Molecular Determinants of High Affinity Binding of \( \alpha \)-Scorpion Toxin and Sea Anemone Toxin in the S3-S4 Extracellular Loop in Domain IV of the Na\(^+\) Channel \( \alpha \) Subunit*

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\( \alpha \)-Scorpion toxins and sea anemone toxins bind to a common extracellular site on the Na\(^+\) channel and inhibit fast inactivation. Basic amino acids of the toxins and domains I and IV of the Na\(^+\) channel \( \alpha \) subunit have been previously implicated in toxin binding. To identify acidic residues required for toxin binding, extracellular acidic amino acids in domains I and IV of the type IIa Na\(^+\) channel \( \alpha \) subunit were converted to neutral or basic amino acids using site-directed mutagenesis, and altered channels were transiently expressed in tsA-201 cells and tested for \( ^{125}\text{I} \)-scorpion toxin binding. Conversion of Glu343 to Ser at the extracellular end of transmembrane segment IVS3 to Arg or His blocked measurable \( \alpha \)-scorpion toxin binding, but did not affect the level of expression or saxitoxin binding affinity. Conversion of individual residues in the IVS3-S4 extracellular loop to differently charged residues or to Ala identified seven additional residues whose mutation caused significant effects on binding of \( \alpha \)-scorpion toxin or sea anemone toxin. Moreover, chimeric Na\(^+\) channels in which amino acid residues at the extracellular end of segment IVS3 of the \( \alpha \) subunit of cardiac Na\(^+\) channels were substituted into the type IIa channel sequence had reduced affinity for \( \alpha \)-scorpion toxin characteristic of cardiac Na\(^+\) channels. Electrophysiological analysis showed that E1613R has 62- and 82-fold lower affinities for \( \alpha \)-scorpion and sea anemone toxins, respectively. Dissociation of \( \alpha \)-scorpion toxin is substantially accelerated at all potentials compared to wild-type channels. \( \alpha \)-Scorpion toxin binding to wild type and E1613R had similar voltage dependence, which was slightly more positive and steeper than the voltage dependence of steady-state inactivation. These results indicate that nonidentical amino acids of the IVS3-S4 loop participate in \( \alpha \)-scorpion toxin and sea anemone toxin binding to overlapping sites and that neighboring amino acid residues in the IVS3 segment contribute to the difference in \( \alpha \)-scorpion toxin binding affinity between cardiac and neuronal Na\(^+\) channels. The results also support the hypothesis that this region of the Na\(^+\) channel is important for coupling channel activation to fast inactivation.

Voltage-gated Na\(^+\) channels are responsible for the conduction of electrical impulses in most excitable tissues (1). The importance of their function is demonstrated by the effects of Na\(^+\) channel-specific neurotoxins that bind to at least six different receptor sites on the Na\(^+\) channel molecule and disrupt its normal behavior (reviewed in Refs. 2 and 3). These natural toxins are powerful tools for understanding and correlating ion channel structure and function, as exemplified by identification of molecular determinants for binding of the pore blocker tetradotoxin, which has provided important information about the structure of the ion selectivity filter and pore (3, 4). Similarly, the identification of molecular determinants for binding of toxins that modify activation or inactivation will likely provide important information about the mechanisms of channel gating.

Neuronal Na\(^+\) channels consist of a 260-kDa \( \alpha \) subunit with two auxiliary subunits, \( \beta \)I and \( \beta \)II (reviewed in Ref. 3). The \( \alpha \) subunit is independently functional when expressed in Xenopus oocytes or mammalian cells, and contains the ion pore and neurotoxin binding sites 1-3 and 5 (reviewed in Refs. 3 and 4). It contains four homologous domains (I-IV) that surround a central ion pore, and each domain contains six transmembrane segments (S1-S6) and a short membrane-penetrating segment (SS1-SS2) between segments S5 and S6. The four short SS1-SS2 segments form the ion selectivity filter and the tetrodotoxin receptor site, and the S4 transmembrane segments act as voltage sensors (3, 4). The intracellular loop between domains III and IV acts as a fast inactivation gate, blocking the conduction pathway following channel activation (3, 4).

Na\(^+\) channel-specific \( \alpha \)-scorpion toxins and sea anemone toxins are distinct families of peptide toxins that share no sequence homology, but slow inactivation by binding to common or overlapping elements of neurotoxin receptor site 3 on the extracellular surface of the Na\(^+\) channel (reviewed in Ref. 2). The three-dimensional structures of \( \alpha \)-scorpion and sea anemone toxins (5-9), the effects of peptide-specific antibodies and chemical modification (10-14), and site-directed mutagenesis (15, 16) indicate that conserved basic amino acid residues of these toxins are important for binding to the sodium channel. The binding affinity of both of these classes of toxins is decreased by depolarization (17-22). The voltage dependence of binding and the specific effect of these toxins on inactivation (19-23) imply that membrane potential affects the structure of neurotoxin receptor site 3, that this region of the channel is important for the coupling of activation to inactivation, and that toxin binding to this site can slow or block a conformational change required for fast inactivation. Photooxidative derivatives of \( \alpha \)-scorpion toxins covalently label both \( \alpha \) and \( \beta \)I subunits (24-26), but \( \alpha \) subunits expressed alone in Chinese hamster ovary cells retain high affinity binding of \( \alpha \)-scorpion toxin (27). Two distinct regions of the Na\(^+\) channel \( \alpha \) subunit have been implicated in \( \alpha \)-scorpion toxin binding by photoaffinity labeling of the S5-SS1 loop in domain I and inhibition of

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toxin binding by site-directed antipeptide antibodies directed against peptides of the S5-S51 and S2-S6 loops in domain I and the S5-S51 loop in domain IV (28, 29).

