Localization of Receptor Sites for Insect-Selective Toxins on Sodium Channels by Site-Directed Antibodies†

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ABSTRACT: Site-directed antibodies corresponding to conserved putative extracellular segments of sodium channels, coupled with binding studies of radiolabeled insect-selective scorpion neurotoxins, were employed to clarify the relationship between the toxins' receptor sites and the insect sodium channel. (1) The depressant insect toxin $LqhIT_2$ was shown to possess two noninteracting binding sites in locust neuronal membranes: a high-affinity (K_{D_1} = 0.9 ± 0.6 nM) and low-capacity (B_{max_1} = 0.1 ± 0.07 pmol/mg) binding site as well as a low-affinity (K_{D_2} = 185 ± 13 nM) and high-capacity (B_{max_2} = 10.0 ± 0.6 pmol/mg) binding site. (2) The high-affinity site serves as a target for binding competition by the excitatory insect toxin AaIT. (3) The binding of $LqhIT_2$ was significantly inhibited in a dose-dependent manner by each of four site-directed antibodies. The binding inhibition resulted from reduction in the number of binding sites. (4) The antibody-mediated inhibition of [^{125}I]AaIT binding differs from that of $LqhIT_2$: three out of the four antibodies which inhibited $LqhIT_2$ binding only partially affected AaIT binding. Two antibodies, one corresponding to extracellular and one to intracellular segments of the channel, did not affect the binding of either toxin. These data suggest that the receptors to the depressant and excitatory insect toxins (a) comprise an integral part of the insect sodium channel, (b) are formed by segments of external loops in domains I, III, and IV of the sodium channel, and (c) are localized in close proximity but are not identical in spite of the competitive interaction between these toxins.

The voltage-sensitive sodium channel is an integral plasma membrane protein responsible for the rapidly rising phase of the action potential (Hille, 1984). Analysis of the amino acid sequence of the vertebrate sodium channel cDNA clones (Noda et al., 1986; Kayano et al., 1988; Catterall, 1988) enabled the construction of topological models of the channel within the plasma membrane. Amino acid sequence analysis reveals the presence of four homologous domains containing six (Noda et al., 1986; Catterall, 1986) or eight (Greenblatt et al., 1985; Guy & Seetharamulu, 1986; Guy & Conti, 1990) transmembrane segments per domain, connected by internally as well as externally located "loops" of amino acid sequences. The extracellular loops are suggested to participate in the formation of binding sites of polypeptide neurotoxins (Thomsen & Catterall, 1989). Recently, a close structural similarity between the vertebrate and insect sodium channels was revealed by analysis of the genomic DNA sequence of *Drosophila* sodium channels (Salkoff et al., 1987; Ramaswami & Tanouye, 1989; Loughney et al., 1989) and chemical characterization of the locust sodium channel (Gordon et al., 1988, 1990; Moskowitz et al., 1991).

As a critical element in nerve excitability, sodium channels serve as specific targets for various neurotoxins included in animal allomonal systems (Catterall, 1980). These toxins were shown to occupy at least four different receptor sites on the sodium channel and to serve as valuable tools for its identification and functional characterization (Catterall, 1986). Among them, the α and β scorpion venom neurotoxins are well-established markers of the voltage-dependent sodium channels (Catterall, 1986; Couraud & Jover, 1984). The α-toxins slow sodium channel inactivation and bind to receptor site 3 while the β-toxins modify activation and bind to receptor site 4 (Catterall, 1986; Couraud & Jover, 1984). The binding site of the α-toxin on the rat brain sodium channel was recently localized to extracellular segments in domains I and IV by combined use of photoaffinity labeling and sodium channel site-directed antibodies (Tejedor & Catterall, 1989; Thomsen & Catterall, 1989).

Scorpion venoms, however, contain two additional groups of neurotoxins which affect sodium conductance, namely, the excitatory and depressant insect toxins. The insect toxins resemble the α- and β-neurotoxins by being single-chain polypeptides of 60–70 amino acids cross-linked by 4 disulfide bridges (Darbon et al., 1982; Zlotkin et al., 1991), but they differ in their unique insect selectivity. Their exclusive activity to insects was demonstrated by toxicity and by electrophysiological and binding assays on in vivo and in vitro preparations derived from various animals (Zlotkin, 1986). Such selectivity may suggest unique structural or functional features of the insect sodium channels when compared to those of vertebrates.

Surprisingly, low concentrations of the depressant toxins are able to completely displace the excitatory toxins from their binding sites in an insect neuronal membrane (Gordon et al., 1984; Zlotkin et al., 1985, 1991) in spite of their contrasting symptomatology (contractile versus flaccid paralysis; Lester et al., 1982), different effects on sodium conductance (enhancement versus suppression of sodium currents; Zlotkin et al., 1985; Pelhate & Zlotkin, 1982; Lester et al., 1982), and obvious differences in their primary structures (Zlotkin et al., 1991). The present study examines whether the excitatory and depressant insect toxins share an identical

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binding site on the insect sodium channel by using site-directed antibodies corresponding to selected external regions of the sodium channel to specifically probe the insect toxins' binding sites in insect neuronal membranes.

