqh\textsubscript{1}IT Binding by Veratridine and Brevetoxin—The binding of Lqh\textsubscript{1}IT to locust neuronal membrane has been demonstrated to be cooperatively increased by veratridine, whereby 100 \text{ mM} veratridine increase both the affinity and capacity of Lqh\textsubscript{1}IT receptor sites (Gordon and Zlotkin, 1993). Most recently we have shown that brevetoxin PbTx-1 causes a 1.4–1.8-fold increase in Lqh\textsubscript{1}IT binding on locust sodium channels (Cestele et al., 1995; see Fig. 6), resembling the increase observed by veratridine. The significant differences in affinity of Lqh\textsubscript{1}IT observed between locust and
cockroach sodium channels (Fig. 5 and Table III) prompt us to compare the allosteric modulations observed recently on Lqh\textsubscript{III} binding on locust sodium channels (Cestele \textit{et al.}, 1995).

In contrast to the situation in locust, neither veratridine nor brevetoxin reveals any significant effect on Lqh\textsubscript{IIII} binding on cockroach sodium channels (Fig. 6). To further examine this discrepancy, we tested the effects of concurrent presence of both lipid-soluble sodium channel activators on the binding of Lqh\textsubscript{IIII} in the two insect neuronal membranes. The effect of veratridine is further enhanced by 2-fold in the presence of 20 nM brevetoxin (over the combined effects of veratridine and brevetoxin; Fig. 6A). Brevetoxin (at 20 nM) alone induces 121 \pm 10\% increase in Lqh\textsubscript{IIII} binding (see Fig. 6B). Thus, veratridine enhances in a synergic manner the binding of Lqh\textsubscript{IIII} at the brevetoxin-modified receptor site in locust sodium channels (Fig. 6A). The synergic effect of veratridine in the presence of 20 nM brevetoxin on Lqh\textsubscript{IIII} binding may be explained by the increase in concentration of Lqh\textsubscript{IIII} receptor sites previously observed in the presence of 100 \mu M veratridine (Gordon and Zlotkin, 1993). All the available receptor sites for Lqh\textsubscript{IIII} are, in turn, modified to a higher affinity state by PbTx-1, resulting in an apparent cooperative increase in Lqh\textsubscript{IIII} binding (see Cestele \textit{et al.} (1995) and Fig. 6B). The effect of brevetoxin on the binding of Lqh\textsubscript{IIII} has been measured in the presence of saturating concentration (100 \mu M) of veratridine. As is demonstrated in Fig. 6B, the effect of PbTx-1 on the veratridine increase in Lqh\textsubscript{IIII} binding is additive (Fig. 6B). Brevetoxin was shown to increase the affinity of Lqh\textsubscript{IIII} with no effect on the receptor concentration (Cestele \textit{et al.}, 1995). No effect is detected on the binding of Lqh\textsubscript{IIII} on cockroach sodium channels under any conditions or combinations tested (Fig. 6). The differences in allosteric modulation of Lqh\textsubscript{IIII} binding indicate the presence of structural differences between locust and cockroach sodium channels.

**DISCUSSION**

The present study examines, for the first time, the interaction of different scorpion neurotoxins, all affecting sodium current inactivation and toxic to mammals, with \textalpha-like scorpion toxin receptor sites on sodium channels in mammals versus insects. Our results suggest that \textalpha- and \textalpha-like (see “Results”) scorpion toxins may be divided into several groups, according to their activity on mammalian and insect sodium channels. Each group may occupy a distinct receptor site on sodium channels and form together a putative macrosite (see below and Fainzilber \textit{et al.} (1995)). This macrosite, which is composed of receptor sites for scorpion toxins that inhibit sodium current inactivation, is very similar on insect and rat brain sodium channels, in spite of the structural and pharmacological differences between them. The sea anemone toxin ATX II is also suggested to bind within this macrosite.