In this study, extracellular amino acids in domains I and IV of the Na+ channel were converted to neutral or basic amino acids, and the resulting mutants were tested for α-scorpion toxin affinity by transient expression in mammalian cells. Glu1613 in the extracellular loop between segments IVS3 and IVS4 was identified as a major determinant of LqTx1 and ATXII binding. Mutation of additional residues within and adjacent to this loop identified residues important for either LqTx1 or ATXII binding, indicating that unique molecular determinants in this region form overlapping binding sites for α-scorpion toxins and sea anemone toxins and contribute to differences in binding of α-scorpion toxins between cardiac and neuronal Na+ channels.

**Molecular Determinants of α-Scorpion and Sea Anemone Toxin Binding**

EXPERIMENTAL PROCEDURES

Materials—α-Scorpion toxin (LqTx) was purified from venom (Lyurus quinquestratus quinquestratus, Latexen) and iodinated as described previously (17, 29). Antibiotics, lactoperoxidase, and poly-d-lysine (70 kDa) were from Sigma, Na+ (NaCl) and [35S]dATP were from New England Biolabs and Boehringer Mannheim. Antigenotoxicidase (Cell Genesis, Foster City, CA). Anti-CD8 antibody-coated microplates (Dynabeads M-450 CDB) were from Dynal (Great Neck, NY).

Cell Culture—CNaI-a-1 cells stably expressing the rIIa Na+ channel α subunit were maintained in RPMI media supplemented with 5% fetal bovine serum (HyClone), as described previously (27). For 125I-LqTx binding, these cells were plated to 24-well (1.6 cm, Falcon) cell culture plates at a density of 20,000 cells/cm2, fed every other day until confluence, then assayed for binding. The tsA-201 cells were maintained in Dulbecco's modified Eagle's medium/F-12 cell culture media were from Life Technologies, Inc./BRL. Restriction endonucleases and other molecular biology reagents were from New England Biolabs and Boehringer Mannheim. Oligonucleotides were synthesized in the Molecular Pharmacology DNA Core Facility at the University of Washington. Molecular cloning vectors and bacterial strains were obtained from the following sources: pCDM vector and J M103, C.J. 236, and MCI061 Escherichia coli bacterial strains (Invitrogen), M13mp18/19 (New England Biolabs), and plBO-PCD-leu2 (CD8 antigen vector, American Type Culture Collection). Human embryonic kidney tsA-201 cells, a simian virus 40 (SV40) large T-antigen expressing derivative of HEK-293 cells, were kindly provided by Dr. Robert Dubridge (Cell Genetics, Foster City, CA). Anti-CD8 antibody-coated microplates (Dynabeads M-450 CDB) were from Dynal (Great Neck, NY).

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Mutagenesis and Assembly of cDNAs Encoding Na+ Channels with Site-directed Mutations—Three M13 constructs containing type IIA Na+ channel α subunit cDNA sequence (30–32) were used for mutagenesis: mp18KXNC, which contained a KpnI/XbaI fragment (nt 23–540); mp19KXal, which contained a XbaI/SmaI fragment (nt 541–1897); and, mp18RVNC, which contained an EcoRV fragment (nt 4729–5997). Ural containing mutagenesis templates were prepared from each of these constructs, and oligonucleotide-directed mutagenesis was performed using the duet "ung" selection procedure (33). Mutations made in the above three M13 mutagenesis constructs were confirmed by sequencing (Sequenase, U.S. Biochemical Corp.), excised by restriction cutting at the sites used for subcloning into the M13 constructs, and isolated by low-melting-point agarose gel electrophoresis and GeneClean. Fragments from mp18KXNC were subcloned into KpnI/XbaI cut pCDM8SalK-NC, and fragments from mp19KXal and mp18RVNC were subcloned into appropriately cut and purified pCDM8Sal-NC. All mutations were then confirmed in the final constructs by DNA sequencing and extensive restriction mapping. Approximately one cDNA clone in 20 was found in this way to have rearranged during bacterial growth and clonal amplification of the plasmids, probably due to recombination of the related cDNA sequences encoding the four homologous domains of the Na+ channel. To be certain that undetected rearrangements did not influence interpretation of our results, multiple independently iso-
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bovine serum albumin essentially as described (38). Transfected cells on 100- or 150-mm dishes were rinsed twice with 5 ml of 1XP (130 mM choline chloride, 50 mM Hepes-Tris (adjusted with Tris base to pH 7.4), 5.5 mM glucose, 0.8 mM MgSO_4, and 5.4 mM KCl), scraped into 1 ml of 1XP, and placed in 15-ml polystyrene tubes (Falcon 2509). Cells were sedimented at 200 × g for 5 min at room temperature in a clinical centrifuge, and the pellet was resuspended in 1 ml of 1XP per 8000 cm^2 of plate area. For a 250-μl binding reaction, 200 μl of cell suspension, 25 μl of 1–100 nM [3H]saxitoxin in 1XP, and 25 μl of 1XP or 100 μM TTX were combined in a 3-ml polystyrene tube (Falcon 2063) and incubated 1 h at 4°C. Reactions were then filtered over GF/C filters (Millipore) under vacuum, washed twice with ice-cold choline wash solution (163 mM choline chloride, 5 mM Hepes-Tris, 1.8 mM CaCl_2, and 0.8 mM MgSO_4, pH 7.4), and bound radioactivity was detected by liquid scintillation counting for the wild-type channel, nonspecific binding accounted for 11–27% of the total binding.