EXPERIMENTAL PROCEDURES

Materials. The insect toxins $LqhIT_2$[1] and AaIT were purified from venoms of the scorpion *Leiurus quinquestriatus hebraeus* and *Androctonus australis* by the methods described in Zlotkin et al. (1991) and Zlotkin et al. (1971), respectively. Ts_{VII} was a generous gift of Dr. L. D. Possani, UNAM, Cuernavaca 62271, Morelos, Mexico.

Antisera directed against synthetic peptides corresponding to different sequences of the α-subunit of the rat brain RII sodium channel were prepared as described (Gordon et al., 1987, 1988). IgGs were isolated from antisera by 50% ammonium sulfate precipitation and resuspension to the original volume of antisera. The site-directed antibodies recognize solubilized sodium channels in rat (Thomsen & Catterall, 1989) and insect (see Figure 4 below) neuronal membranes, as demonstrated by immunoprecipitation and phosphorylation assays performed according to a previously described method (Gordon et al., 1988, 1990; Moskowitz et al., 1991; see below).

Radioiodination. The insect toxins were iodinated by iodogen (Pierce Chemical Co., Rockville, MD) with minor modifications of the method described by Lima et al. (1989) using 0.5 mCi of carrier-free $Na^{125}I$ (0.3 nmol) (Nuclear Research Center, Negev, Israel) and 5 μg (0.7 nmol) of $LqhIT_2$ or AaIT. The monoiodotoxins were purified by a Beckman Ultrapore C3 RPSC column (4.6 × 75 mm) using a gradient of 10–80% solvent (solvent A = 0.1% TFA; solvent B = 50% 2-propanol, 50% acetonitrile, and 0.1% TFA) at a flow rate of 0.5 mL/min. The radioactivity and the protein were simultaneously monitored on line during the elution process using a solid scintillator cell (radioactive flow detector CT Flow-One, Radiomatic Instrument Co.) and the absorbance at 280 nm (using a Varian V UV-10 UV detector), respectively, with the aid of a Merck Hitachi HPLC and L-5000 LC controller. The monoiodotoxin was eluted as the first peak of radioactive protein (retention time 65–67 min at about 30% solvent B) following the peak of the native toxin (retention time 62–64 min at about 28% solvent B). The purification procedure gave the same results as in Lima et al. (1989), who characterized the first peak as monoiodotoxin. The top fraction of the monoiodinated peak was used in binding studies. The concentration of radiolabeled toxin was estimated according to the specific radioactivity of ^{125}I and corresponded to 2424 dpm/fmol of monoiodotoxin.

Insect Neuronal Membranes. Locust (*Locusta migratoria*) central nervous system synaptosomal membranes (LP_2L preparation) were prepared according to established methods (Gordon et al., 1984, 1990).

Binding Assays. These were performed in the form of equilibrium saturation assays using increasing concentrations of the cold toxin in the presence of a constant low concentration of the labeled toxin. In order to obtain saturation curves, the specific radioactivity and the amount of bound toxin were calculated and determined for each toxin concentration. Analysis of all binding assays was performed using the iterative computer program LIGAND (P. J. Munson and D. Rodbard, modified by G. A. McPherson, 1985).

Locust neuronal membranes were suspended in binding medium containing 0.13 M choline chloride, 1 mM EDTA, 20 mM Hepes/Tris, pH 7.4, and 5 mg/mL BSA. Following incubation, the reaction mixture was diluted with 2 mL of ice-cold wash buffer (150 mM choline chloride, 5 mM Hepes/Tris, pH 7.4, 1 mM EDTA, and 5 mg/mL BSA) and filtered over GF/F filters (Whatman, U.K.) under vacuum, followed by two more washes of the filters. Nonspecific toxin binding was determined in the presence of 1 μM unlabeled toxin and corresponded to 30–50% of the total binding.

In assays of the inhibition of toxin binding by site-directed antibodies, the membranes were preincubated for 16–20 h at 4 °C with the indicated site-directed antibody (1–10 μL) in the presence of high amounts of the nonimmune IgG (20 μL). The maximal specific binding (100%) was determined in the presence of 20–40 μL of the nonimmune IgG. In that range of volumes, the specific binding was not affected by the nonimmune IgG. Following the preincubation period with the antibodies, the radiolabeled toxin was added for 1 h at 4 °C. Nonspecific binding was determined in the presence of 1 μM nonlabeled toxin. Termination and washing were performed as above.