Several Groups of \textalpha-like Toxins Are Revealed by Activity in Vivo and in Vitro—The \textalpha- and \textalpha-like scorpion toxins are classified into several groups, according to their relative activity on mammals and insects. The first group comprise the classical \textalpha-toxins highly active on mammals, AaH I, AaH II, AaH III, and Lqq V. These toxins demonstrate the highest affinity to vertebrate sodium channels and the lowest affinity to insect neuronal membranes (Table III, Fig. 5). The second group is represented by Lqq IV, shown to be very weakly active on insects; however, it is 54-fold less effective on mammals than AaH II (by intracerebroventricular injection, Table II). This toxin have been demonstrated to competitively inhibit the binding of AaH II to rat brain synaptosomes, as well as the binding of Lqh\textsubscript{IIII} to insect sodium channels. Lqq IV may represent an intermediate scorpion toxin group, which binds with moderate affinities to both mammal and insect sodium channels but express its toxic activity mainly on mammals.

The third group consists of Bom III and IV, which are shown to be active on both insect and mice and compete at nanomolar concentrations for the binding of Lqh\textsubscript{IIII} to insect sodium channels. Bom III and IV are substantially similar active on mice and on insects (Table III) and inhibit sodium current inactivation in both rat neuronal cells (Fig. 3) and in cockroach axon (Fig. 4). The fourth group consists of Lqq III and Lqh\textsubscript{IIII}. These two homologous toxins demonstrate the highest affinity to insects, as opposed to the very low affinity to rat brain sodium channels (Table III). The activity of Lqh\textsubscript{IIII} is very similar to that of Lqq III, but it reveals slightly higher specificity to insects versus mammals, which is also reflected by its lower ability to inhibit the binding of AaH II in rat brain membranes (as compared to Lqq III, Table III). Thus, Lqh\textsubscript{IIII} and Lqq III are considered anti-insect \textalpha-scorpion toxins.

\textalpha Scorpion Toxins Receptor Sites Are Homologous But Not Identical on Mammal and Insect Sodium Channels—The existence of receptor site 3 (Catterall, 1980, 1986), which binds the classical \textalpha-scorpion toxins on mammalian sodium channels, could not be demonstrated on insects by direct binding studies, since no specific binding of \textalpha\textsubscript{25}G\textsubscript{AaH II} has been detected in locust neuronal membranes (Gordon \textit{et al.}, 1984), probably due to the very low affinity of this anti-mammal toxin to insects. It was demonstrated that high doses of AaH II were completely
Differences in α-Scorpion Toxin Receptor Sites

TABLE III
Activity of different α- and α-like scorpion toxins in insects and mammals

| Structural group | Toxin       | IC50 a (nM) | IC50 (nM) | Toxicity (LD50) |
|------------------|-------------|-------------|-----------|-----------------|
|                  | Locust central nervous system | Cockroach central nervous system | Rat brain | To mice | To insect |
|                  |             |             |           | s.c. pmole/g body weight | B. germanica pmole/g body weight |
| Sea anemone      | ATX II      | 5.9 ± 1.2d  | 0.53 ± 0.03 | 200a           | 22f  | 2.5 |
|                  |            |             |           |                 |       |       |
| I                | AaH I       | 2210 ± 1400 | 160.5 ± 59.6 | 4.5a           | 2.4a  | 276 |
|                  | AaH III     | 1360 ± 260  | 325.6 ± 120.2 | 3.0a           | 3.4a  | 1490 |
| II               | AaH II      | >>1000h     | 58.8 ± 12.6  | 0.2-0.3k        | 1.7a  | 897 |
| II               | Lqq V       | 500-1000    | 120.4 ± 10.1 | 1.0a           | 3.4a  | 2317 |
| III              | LqhαααT     | 0.2 ± 0.1   | 0.02 ± 0.01  | >>1000h         | 8.3   | 2.5 |
| III              | Lqq III     | ND          | 0.03 ± 0.01  | 700k           | 6.9f  | 8.3f |
| III              | Bom III     | 39.1 ± 15.9 | 29.3 ± 8.1   | >>1000αααL     | 19.7a | 52.6 |
| III              | Bom IV      | 14.7 ± 5.7  | 4.6 ± 1.6    | >>1000αααL     | 5.5m  | 19.7 |
| IV               | Lqq IV      | 15.0 ± 4.6  | 77.8 ± 14  | 49 ± 21        | 9.6l  | 1230.0 |

a Competition for 125I-LqhαααT binding in insect central nervous system membranes.