Ligand Binding Analysis—Results of displacement or saturation binding experiments were analyzed using the iterative fitting programs EBDA and LIGAND (Elsevier Biosoft, UK). A Student’s t-test (paired or unpaired as appropriate) was used for statistical comparisons of toxin affinities, using p < 0.05 as the criterion of statistical significance.

Electrophysiological Analysis—Whole cell voltage clamp experiments were performed as described previously (39) using solutions that contained 90 mM CsF, 50 mM CsCl, 10 mM CsEGTA, 10 mM NaF, 2 mM MgCl_2, 10 mM Hepes (pH 7.4) in the pipette and 70 mM NaCl, 70 mM N-methyl-D-glucamine, 5 mM CsCl, 1.8 mM CaCl_2, 1 mM MgCl_2, 10 mM glucose, and 10 mM Hepes (pH 7.4) in the bath. Except where noted in the figure legends, LqTx was incubated with the cells for 30 min at 37°C before the beginning of the experiment. Data collection was initiated 10 min after breaking the cell membrane to obtain the whole cell voltage clamp configuration.

RESULTS

α-Scorpion Toxin Binding to Stably and Transiently Expressed Na^+ Channels—Previous experiments have shown that mammalian cells stably expressing only the α subunit of the type IIa Na^+ channel bind LqTx with a 2- to 3-fold lower affinity than found with rat brain synaptosomes or neuroblastoma cells in culture (27). These non-neuronal cells have a more positive resting membrane potential than neuroblastoma cells.7 As LqTx binding is inhibited by depolarization, gramicidin was used to increase the resting membrane potential and binding affinity. In the Na^-free, choline-substituted isotonic solution used for binding assays, gramicidin allows K^+ efflux without choline influx (40) to increase the negative membrane potential. Addition of gramicidin increased the receptor occupancy at 0.2 nM LqTx by reducing the apparent K_d from 5.4 ± 3.1 nM to 2.0 ± 0.6 nM in CNAILa-1 cells (mean ± S.D.; Fig. 1A). This reduction in apparent K_d is consistent with a coincident increase in membrane potential and binding affinity, and results in an LqTx affinity identical to that seen in neuroblastoma cells (17).

For analysis of mutations, Na^+ channels were expressed in the tsa-201 subclone of human embryonic kidney cells (HEK 293) (35). Fig. 1B illustrates LqTx binding to cells transfected with an expression vector containing no Na^+ channel sequence or containing the rIIa α subunit cDNA. Cells transfected with the empty vector exhibited no specific binding of 125I-LqTx, whereas cells expressing wild-type or modified channels specifically bound 0.7–1.25 fmol of 125I-LqTx per well in the presence of 0.5 nM labeled toxin and gramicidin. A Scatchard transformation of these results yielded K_d values for LqTx binding to wild-type or selected mutant channels between 2.6 and 3.9 nM and B_max values of 28–50 pm, corresponding to 5.6–10 fmol/culture well (Fig. 1B, inset). In this transient expression system, wild-type Na^+ channels and those containing site-directed mutations are expressed with varying efficiency depending on the mutation, plasmid preparation, and preparation of the calcium phosphate precipitate. Analysis of LqTx binding to the wild-type Na^+ channel at levels ranging from 0.1 to 6 fmol of specific 125I-LqTx binding per well (0.2–10 fmol/mg of cell protein) indicated no effect of expression level on LqTx affinity.

Depolarization with a high extracellular K^+ concentration would be expected to decrease binding affinity by decreasing the K^+ equilibrium potential and the resting membrane potential. To test this, cells transiently expressing the wild-type α subunit were tested in an isotonic binding solution containing gramicidin and either 5.4 or 60 mM extracellular K^+ (Fig. 1B).
Depolarization with high extracellular K⁺ increased the apparent Kᵦ of wild-type Na⁺ channels expressed in tsA-201 cells from 3.2 ± 0.2 to 8.3 ± 0.7 nM.