Immunoprecipitation and Phosphorylation. The insect neuronal sodium channels were solubilized from the membranes by 3% Triton X-100 and immunoprecipitated by the site-directed antibodies as described (Gordon et al., 1988, 1990). The sodium channel–antibody complexes, after sedimentation by Sepharose-bound protein A, were radiophosphorylated using [γ-^{32}P]ATP and the catalytic subunit of cAMP-dependent protein kinase (Sigma, St. Louis, MO). The sodium channel polypeptides were separated by SDS–PAGE and visualized by autoradiography, as described (Gordon et al., 1990).

Protein Determination. Membrane protein was determined according to Peterson (1977) using BSA as standard.

RESULTS

The Depressant Toxin $LqhIT_2$ Is Competitively Displaced by the Excitatory Toxin AaIT. As previously shown, AaIT selectively binds to a single class of noninteracting binding sites of high affinity (K_D = 1.2–3 nM) in various insect neuronal membranes (Gordon et al., 1984, 1985) and is competitively displaced from its binding sites by various depressant toxins, including $LqhIT_2$ (Zlotkin et al., 1985, 1991; Gordon et al., 1984, 1985). With this background, the binding properties of radioiodinated $LqhIT_2$ and its competitive displacement by AaIT were studied.

Scatchard analysis of a saturation binding curve of [^{125}I]-$LqhIT_2$ (Figure 1) reveals that the depressant $LqhIT_2$ possesses two distinct binding sites (Hill no. = 0.996) in the locust neuronal membranes. As shown (Figure 1), the equilibrium dissociation constant (K_D) and the capacity (B_{max}) of the high-affinity site are lower by 2 orders of magnitude than the second, low-affinity site (K_{D_1} = 0.9 ± 0.6 nM, B_{max_1} = 0.1 ± 0.07 pmol/mg of protein; K_{D_2} = 185 ± 13 nM, B_{max_2} = 10.0 ± 0.6 pmol/mg, respectively). The occurrence of two distinct binding sites for $LqhIT_2$ was also obtained using a different insect neuronal preparation derived from fly heads (Moskowitz et al., unpublished results). To elucidate which of the two binding sites is affected by the excitatory AaIT, competitive displacement assays of the two toxins were performed (Figure 2). As shown (Figure 2A), AaIT is able to displace only half

[1] Abbreviations: AaHII, α-scorpion toxin II affecting mammals from the venom of *Androctonus australis*; AaIT, excitatory insect-selective toxin from the venom of *Androctonus australis*; $LqhIT_2$, depressant insect-selective toxin from the venom of the scorpion *Leiurus quinquestriatus hebraeus*; Ts_{VII}, β-mammal toxin 7 from the venom of the scorpion *Tityus serrulatus*.

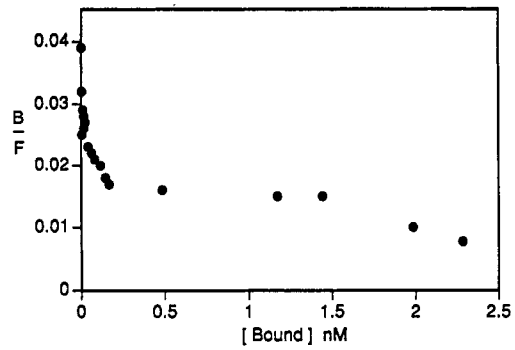

FIGURE 1: Scatchard analysis of a saturation curve of $LqhIT_2$ binding to insect neuronal membranes. Locust neuronal membranes (155 μg of protein) were incubated with 72.5 pM [^{125}I]$LqhIT_2$ and increasing concentrations of unlabeled $LqhIT_2$ for 30 min at 22 °C in 0.4 mL of binding buffer, and the amount of specifically bound [^{125}I]$LqhIT_2$ was determined following rapid filtration (see Experimental Procedures). Nonspecific binding (30–50% of total binding) was determined in the presence of 1 μM $LqhIT_2$ and was subtracted from all data points. The concentration of $LqhIT_2$ up to 5 μM did not reduce nonspecific binding. The specific radioactivity and the amount of bound toxin were recalculated for each toxin concentration, and the Scatchard plot was drawn using the computer program LIGAND (see Experimental Procedures). Scatchard plot analysis yielded the best fit ($P < 0.05$) using a two binding site model. The K_D values, obtained from three separate experiments, are $K_{D_1} = 0.9 \pm 0.6$ nM, $B_{max_1} = 0.1 \pm 0.07$ pmol/mg of protein and $K_{D_2} = 185 \pm 13$ nM, $B_{max_2} = 10.0 \pm 0.6$ pmol/mg of protein. The total concentration of binding sites (R_T) used in the Hill plot was determined from Scatchard analysis. $n_H = 0.996$.