b Competition for 125I-AaH II binding in rat brain synaptosomes.

c Subcutaneous injection.

d Gordon and Zlotkin, (1993).

e Couraund et al. (1982).

f Barhanin et al. (1981).

g See Martin-Eauclaire et al. (1992) for references.

h No significant inhibition was detected at 1 µM toxin.

i Fig. 2 (upper inset).

j Not determined.

k Kopeyan et al. (1993).

l Kopeyan et al. (1985).

m Vargas et al. (1987).

inactive when injected to fly larvae (Zlotkin et al., 1971, 1972) and LD50 to cockroach is achieved at doses 350 times higher than LqhαααT (Table III), establishing the anti-mammal specificity of AaH II. Our results demonstrate that the highly active toxins on mammals, like AaH II, possess a receptor site also on insect sodium channels, as the classical α-scorpion toxins are able to compete for LqhαααT binding in insect neuronal membranes (Fig. 5 and Table III). The inhibition of sodium inactivation by high concentration of AaH II in an isolate axon of a cockroach (Pélathe and Zlotkin, 1982) indicates that the α-scorpion toxin binding on insect sodium channels is pharmacologically active and its receptor site might be homologous to receptor site 3 on rat brain sodium channels.

The positive cooperative interaction observed between veratridine and α-scorpion toxins (Lqq V and AaH II) on rat brain sodium channels (Ray et al., 1978; over et al., 1980; Cestele et al., 1995), comparable to the cooperativity detected between veratridine and LqhαααT binding on locust sodium channel (Fig. 6) (Gordon and Zlotkin, 1993; Cestele et al., 1995), further support the similarity in the α-scorpion toxins receptor sites on insect and rat brain sodium channels.

The low affinity revealed by the α-mammal toxins on insects is in contrast of the high affinity observed on rat brain sodium channels, indicating differences in receptor site structures on mammal versus insect sodium channels. However, the complete inhibition of LqhαααT binding, especially on cockroach sodium channels and the shift in affinity detected in cockroach versus locust (which correspond to a concentration change of about 1 order of magnitude between LqhαααT binding inhibition in cockroach as compared to locust neuronal membranes; Fig. 5 and Table III), which conforms with the shift in affinity of LqhαααT on these insect sodium channels (Fig. 5D), supports that the competition may result from binding to homologous, similar or overlapping receptor sites.

The sea anemone toxin ATX II and the α-scorpion toxins AaH II and Lqq V have been shown to compete on binding to vertebrate excitable cells and to have similar pharmacological and electrophysiological activities (COURAUND ET AL., 1982; Jover et al., 1978; Catterall and Beress, 1978; Salgado and Kem, 1992). On this basis they were considered to bind to a common receptor site on mammalian sodium channels. The competition of ATX II for α-mammal toxins binding on rat brain as well as for LqhαααT binding on insect sodium channels (Gordon and Zlotkin, 1993) (Fig. 5C and Table III) strongly suggests that these α-scorpion toxins, having different specificity to mammal versus insect sodium channels, may bind to closely related receptor sites, which might also (at least partially) overlap with ATX II in the different sodium channel subtypes.