Charge Mutations in the α Subunit of the rIIa Na⁺ Channel—Extracellular loops in domains I and IV of the Na⁺ channel α subunit have been implicated in α-scorpion toxin binding (28, 29), and basic amino acids of α-scorpion and sea anemone toxins are thought to be important for interaction with the receptor site (10–16). In order to test the role of acidic amino acids in the extracellular loops of these two domains in toxin binding, conserved extracellular acidic residues were altered individually or in clusters to neutral or positively charged residues using oligonucleotide-directed site-specific mutagenesis (Table I). The extracellular acidic residues Asp1558 and E1613 are conserved in cloned rat brain Na⁺ channels (38). Thus, this mutation is specific in that it disrupts α-scorpion toxin binding and saxitoxin binding to mammalian cells stably expressing the rIIa subunit (30–32) and is an Asp in the cardiac Na⁺ channel (34) as well as in the skeletal muscle, Drosophila para, eel electrophys, and squid Na⁺ channels. Most alignments (e.g. Refs. 30, 32, and 34) predicted Glu1613 to be within the IVS3 transmembrane segment, but two analyses predicted that this residue is located at the extracellular end of the IVS3 transmembrane segment (43, 44). We found that analysis of the rIIa sequence from Thr1591 to

| Mutant | Loop | ΔQ | Apparent Kᵦ (nM) | S.D. | n |
|--------|------|----|------------------|------|---|
| Wild type | | | 2.8 ± 0.3 | 18 |
| S149H/N150D/D153P/N157Y | IS1-2 | 0 | 2.3 | 3 |
| E206R | IS3-4 | +2 | 2.5 | 3 |
| D209R | IS3-4 | +2 | 2.9 | 3 |
| R217E | IS4 | -2 | 2.9 | 3 |
| D317N/E318K/E321K/D322N | IS5-S51 | +6 | 3.5 | 3 |
| E330K/Q332K/D334K | IS5-S51 | +5 | 2.6 | 3 |
| D343N | IS5-S51 | +1 | 2.5 | 3 |
| E349K | IS5-S51 | +2 | 1.9 | 3 |
| D369N | IS5-S51 | +1 | 3.8 | 3 |
| E378Q | IS2-S6 | +1 | 2.8 | 3 |
| E1551R | ISV1-2 | +2 | 2.4 | 3 |
| D1553R | ISV1-2 | +2 | 2.8 | 3 |
| D1554R | ISV1-2 | +2 | 3.0 | 3 |
| E1558R | ISV1-2 | +2 | 2.7 | 3 |
| E1613R | ISV3-4 | +2 | NB | 3 |
| E1613H | ISV3-4 | +1 | NB | 3 |
| E1616R | ISV3-4 | +2 | 1.6 | 3 |
| R1626Q | ISV4 | -1 | 2.0 | 3 |
| R1626E | ISV4 | -1 | 2.4 | 3 |
| R1678A/V1688A/V1691A | ISV5-S51 | +2 | 3.5 | 3 |
| D1692N/D1693K/E1698Q | ISV5-S51 | +4 | 2.3 | 3 |
| A17215/S1726T/D1730Y/E1734N/K1735L/D1736P/N1737N/P1738S/G1793N/S1710G/K1743R/D1745N/S1750A | ISV5-S51 | +3 | 2.3 | 6 |
| D1730N/D1732N | ISV5-S51 | +2 | 2.7 | 3 |
Arg by the Predict Protein program (45) also predicted Glu to be on the extracellular surface.

Importance of Charged Amino Acids in the IVS3-S4 Extracellular Loop for \( \alpha \)-Scorpion Toxin and ATX II Binding—To test the specificity of interaction between Glu and \( \alpha \)-scorpion toxin, Glu was changed to either Asp or Gln and affinity was determined. Although neither of these mutations caused a statistically significant change in LqTx binding affinity (43, Fig. 3A), some mutations significantly decreased LqTx affinity (43, Fig. 3A).

Since \( \alpha \)-scorpion toxins and sea anemone toxins share a common binding site (18), the ATX II affinity of these mutant channels was also tested by competition for \( ^{125} \text{I}-\text{LqTx} \) binding with unlabeled ATX II (43, open squares). The results were analyzed with LIGAND/EBDA and plotted. Apparent affinities from this Scatchard plot were 0.28 nM for the wild-type channel and 0.29 nM for the E1613R channel.