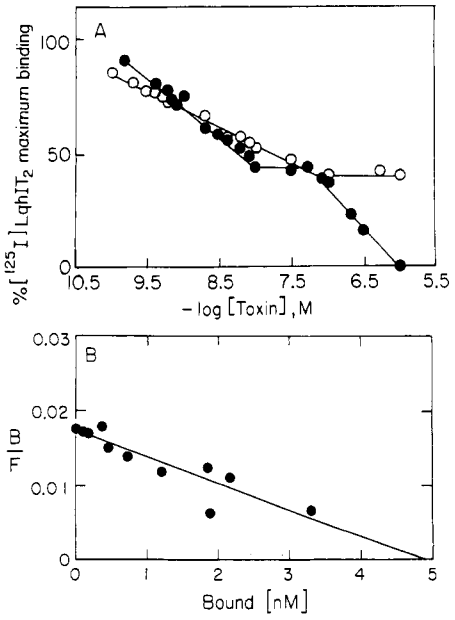

FIGURE 2: Displacement of [^{125}I]$LqhIT_2$ binding by $LqhIT_2$ and AaIT. Locust neuronal membranes were incubated for 30 min at 22 °C in the presence of 72.5 pM [^{125}I]$LqhIT_2$ and increasing concentrations of $LqhIT_2$ (●, 150 μg of membrane protein) or AaIT (○, 114 μg of membrane protein). Nonspecific binding of [^{125}I]-$LqhIT_2$, determined in the presence of 1 μM $LqhIT_2$, was subtracted. The nonspecific binding was not affected by higher $LqhIT_2$ concentrations, up to 5 μM. (A) Plots of [^{125}I]$LqhIT_2$ bound as a function of log toxin concentration. (B) Scatchard analysis of a separate experiment in which 72.5 pM [^{125}I]$LqhIT_2$ and 0.5 μM unlabeled AaIT (to saturate the high-affinity binding sites of $LqhIT_2$) were incubated with 191 μg of locust membrane protein in the presence of increasing concentrations of unlabeled $LqhIT_2$. The data were analyzed using the computer program LIGAND (see Experimental Procedures). The binding parameters of $LqhIT_2$, representing the second low-affinity binding site, are $K_D = 194 \pm 36$ nM and $B_{max} = 13.8 \pm 6.9$ pmol/mg of protein.

of the specifically bound [^{125}I]$LqhIT_2$. The K_D for AaIT is 1.1 ± 0.8 nM. In the presence of a saturating concentration

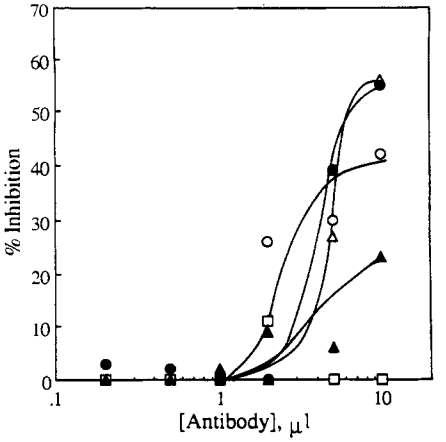

FIGURE 3: Concentration dependence of the inhibition of [^{125}I]$LqhIT_2$ binding by site-directed antibodies. Locust neuronal membranes (110 μg of protein) were preincubated for 20 h at 4 °C in 0.2 mL of the binding buffer, containing 20 μL of nonimmune rabbit antibodies (IgG) and the indicated amounts (0.1–10 μL) of the site-directed antibodies (see Experimental Procedures). [^{125}I]$LqhIT_2$ (0.22 nM) was added and the incubation continued for 1 h at 4 °C. The calculation of the percent of binding inhibition was based on controls including only nonimmune IgG which represented 100% binding. Each data point represents the mean of three experiments, where triplicate determinations were made at each antibody concentration. The antibodies used were Ab 355–371 (▲), Ab 382–400 (●), Ab 1429–1449 (△), Ab 1686–1703 (□), and Ab 1729–1748 (○). The maximal inhibition of toxin binding was not affected by amounts of the site-directed antibodies in the range of 10–20 μL.

(0.5 μM) of AaIT, $LqhIT_2$ binds only to the second, low-affinity site (Figure 2B). Thus, AaIT competes only for binding to the high-affinity $LqhIT_2$ site. A scorpion β-toxin (Ts_{VII}), which was previously shown to compete with the AaIT on its binding sites in fly and cockroach CNS neuronal membranes (Lima et al., 1986, 1989), as well as with other β-toxins in rat brain membranes (Barhanin et al., 1982), was presently shown to displace the [^{125}I]$LqhIT_2$ from its high-affinity binding sites in the locust preparation also (data not shown). Thus, the two different insect-selective neurotoxins are able to mutually displace each other from a common receptor site, the high-affinity site of $LqhIT_2$, which is also shared by the β-toxin Ts_{VII}.

Inhibition of $LqhIT_2$ Binding by Site-Directed Antibodies. To probe the binding sites of the depressant and excitatory toxins, we employed five site-directed antibodies that recognize different extracellular regions of the rat brain RII sodium channel α-subunit corresponding to amino acid residues 355–371, 382–400, 1429–1449, 1686–1703, and 1729–1748 (Thomsen & Catterall, 1989) (Figure 7). The previously described anti-SP19 antibody, corresponding to the intracellular sequence 1491–1508 (Gordon et al., 1988, 1990; Vassilev et al., 1988), was also employed. These sequences resemble the corresponding amino acid segments in the sodium channel polypeptide from *Drosophila para* locus (Loughney et al., 1989), having 67, 79, 50, 56, 40, and 83% identity, respectively.