Our results demonstrate that ATX II and AaH II reveal inverse affinities toward insect and mammal sodium channels, as detected by their competitive inhibition on LqhαααT binding: the IC50 of AaH II on insect sodium channels is increased by about 2 orders of magnitude, in contrast to a similar decrease in IC50 of ATX II (Table III). These contrary affinities may indicate that at least some of the recognition sites that are involved in the high affinity binding of these two different toxins might be chemically different on mammal and insect sodium channels. The comparable shift in IC50 values between ATX II and LqhαααT in cockroach versus locust (Fig. 5C) conforms that the receptor site for ATX II is highly similar to that of LqhαααT on the two insect sodium channels, but different (at least in part) from the one of AaH II. The membrane potential-independent binding of LqhαααT is comparable to the ability of ATX II to compete in a potential-independent manner with LqhαααT for binding in locust neuronal membranes (Gordon and Zlotkin, 1993), further supporting the notion that ATX II receptor site might be very similar to that of LqhαααT on insect sodium channels. These and previous (Catterall and Beress, 1978; Catterall and Coppersmith, 1981; Frelin et al., 1984; Renaud et al., 1986) results suggest that ATX II and α-scorpion toxins may not bind to identical receptor site on mammalian
sodium channels, but rather to overlapping (at least in part) sites. The specificity and differences in the insect versus mammal activity of the $\alpha$- and $\alpha$-like scorpion toxins may be attributed, in part, to structural differences among both the toxins and the homologous receptor sites on insect and mammalian sodium channels. Clarification of the structural basis for selectivity in action of toxins will require three-dimensional structural knowledge of the toxins coupled with molecular localization of the amino acids directly interacting with the recognition points within the receptor site structure and are important areas of future studies.

Other Receptor Sites Are Revealed by $\alpha$-Like Toxin Binding—The expanding number of selective toxin ligands with similar apparent physiological activity (inhibition of sodium current inactivation) urged us to examine their interactions with the known probes of receptor site 3 on several sodium channel preparations. However, binding experiments may reveal competitive inhibition between toxins that do not bind to the same or overlapping receptor sites, as have been demonstrated for a number of toxins that compete on binding, but by various criteria cannot share precisely the same binding sites (Adams and Olivera, 1994; Gordon et al., 1992; Fainzilber et al., 1994, 1995). Such competition may result from steric interference (hindrance) between toxin molecules upon binding to their distinct receptor sites. Electrostatic repulsion between highly charged molecules may further contribute to this interference. The interference may be related to the three-dimensional structure and flexibility of a toxin, and to the surface of its receptor site. As a practical approximation, we suggest to refer to a toxin “binding area,” which represents the surface of projection of a toxin bound on the sodium channel surface. Such a binding area may be largely responsible for the apparent competitive inhibition observed in binding studies.

**Fig. 5.** Competitive inhibition curves for $^{125}$I-Lqh$\alpha$IT binding by $\alpha$- and $\alpha$-like scorpion toxins. Insect neuronal membranes were incubated with $^{125}$I-Lqh$\alpha$IT and increasing concentrations of the other toxins (as described under “Experimental Procedures”). The amount of $^{125}$I-Lqh$\alpha$IT bound is expressed as the percentage of the maximal specific binding in the system without additional toxins. All curves were analyzed by LIGAND program, and IC$_{50}$ values were calculated using DRUG analysis. The lines are drawn by hand. A, cockroach neuronal membranes (1 $\mu$g of protein) were incubated with 30–60 pM of the labeled toxin. Inset, Scatchard analysis of a saturation binding curve. The membranes were incubated for 1 h at 22 °C with increasing concentrations of $^{125}$I-Lqh$\alpha$IT ("hot" saturation), as described under “Experimental Procedures.” Equilibrium binding constants, obtained by the computer program analysis (LIGAND) were as follows: $K_d = 32.9 \pm 8.2$ pm; $B_{max} = 1.85 \pm 0.62$ pmol/mg protein. There was a very good agreement between the binding constants obtained by “cold” and “hot” saturation curves (0.03 $\pm$ 0.01 nM, $n = 4$). B. Locust neuronal membranes (15 $\mu$g of protein) were incubated with 0.1 nM of $^{125}$I-Lqh$\alpha$IT. Inset, Scatchard analysis of a "cold" saturation binding curve (see "Experimental Procedures"). The equilibrium binding constants, obtained as in A, were $K_d = 0.46 \pm 0.34$ nM; $B_{max} = 0.33 \pm 0.05$ pmol/mg. The IC$_{50}$ values are presented in Table III. C–E, comparison between $^{125}$I-Lqh$\alpha$IT binding inhibition by various neurotoxins on cockroach (black symbols) and locust (empty symbols) neuronal membranes. Note the shifts in the competition curves obtained by the different inhibitors in locust versus cockroach membranes (see text). The IC$_{50}$ values are presented in Table III.
Receptor Site of Lqq IV—Examination of competitive binding interactions of Lqq IV with Lqh IT in locust and cockroach neuronal membranes revealed that Lqq IV is able to inhibit the binding of Lqh IT in insect sodium channels; however, the IC$_{50}$ for Lqq IV is 5-fold higher on cockroach than on locust (Fig. 5E and F, Table III), in contrast to the situation with Lqh IT and ATX II (Fig. 5, C and D). These data suggest that Lqq IV binds to a different receptor site that Lqh IT on insect sodium channels. The very weak activity of Lqq IV in insects, about 500-fold higher L.D$_{50}$ than Lqh IT (Table III), may indicate that the binding of Lqq IV results in very limited functional activity, suggesting a very low efficacy of this toxin action in cockroaches (Table III).