Fig. 2. \([\text{H}]\text{Saxitoxin binding to transiently expressed Na}^+ \text{ channels. A, } \text{t}5\text{A-201 cells were transfected with empty pCDM8Sal, pCDM8Sal.NC, or three constructs containing site-directed mutations that showed no specific } ^{125}\text{I}-\text{LqTx} \text{ binding. Each construct was tested two to four times in a total of five experiments. To correct for different expression efficiencies between experiments, binding results from each experiment were normalized to the total } [\text{H}]\text{Saxitoxin binding of cells expressing the wild-type rIIa Na}^+ \text{ channel in that experiment. The normalized results were averaged between experiments and plotted (mean = S.D.). B, saturation binding of 0.1 to 11 nM } [\text{H}]\text{Saxitoxin to } \text{t}5\text{A-201 cells transiently expressing wild type (solid line) or E1613R (dotted line) Na}^+ \text{ channels was carried out for 1 h at 4 }^\circ \text{C in the absence or presence of 500 nM TTX. Results were analyzed with LIGAND/EBDA and plotted. Apparent affinities from this Scatchard plot were 0.28 nM for the wild-type channel and 0.29 nM for the E1613R channel.}

 Importance of Charged Amino Acids in the IVS3-S4 Extracellular Loop for \( \alpha \)-Scorpion Toxin and ATX II Binding—To test the specificity of interaction between Glu and \( \alpha \)-scorpion toxin, Glu was changed to either Asp or Gln and LqTx affinity was determined. Although neither of these mutations caused a statistically significant change in LqTx binding affinity (43, Fig. 3A), some mutations significantly decreased LqTx affinity (43, Fig. 3A).

Since \( \alpha \)-scorpion toxins and sea anemone toxins share a common binding site (18), the ATX II affinity of these mutant channels was also tested by competition for \( ^{125} \text{I}-\text{LqTx} \) binding with unlabeled ATX II (43, open squares). The results were analyzed with LIGAND/EBDA and plotted. Apparent affinities from this Scatchard plot were 0.28 nM for the wild-type channel and 0.29 nM for the E1613R channel.

residues, Leu1614 through Tyr1618, individually with Ala re-

found to significantly increase affinity for Leu1614, Lys1617, Ser1621, and Lys1618 as well. Replacement of five consecutive charged residues in the IVS3-S4 Loop—

were important for a- scorpion toxin binding in wild type (Fig. 2A), indicating that this residue is probably important in channel assembly or folding.

Amino Acid Residues which Cause Differences in a-Scorpion Toxin Binding to Brain and Cardiac Na+ Channels—Cardiac Na+ channels bind a-scorpion toxin with lower affinity than brain Na+ channels (46). Rat brain type IIa Na+ channels expressed in tsA-201 cells have a Kd for LqTx binding of 2-5 nM (Figs. 1 and 5), whereas rat cardiac rH1 Na+ channels have a Kd of 18 nM (Fig. 5, B and C). These two Na+ channel a subunits have several differences in amino acid sequence in transmembrane segment IVS3 and in the IVS3-S4 extracellular loop (Fig. 5A). We constructed chimeric Na+ channels in which the amino acid residues in the IVS3-IVS4 segment of the rat brain type IIa subunit were converted to those in the cardiac isoform (Fig. 5A). Conversion of two extracellular residues (Chim1) or four extracellular residues (Chim2) did not significantly affect LqTx affinity (Fig. 5, B and C). The lack of significant effects of these extracellular mutations on a-scorpion toxin affinity is consistent with the results of alanine-scanning mutants (Fig. 3). In contrast, conversion of seven amino acid residues in this region, including three residues predicted to be in transmembrane segment IVS3, reduced LqTx affinity to a level similar to that of the cardiac Na+ channel (Chim3, Kd = 24 nM, Fig. 5, B and C). This result suggests that residues at the extracellular end of the IVS3 transmembrane segment confer isoform-specific LqTx binding properties on the a-scorpion toxin receptor site. We also analyzed the binding of LqTx to chimeras in which each of the other 15 extracellular loops of the rIIa a subunit had been individually replaced with the corresponding amino acids from the rH1 a subunit. All of these chimeras had Kd values for LqTx which were identical to rIIa channels, indicating that the amino acid residues near the extracellular end of the IVS3 transmembrane segment may be primarily responsible for differences in LqTx binding between cardiac and neuronal Na+ channels.

Electrophysiological Properties of Na+ Channels Containing Mutations of Glu1613—To determine the toxin affinity and electrophysiological properties of the E1613R and E1613H mutants, wild-type and mutant channels were transiently expressed in tsA-201 cells and analyzed by whole cell voltage clamp. The Na+ currents elicited by depolarization of cells expressing wild-type, E1613R (Fig. 6), or E1613H (not shown) appeared identical in time course without LqTx present. The voltage eliciting half-maximal activation (V1/2) from a holding potential of -140 mV was -19.3 ± 3 mV for wild-type rIIa channels (n = 3), -20.9 ± 2.3 mV for E1613R (n = 5), and -30.5 ± 3.7 mV for E1613H (n = 3) (data not shown). The V1/2 for steady-state inactivation was -59.0 ± 2.9 mV (n = 5) for wild-type channels, -61.4 ± 2.7 mV for E1613R (n = 5), and -74.0 ± 0.4 mV for E1613H (n = 3) (data not shown). Thus, the voltage dependence of channel activation and inactivation was very similar for wild type and E1613R, but both of these parameters were shifted in the hyperpolarized direction for E1613H.