Preincubation of the locust neuronal membrane with four out of the five externally directed antibodies (Ab) inhibited [^{125}I]$LqhIT_2$ binding in a concentration-dependent manner (Figure 3). Maximum inhibition of about 50% was obtained with Ab 382–400 (55 ± 17%), Ab 1429–1449 (56 ± 18%), and Ab 1729–1748 (42 ± 20%). Ab 355–371 inhibits [^{125}I]-$LqhIT_2$ binding by only 23 ± 5% (Figures 3 and 6).

There was no correlation between the ability of the various site-directed antibodies to recognize the insect sodium channel polypeptide by the immunoprecipitation–radiophosphorylation method (see Experimental Procedures and Figure 4) and

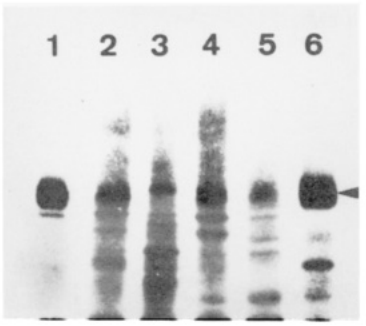

FIGURE 4: Identification of insect sodium channel polypeptides by immunoprecipitation and phosphorylation using site-directed antibodies. Locust neuronal membranes were solubilized by 3% Triton X-100 and immunoprecipitated with 15 μL of Ab 382–400 (lane 1), Ab 1429–1449 (lane 2), Ab 1729–1748 (lane 3), Ab 1686–1703 (lane 4), and Ab 355–371 (lane 5) and 10 μL of Ab 1491–1508 (anti-SP19, lane 6), under conditions as previously described (see Experimental Procedures). Briefly, after radioactive labeling of the immunoprecipitated sodium channel polypeptides by reaction with the catalytic subunit of cAMP-dependent protein kinase and [γ-^{32}P]ATP, the phosphorylated sodium channels were resolved by SDS–PAGE after reduction of disulfide bonds as previously determined (Gordon et al., 1990; Moskowitz et al., 1991). Two hundred femtomoles of sodium channels, as assessed by saxitoxin binding assay of the membranes, was used in each lane. The arrow marks the migration position of the α-subunit of locust sodium channel polypeptides (250 kDa). Illustrative lanes are presented.

their ability to inhibit toxin binding to insect neuronal membranes. For example, Ab 382–400 significantly inhibited toxin binding (Figures 3 and 6) and recognized the sodium channel polypeptide by the immunoprecipitation–phosphorylation method (Figure 4). Ab 1686–1703, on the other hand, did not reveal any significant inhibition of toxin binding to the locust neuronal membranes (Figures 3 and 6) but immunoprecipitated the sodium channel polypeptide similarly to Ab 1491–1508 (SP19) (Figure 4). These results are consistent with specific inhibition of toxin binding by a subset of our antibodies that block the toxin receptor site.

We cannot rule out the possibility that conformational changes (which might occur during membrane solubilization) may partly account for the lack of correlation between inhibition of toxin binding and immunoprecipitation of the sodium channel polypeptide. However, the anti-peptide antibodies used are a polyclonal population of antibodies that are likely to recognize all accessible conformations of the peptide antigen in the intact membranes as well as in the solubilized form. It is noteworthy that similar results concerning the immunoprecipitation and inhibition of $LqhIT_2$ binding by site-directed antibodies were obtained also using the fly head neuronal preparation (Moskowitz et al., unpublished results). The SP19 antibody was previously shown to specifically recognize sodium channel polypeptides in various insects (Gordon et al., 1988, 1990; Moskowitz et al., 1991).

The inhibitory effect of the site-directed antibodies on the binding of [^{125}I]$LqhIT_2$ may result from a reduction in the number of toxin binding sites or a decrease in the toxins' affinity. Figure 5 shows Scatchard analysis of saturation curves of [^{125}I]$LqhIT_2$ binding to locust neuronal membranes with and without Ab 382–400. No significant change in K_D values was seen (Figure 5). The toxin was used in a low concentration range in order to occupy mainly the high-affinity sites of $LqhIT_2$. Under these conditions, in the presence of a half-maximal concentration of Ab 382–400, about 25% reduction in the number of binding sites was obtained (Figure 5).