The lack of correlation between toxicity and IC$_{50}$ of Lqq IV in mammals (Tables II and III) suggest that this structurally different toxin (Fig. 1) may bind to a distinct receptor site also on rat brain sodium channels. The relatively lower toxicity ratio as compared to the IC$_{50}$ ratio (Table II) suggest that Lqq IV's relatively weak competitive inhibition on AaH II binding is due to a steric interference between their binding areas, suggesting the presence of distinct receptor site for each. Presently, no direct binding data are available on Lqq IV, making it difficult to relatively localize its binding area. It is suggested to occupy a closely related area to those of AaH II and Lqh IT.

Receptor Site for Bom III and IV—Bom III and IV, shown to induce similar inhibition of sodium current inactivation on rat brain neurons (Fig. 3) as well as on cockroach axon (Fig. 4), are the most peculiar in their action. These toxins were shown to be toxic to mice both by intracerebroventricular and by subcutaneous injection, but reveal no competition with AaH II binding on rat brain synaptosomes (Table II). This may result either from binding to different receptor sites than AaH II on the same sodium channels or from binding to different sodium channel subtypes. It is also possible that Bom III binds and acts on sodium channel subtypes that are not abundant in rat brain synaptosomes, thus explaining the lack of competition with AaH II in this preparation. At present, we cannot discriminate between these possibilities, and further study is required to clarify this phenomenon.

In contrast to the lack of interaction between AaH II and Bom III and IV on rat brain synaptosomes, the binding of Lqh IT to insect sodium channels is inhibited by nanomolar concentrations of these toxins (Fig. 5). The two toxins reveal similar IC$_{50}$ values in locust and cockroach, in contrast to the marked shift in IC$_{50}$ detected with other toxins (Fig. 5E and Table III). These results suggest that Bom III and IV may bind to a separate receptor site than Lqh IT on insect sodium channels. Unlike the situation in rat brain synaptosomes, the receptor sites for α-scorpion toxins and Bom III and IV must be present on the same insect sodium channel population. Bom III receptor site (or binding areas) may partially overlap or be in a close proximity to that of Lqh IT.

These results may suggest that each α-like toxin group binds to a different receptor site on the sodium channel extracellular surface. The competitive binding interactions observed among the most specific α-scorpion toxins to mammal and insect sodium channels, AaH II and Lqh IT, respectively, suggest that all the α-like scorpion toxins may bind to a common area, or a macrosite, present on sodium channels in the different animal phyla, and shared also by the sea anemone toxin ATX II. Interestingly, the δ-conotoxins (Fainzilber et al., 1994, 1995) may occupy a different area, or macrosite on the sodium channel surface (see Fainzilber et al. (1995) for a tentative model). All these peptide toxins reveal similar apparent electrophysiological effect, namely inhibition of sodium current inactivation, with different specificity to various animal groups (iTx-VIA is active only on mollusk sodium channels; Lqh IT and AaH II are preferably active on insect and mammalian sodium channels, respectively).