Affinity of Na+ Channels Containing Mutations at Glu1613 for LqTx and ATX II—In the absence of toxin, Na+ conductance through wild-type Na+ channels decays to approximately 5% of the peak within 2 ms. In contrast, after addition of a saturating concentration of LqTx (20 nM) up to 70% of the current remains after 2 ms (Fig. 6A). Mutant E1613R was more than 10-fold less sensitive to a-scorpion toxin, since 200 nM toxin caused less slowing of inactivation for the mutant than 20 nM did for

FIG. 4. Displacement of bound 125I-LqTx from alanine-scanning mutants of the rIIa channel IVS3-S4 loop with unlabeled ATXII. TSA-201 cells transiently expressing wild type ( ), L1614A ( ), E1616A ( ), K1617A ( ), and Y1618A ( ) were incubated for 1 h at 37 °C with 0.5 nM 125I-LqTx and increasing concentrations of unlabeled ATXII. Unbound toxin was then removed by several washes as described under “Experimental Procedures,” and bound toxin was determined by γ-sciillation spectrophotometry. Data was normalized to maximal binding (100%) and fit using the equation, Bound = B∞(1 + Kd/[LqTx]) + c, where c corresponds to the nonspecific binding component. Apparent IC50 values for inhibition of LqTx binding by ATXII calculated from these fits were 101 fmol for wild type ( ), 125 fmol for L1614A ( ), 275 fmol for rIIa-K1617A ( ), and 85 fmol for rIIa-Y1618A ( ) channels. In the presence of 0.5 nM 125I-LqTx, maximal labeled toxin bound per well was 3.22 fmol for wild type, 2.56 fmol for L1614A, 1.39 fmol for K1617A, and 1.35 fmol for Y1618A channels.
To determine the $K_D$ for LqTx, transiently expressed wild-type or mutant Na$^+$ channels were incubated with varying concentrations of LqTx for 30 min at 37°C. Cells were then voltage clamped at a holding potential of $-140$ mV, and currents were elicited by a depolarizing pulse to $-10$ mV. The fraction of conductance remaining 2 ms after the peak is proportional to the number of channels modified by $\alpha$-scorpion toxin, and this fraction can be used to estimate receptor occupancy and toxin affinity according to the formula,

$$K_D = \frac{F_G}{F_G - F} \cdot \frac{1}{F} \text{ (Eq. 1)}$$

where $F_G$ is the fraction of Na$^+$ current remaining 2 ms after the beginning of the pulse, and $F$ is the maximum fraction of current 2 ms after the beginning of the pulse in the presence of a saturating concentration of $\alpha$-scorpion toxin (1 $\mu$M, Ref. 47).

From this analysis, the $K_D$ for LqTx binding was 1.7 nM for the wild-type channel, 14 nM for E1613H, and 106 nM for E1613R channels (Table II).

Similar experiments were carried out for ATX II binding to mutant E1613R. Addition of 1 nM ATX II slowed the inactivation of a significant fraction of wild-type Na$^+$ channels, and 10 nM slowed inactivation of most wild-type Na$^+$ channels (Fig. 6C). Averaged results indicated a $K_D$ value at $-140$ mV of 3.3 nM (Table II). A concentration of 10 nM ATX II was much less effective in slowing inactivation of E1613R than 10 nM ATX II was in slowing wild-type (Fig. 6D), and averaged results indicated a $K_D$ value of 270 nM for E1613R (Table II). Thus, the E1613R mutation reduces the affinity for LqTx 62-fold and the affinity for ATX II 82-fold. These results are consistent with the conclusion that Glu$^{1613}$ is an integral component of overlapping binding sites for both $\alpha$-scorpion toxins and sea anemone toxins.

Electrophysiological Determination of the Kinetics of LqTx Binding to Wild-type, E1613R, and E1613H Channels—Binding of $\alpha$-scorpion toxins is reversible, and the difference in toxin
The indicated amino acids were substituted for Glu1613 by oligonucleotide-directed mutagenesis, the resulting mutant Na\(^+\) channels were expressed in tsA-201 cells, and the \(K_p\) values for binding of LqTx and ATX II were measured as described in Fig. 6 and the text.

| Mutant   | Loop       | \(\Delta Q\) | \(K_p\) (LqTx) \(\text{nm}\) | \(n\) | \(K_p\) (ATX II) \(\text{nm}\) | \(n\) |
|----------|------------|---------------|-------------------------------|------|-------------------------------|------|
| Wild type| IVS3-S4    | 0             | 1.71 ± 1.1                   | 5    | 3.3 ± 2.0                     | 6    |
| E1613H   | IVS3-S4    | +1            | 14.0 ± 4.0                   | 3    |                               |      |
| E1613R   | IVS3-S4    | +2            | 106 ± 14                     | 6    | 268 ± 160                     | 6    |

The table shows the effects of mutations of Glu1613 on binding of LqTx and ATX II. The indicated amino acids were substituted for Glu1613 by oligonucleotide-directed mutagenesis, the resulting mutant Na\(^+\) channels were expressed in tsA-201 cells, and the \(K_p\) values for binding of LqTx and ATX II were measured as described in Fig. 6 and the text.