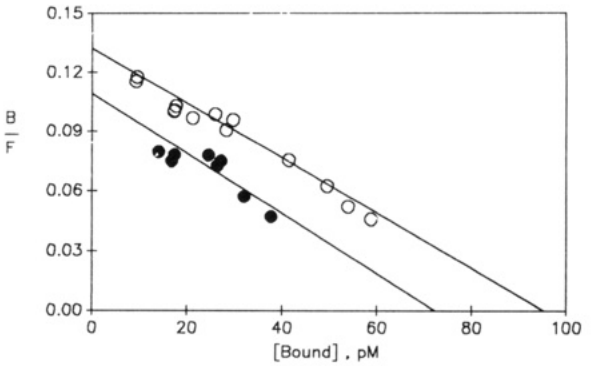

FIGURE 5: Scatchard plots of saturation curves of $LqhIT_2$ binding in the presence or absence of site-directed antibody. Locust neuronal membranes (182 μg of protein) were preincubated for 20 h at 4 °C with 20 μL nonimmune antibodies in 0.4 mL of binding medium (control, ○) or in the presence of 10 μL of antibody 382–400 in addition to 20 μL of nonimmune antibodies (●). Increasing concentrations of $LqhIT_2$ up to 1.25 nM were added to the 89 pM [^{125}I]$LqhIT_2$, to occupy the high-affinity receptor site of $LqhIT_2$ (see Figures 1 and 2). Scatchard plots were prepared by the computer program LIGAND (see Experimental Procedures). Note that the antibody 382–400 concentration was half of the maximal concentration used in the assays presented in Figures 3 and 6. Each toxin concentration was determined in triplicate. The K_D and B_{max} values obtained in controls (○) were 0.72 nM and 0.21 pmol/mg of protein, respectively, and in the presence of Ab (●) 0.67 nM and 0.16 pmol/mg of protein, respectively. Two separate experiments yielded similar results.

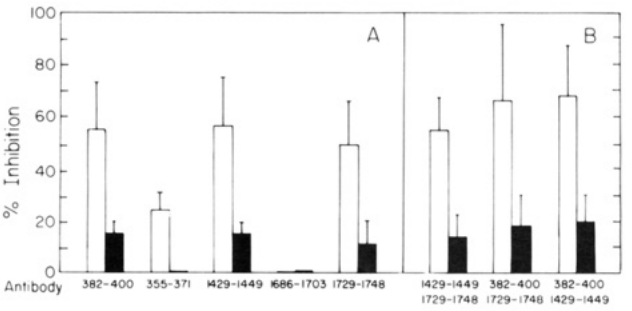

FIGURE 6: Inhibition of [^{125}I]$LqhIT_2$ and [^{125}I]AaIT binding by the site-directed antibodies. Locust neuronal membranes (0.5 mg/mL) were preincubated with 20, 30, and 40 μL of nonimmune antibodies (control, 100% binding) or 20 μL of the nonimmune antibodies together with 10 μL of each of the indicated site-directed antibodies (A). In the combined applications of two site-directed antibodies, each antibody was applied in 10 μL in the presence of 20 μL of nonimmune antibodies (B). Each bar represents the mean ± SD of three to five independent experiments, where triplicate determinations were made for each antibody. Toxin binding was determined as described under Experimental Procedures. The concentrations of labeled toxins used were 224.7 ± 12.3 pM for [^{125}I]$LqhIT_2$ (open bars) and 249.6 ± 80.4 pM for [^{125}I]AaIT (solid bars).

Distinction between the Binding Sites of the Depressant and Excitatory Insect Toxins. The competitive interaction observed between the two different insect toxins could be interpreted as either competition at an identical binding site, steric interference between adjacent binding sites, or distant allosteric interaction. In the first possibility, the various site-directed antibodies would be expected to inhibit the binding of the two ^{125}I-labeled toxins to the same extent. Figure 6 presents antibody-mediated inhibition of [^{125}I]$LqhIT_2$ and [^{125}I]AaIT binding to insect neuronal membranes. The membranes were preincubated in the presence of maximal amounts of each Ab, and the binding of the two toxins was measured under similar conditions. Antibodies 382–400, 1429–1449, and 1729–1748, which cause over 50% inhibition