Comparison between Locust and Cockroach Sodium Channels—Sodium conductance in cockroach axonal membranes is affected by different neurotoxins like veratridine, brevetoxin, TTX, and the α-scorpion toxins AaH II and Lqh IT in a comparable manner to that in vertebrate electrophysiological preparations (Pelhate and Sattelle, 1982; Cestele et al., 1990, 1994; Pelhate and Zlotkin, 1982; Eitan et al., 1990). Locust and cockroach sodium channels revealed some pharmacological similarity, demonstrated by comparable binding and similar mutual competitive inhibition of excitatory (AaIT) and depressant (LqhIT$_2$) insect-selective toxins, that markedly differed from the competitive interactions revealed on other insect neuronal membranes (Gordon et al., 1992; Moskowitz et al., 1994). However, structural and pharmacological differences between locust and cockroach sodium channels may be inferred from our previous (Gordon et al., 1990; Moskowitz et al., 1994; Cestele et al., 1995) and present results.

The affinity of Lqh IT is 10-fold higher in cockroach as compared to locust sodium channels (Table III, Fig. 5). Similar change in affinity is revealed by ATX II and some α-mammal scorpion toxins (Table III, Fig. 5). These differences in binding interactions observed with the various toxins indicate that the receptor sites for Lqh IT, that may be shared (or partially overlap) also by these other toxins, may differ in structure on the two insect sodium channels. The cockroach sodium channels form receptor sites with the highest affinity.
Allosteric interactions between brevetoxin, veratridine, and Lqh IT receptor sites provide further evidence for the structural differences between sodium channels in locust and cockroach central nervous system. Both lipophilic sodium channel activators (brevetoxin and veratridine) cooperatively enhance the binding of Lqh IT to locust sodium channels (Cestele et al., 1995) (Fig. 6), but reveal no effect on Lqh IT binding to cockroach sodium channels, not even under concurrent presence of both allosteric modulators (Fig. 6). It may be assumed that the receptor site for α-scorpion toxins in cockroach sodium channels is at its most favorable, high affinity conformational state for the toxin binding, and therefore it cannot be further positively modified by the allosteric interactions induced on the channel by brevetoxin and/or veratridine binding. Hence, the lack of allosteric interaction between these receptor sites on cockroach sodium channels may indicate some structural/functional difference between cockroach and locust sodium channels, perhaps also in the coupling between receptor sites of Lqh IT and brevetoxin and veratridine.

The differences revealed by α-scorpion toxin binding between locust and cockroach sodium channels are in accordance with previous biochemical examination of various insect neuronal sodium channel polypeptides. Sodium channel proteins immunoprecipitated from various insect central nervous systems revealed variations in their molecular mass and partial proteolytic peptide maps, indicating the presence of structural differences among them (Gordon et al., 1990, 1993; Moskowitz et al., 1994).

Our results suggest that the structurally related α-like scorpion toxins may be classified according to their relative specificity in action and binding to mammals and insect sodium channels. Despite the competitive binding interaction, each toxin group is suggested to bind to a distinct, different receptor site, which together may confine a large macromolecule on the extracellular surface on sodium channels. Such a macromole, which preferentially bind scorpion toxins affecting current inactivation and is shared also by ATX II, is suggested to be present on both rat brain and insect sodium channels, despite the structural and pharmacological differences among them.

Our study emphasizes the lack of structural information on the molecular level on these receptor sites. Localization of the attachment points comprising these receptor sites may shed light on the mechanism of action of toxins that modify sodium channel gating. Use of known selective sodium channel neurotoxins as pharmacological sensors for minor, subtle differences in their receptor sites on sodium channels in different animal phyla may provide a rational approach to this complex problem, and contribute to the elucidation of the structural basis for their selectivity and to structure-function relationship in sodium channels.

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