The table shows the effects of mutations of Glu1613 on binding of LqTx and ATX II. The indicated amino acids were substituted for Glu1613 by oligonucleotide-directed mutagenesis, the resulting mutant Na\(^+\) channels were expressed in tsA-201 cells, and the \(K_p\) values for binding of LqTx and ATX II were measured as described in Fig. 6 and the text.

The rates of toxin association were assessed for the wild-type and mutant channels using a 200-ms prepulse to +100 mV to cause toxin dissociation followed by progressively longer hyperpolarizing prepulses to follow the time course of toxin re-binding and action. Fig. 8A shows the stimulus protocol and the cumulative slowing of inactivation as toxin reassociates with the wild-type channel. The first Na\(^+\) current recorded 102 ms after repolarization to −120 mV was rapidly inactivating; more slowly inactivating currents were recorded following progressively longer hyperpolarizing prepulses as toxin binding approached equilibrium. The time courses of toxin association at several different potentials were determined for wild-type and mutant channels using this protocol. The association kinetics were fit with a single exponential time constant, which was 548...
...rates were much faster for the E1613R channels and were strongly voltage-dependent, with more depolarized potentials significantly accelerating dissociation (Fig. 9B). A semilog plot of the voltage dependence of toxin dissociation from E1613R channels was linear between 0 and +100 mV and indicated an e-fold increase in toxin dissociation rate for every 36.4 mV of depolarization. Similar experiments with the wild-type channel were more difficult as the dissociation rate was much slower and could only be measured above +60 mV. However, using the dissociation rates at +60, +80, and +100 mV (Fig. 9B), the toxin dissociation rate for wild-type channels was found to increase e-fold for every 22–25 mV. Although the narrow voltage range examined and the much slower dissociation rate for the wild-type channel make the comparison difficult, it appears that the voltage dependence of the rate of toxin dissociation is less steep for mutant E1613R than for wild type in this positive voltage range.

Voltage Dependence of Toxin Binding at Equilibrium—Previous models describing the voltage dependence of α-scorpion toxin binding have proposed two receptor states with high and low toxin affinities. These states are in reversible equilibrium described by a voltage-dependent, allosteric equilibrium constant (17, 19, 22). A negative membrane potential favors the high affinity conformation, de-allosterization favors the low affinity conformation, and intermediate voltages reveal a distribution of receptor affinities as described by the Monod-Wyman-Changeux model for allosteric modulation of oxygen binding to hemoglobin (17, 19, 48). Mutations of Glu1613 may cause a reduction in binding affinity by directly disrupting binding, or by indirectly shifting the voltage-dependent allosteric equilibrium constant. By measuring the extent of toxin association at equilibrium at a range of negative membrane potentials, the $K_D$ values for LqTx binding at voltages between −120 mV and 0 mV for wild-type and E1613R channels were determined (Fig. 10, A and B, circles). With both channels, the voltage-dependent affinity change was sigmoidal, with the conversion between high and low affinity states occurring between −80 and −40 mV. Fitting with the Boltzmann equation yielded a $V_{1/2}$ value for the affinity change of $−55 ± 5.9$ mV ($n = 3$) and a slope factor of e-fold per 4.6 ± 1.7 mV for wild-type channels and a $V_{1/2}$ of $−51.6 ± 1.8$ mV ($n = 3$) and slope factor of e-fold per 4.2 ± 0.4 mV for E1613R channels (Fig. 10, legend). Similar results were obtained with mutant E1613R when the extent of toxin dissociation at equilibrium was measured (Fig. 10B, squares). At potentials more negative than −100 mV, the $K_D$ values for wild-type and E1613R channels were 1.7 and 106 mN, respectively. At +40 to +100 mV, the $K_D$ values were 26 and 800 mN, respectively. These results indicate that, although the binding affinities of these channels differ by over 60-fold at −100 mV, the voltage dependence of LqTx binding to these channels at equilibrium is similar.

By combining the equations $K_D = k_d/k_a$, and $\tau = (k_d + k_1)^{-1}$, the $K_D$ values determined at each potential, and the association and dissociation time constants determined above, the corresponding dissociation ($k_1$) or association ($k_d$) rate constants could be calculated using the equations

$$k_1 = (\tau_{\text{assoc}}[\alpha - \text{ScTx}](1 + K_D))^{-1}$$

(Eq. 2)

and

$$k_1 = (\tau_{\text{assoc}}[1 + K_D^{-1}])^{-1}$$

(Eq. 3)

At negative potentials, the $K_D$ and association time constants for LqTx binding to wild-type and E1613R channels were approximately constant between −120 and −80 mV (Figs. 9 and 10), indicating that the dissociation rate did not change substantially over this voltage range. Using the $\tau_{\text{assoc}}$ rates from several experiments at −100 mV and $K_D$ values at −100 mV...