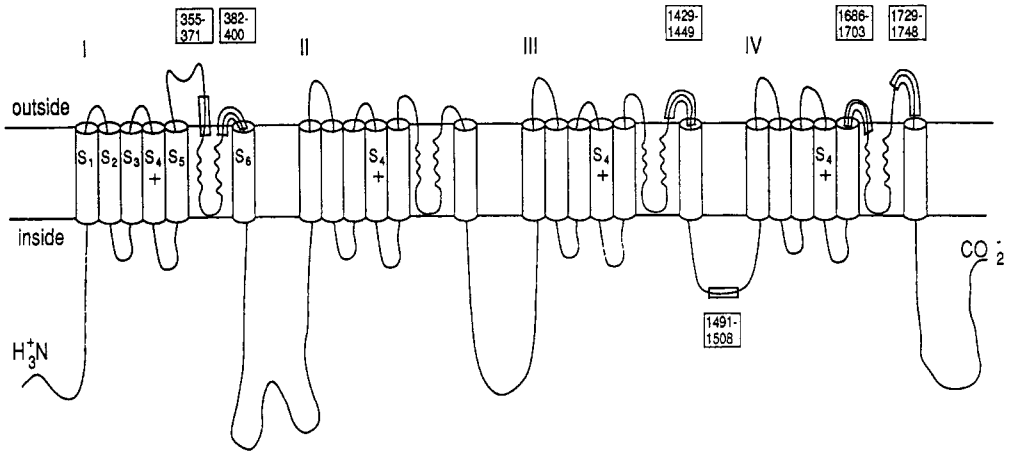

FIGURE 7: Two-dimensional model of the proposed transmembrane folding of a sodium channel α-subunit depicting the sites of interaction of the site-directed antibodies (wide boxes). The framed numbers indicate the various site-directed antibodies used. Antibodies 382–400, 1429–1449, and 1729–1748 inhibit $LqhIT_2$ and AaIT binding by about 50% and 20%, respectively. Ab 1491–1508 corresponds to anti-SP19 antibody, and represents part of the inactivation site.

of [^{125}I]$LqhIT_2$ binding, were shown to inhibit [^{125}I]AaIT binding by less than 20%. Antibody 355–371, which inhibits the depressant toxin by 23%, did not reveal any significant effect on the binding of the excitatory ^{125}I-toxin, similarly to Ab 1686–1703 which did not affect the binding of either toxin (Figure 6A).

The data presented in Figure 6B demonstrate that the combined application of two Abs, each effective in toxin binding inhibition, was not additive. These results suggest that (a) each antibody was given in a saturable amount (see also Figure 3) and (b) the partial inhibition of toxin binding (Figures 3 and 6) indicates that all the antibodies used identify and bind to the same population of sodium channels. Otherwise, the combined presence of two effective antibodies would result in an additive effect (Figure 6B). It is noteworthy that the possibility of steric hindrance in the binding of the combined antibodies to a single sodium channel is not ruled out by our data. However, it would not alter our suggested interpretation of Figure 6B. The main conclusion, however, demonstrated in Figure 6 is the ability of the various site-directed Abs to quantitatively differentiate between the binding of the $LqhIT_2$ and AaIT. This indicates that their binding sites are not identical although located on the same insect sodium channel.

DISCUSSION

The Insect Sodium Channel as the Receptor for the Two Insect Toxins. The amino acid sequence within the four homologous domains (I–IV) of rat brain sodium channels is conserved in the deduced amino acid sequence of the insect sodium channel (59%, 66%, 58%, and 62% identity, respectively; Loughney et al., 1989). The identification of the insect neuronal sodium channel polypeptides by the various site-directed antibodies employed in the present study (Figures 3, 4, and 6) further emphasizes the similarity among mammalian and insect sodium channels (Loughney et al., 1989; Gordon et al, 1988, 1990; Moskowitz et al., 1991; Gordon, 1990). Unique structural and/or functional features of insect sodium channels are implied by their ability to serve as the receptors of the insect-selective scorpion neurotoxins which modify the sodium conductance exclusively in insect neuronal preparations (Zlotkin, 1986). Thus, identification of the receptors of these neurotoxins may contribute to the understanding of the organization of the insect sodium channels through the identification of specific regions within the sodium channel polypeptide that cause the functional effects of these toxins.

The depressant $LqhIT_2$ toxin was shown to possess two binding sites on the locust neuronal membranes (Figure 1). The nature of the low-affinity and high-capacity $LqhIT_2$ binding sites is presently unknown. The high-affinity binding sites, however, were shown to serve as a target for the mutual competitive binding of the depressant and the excitatory toxins (Figure 2) and for binding inhibition by several of the site-directed antibodies (Figure 3, 5, and 6) corresponding to specific external segments in the sodium channel polypeptide. These results indicate that the high-affinity binding sites of the insect toxins are indeed located on the insect sodium channel.

This conclusion is supported by the specificity of the effect of the site-directed antibodies as indicated by (a) the absence of any inhibitory effect by the nonimmune antibodies, (b) the selectivity in action of the various site-directed antibodies, i.e., the absence of inhibition by the anti-SP19 antibody directed against an intracellular segment and by Ab 1686–1703 directed against an extracellular segment of the sodium channels, even though both antibodies were shown to recognize the insect sodium channel polypeptide (Figure 4 and Gordon et al. (1990) and Moskowitz et al., (1991)], (c) the differential ability of the antibodies directed against external regions of the sodium channel to inhibit $LqhIT_2$ binding, i.e., Ab 355–371 ($\approx$30%) and Abs 382–400, 1429–1449, and 1729–1749 ($\approx$50%), and (d) the ability of the antibodies to differentially affect the binding of AaIT when compared to $LqhIT_2$ as demonstrated by antibodies 382–400, 1429–1449, and 1729–1748 (Figure 6). The distinction between the binding sites of the two toxins by the antibodies strongly suggests that they bind to related, but not identical, binding sites located on the insect sodium channel.

The antibody-mediated inhibition of the binding of [^{125}I]-$LqhIT_2$ was shown to be partial (about 50%) even at saturating concentrations of the effective antibodies, applied either separately (Figures 3 and 6A) or in combination (Figure 6B). That is, only a fraction of the sites identified by the toxin are occupied by the effective antibodies. Assuming that the toxin binds exclusively to sodium channels, the observed partial inhibition could suggest that only a fraction of the channels identified by the toxin is accessible to the antibodies. However, the data presented in Figures 1 and 2 reveal the presence of two binding sites for $LqhIT_2$. Only the high-affinity (and low-capacity) one is recognized by AaIT (Figure 2). This high-affinity site was shown (Figure 5) to serve as a target for binding inhibition by the antibodies. It is noteworthy that

the degree of binding inhibition of [^{125}I]LqhIT$_2$ by AaIT (about 50%, Figure 2) is very similar to that induced by the maximal concentrations of the effective antibodies (Figures 3 and 6). Thus, it may be suggested that [^{125}I]LqhIT$_2$ binding in the presence of the antibodies (Figure 6) corresponds to the occupancy of the low-affinity and high-capacity binding sites as revealed in the presence of AaIT (Figure 2B). We therefore assume that the effective antibodies are accessible to the same population of sodium channels recognized by the toxins. This assumption supports the significance of the distinction between the binding sites of the two toxins as revealed by the effect of the antibodies.

The Insect Toxins' Receptors Are Formed by Multiple Extracellular Segments of Domains I, III, and IV. As shown (Figures 3 and 6), the inhibitory effect on the binding of the two insect toxins was exerted by antibodies corresponding to extracellular regions in domains I (Ab 382–400), III (Ab 1429–1449), and IV (Ab 1729–1748) of the insect sodium channel (Figures 6 and 7). Moreover, each one of the several effective antibodies is sufficient to induce maximal binding inhibition (Figure 6). Assuming that each of the antibodies prevents toxin binding by occupying their corresponding amino acid sequence epitope, our results indicate that amino acid sequences from distant parts of the primary structure of the sodium channel participate in the formation of the insect toxins' receptor. This conclusion supports the widely accepted concept on the organization of the four homologous domains of the sodium channels in a pseudosymmetric array around a central pore and its general topological organization suggested by current models (Catterall, 1988; Guy & Conti, 1990) (Figure 7). A similar conclusion was drawn from a recent study (Thomsen & Catterall, 1989) of the localization of the receptor site for α scorpion toxin on rat brain sodium channels. About 50% inhibition of α scorpion toxin binding was obtained by Ab 355–371 and Ab 1686–1703, which correspond to extracellular segments in domains I and IV, respectively, in the rat brain sodium channel (Thomsen & Catterall, 1989). However, no inhibitory effects on α scorpion toxin binding were observed using Ab 1429–1449 and Ab 1729–1748. These results further emphasize the specificity in the antibodies' effects.

Toxins Binding Requires Several Attachment Sites. The present results are in concert with the previously suggested concept of multipoint attachment of scorpion toxins. This concept was based on chemical modifications of selected amino acid residues, localized on various regions of the primary structure of the α scorpion toxins (AaHII; Kharrat et al., 1989) as well as the insect toxins (AaIT; Loret et al., 1990). Thus, it may be suggested that the different regions of the toxin essential for its attachment are complemented by several points of attachment in the receptor molecule, all simultaneously required to carry out the high-affinity binding reaction. This hypothesis, however, deserves further study.

The requirement of several segments of the sodium channel for toxin binding may reasonably explain the phenomenon of binding competition among chemically and pharmacologically distinct toxins such as the insect and β scorpion toxins (see Results). Thus, a partial overlap, even in one of the several attachment points of the various toxins to the receptor, may be sufficient to inhibit toxin binding. This notion is supported by the recent finding of a new scorpion toxin, AaHIT$_4$ (Loret et al., 1991), which has a low homology with other scorpion toxins. AaHIT$_4$, however, displaces both α- and β-toxins, which bind to distinct receptor sites (Catterall, 1986; Couraud & Jover, 1984), from rat brain sodium channels and also displaces the insect toxin AaIT from insect sodium channels.

With this background, we propose that the competitive interaction between the depressant and excitatory insect toxins is a consequence of a partial overlap in their points of attachment in the external segments of the insect sodium channel. Thus, the two groups of toxins may have closely localized, but not identical, binding sites. It may be further suggested that the insect selectivity of the insect toxins may correspond to the recognition of minor modifications in the insect sodium channel when compared to its vertebrate counterpart, in one or more of the several points of attachment required for toxin binding. The difference may be expressed on the amino acid sequence and/or conformational levels. Further clarification of the structural basis of insect selectivity on the level of the toxins as well as their receptors may lead to the design of selective insecticides in the future (Zlotkin, 1991).

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