FIG. 8. Determination of rates of LqTx association to transiently expressed Na$^+$ channels at different membrane potentials. A, cells transiently expressing the wild-type Na$^+$ channel were incubated in 100 nM LqTx for 10 min at a holding potential of −120 mV to establish binding. A 200-ms depolarizing step to +100 was then used to dissociate all of the bound toxin and the cell was returned to −120 mV for increasing durations (102, 202, 402, 602, 1102, 2102, 3102, or 4102 ms, see diagram) before testing the extent of inactivation removal with a 10-ms test pulse to 0 mV. The pulse following 102 ms at −120 mV elicited a rapidly inactivating current. Subsequent cycles with longer re-binding periods at −120 mV elicited Na$^+$ currents with successively slower inactivation kinetics until equilibrium was reached. Re-binding periods at different potentials (e.g. −100 and −80 mV) were used to assess the voltage dependence on LqTx association. B, kinetics of association of 100 nM LqTx to cells expressing the wild-type Na$^+$ channel using the same protocol at conditioning potentials of −80 mV (●) and −100 mV (○). The ratio of current 2 ms after the depolarizing step relative to peak current was plotted in order to normalize for the change in peak current and to determine the extent of inactivation removal by LqTx. The association time courses were fit by single exponential time constants of $\tau_{−80\text{ mV}} = 639.6$ ms and $\tau_{−100\text{ mV}} = 550.5$ ms. C, association rates for LqTx binding to cells transiently expressing E1613R at −60 mV (▼), $\tau = 695.8$ ms, −70 mV (▲), $\tau = 520.8$ ms, −80 mV (▲), $\tau = 388.1$ ms, −90 mV (□), $\tau = 506.1$ ms, −100 mV (●), $\tau = 440.9$ ms, −110 mV (□), $\tau = 463.4$ ms, and −120 mV (○), $\tau = 485.5$ ms. The LqTx occupancy at −40 mV (▼) was too low to fit. ±208 ms for wild-type at −100 mV ($n = 4$, Fig. 8B), 429 ± 49 ms for E1613R ($n = 3$, Fig. 8C), and 365 ± 204 ms for the E1613H ($n = 5$, data not shown). By comparing the time constants measured at a range of voltages (Fig. 8, legend), the rate of toxin association at negative potentials was found to be voltage-independent, and similar for wild-type and mutant channels.

Fig. 9 compares the voltage dependence of time constants for LqTx association and dissociation. The time constants for association of LqTx between −120 and −60 mV appeared unaffected by voltage and were similar for both of these channels (Fig. 9A) and for E1613H (not shown). In contrast, the dissociation rates were much faster for the E1613R channels and were...

...mS for E1613R (Fig. 9B, legend), the rate of toxin association at negative potentials was found to be voltage-independent, and similar for wild-type and mutant channels.

Fig. 9 compares the voltage dependence of time constants for LqTx association and dissociation. The time constants for association of LqTx between −120 and −60 mV appeared unaffected by voltage and were similar for both of these channels (Fig. 9A) and for E1613H (not shown). In contrast, the dissociation rates were much faster for the E1613R channels and were strongly voltage-dependent, with more depolarized potentials significantly accelerating dissociation (Fig. 9B). A semilog plot of the voltage dependence of toxin dissociation from E1613R channels was linear between 0 and +100 mV and indicated an e-fold increase in toxin dissociation rate for every 36.4 mV of depolarization. Similar experiments with the wild-type channel were more difficult as the dissociation rate was much slower and could only be measured above +60 mV. However, using the dissociation rates at +60, +80, and +100 mV (Fig. 9B), the toxin dissociation rate for wild-type channels was found to increase e-fold for every 22–25 mV. Although the narrow voltage range examined and the much slower dissociation rate for the wild-type channel make the comparison difficult, it appears that the voltage dependence of the rate of toxin dissociation is less steep for mutant E1613R than for wild type in this positive voltage range.
Steady-state binding following dissociation was monitored at positive potentials with E1613R because of the rapid dissociation, low affinity, and of LqTx to cells expressing E1613R was assessed using steady-state binding with both the on-rate (Fig. 8) and off-rate (Fig. 7) protocols. For determination of steady-state binding at each potential, a 2000-ms pulse to the indicated binding potential was repeated 11 or 16 times in order to reach steady-state at each binding potential. A 2000-ms pulse to allow LqTx to bind the channel, followed by a 100-ms period at −120 to recover from fast inactivation and a 10-ms test pulse to 0 mV. This was repeated 11 or 16 times in order to reach steady-state at each binding potential. $k_0$ was calculated with the equation $k_0 = [LqTx] [IF_{LqTx} / F_0 - 1]$ as described in the text and plotted as a function of the voltage at which binding was monitored. The values of $k_0$ were fit with a Boltzmann equation (solid line) of the form $K_0 = 1/[1 - \exp(-V/V_{1/2})]$, where $V_{1/2}$ is the potential giving half-maximal affinity and $k$ is the slope factor. For this cell the $V_{1/2}$ was −55 mV and the slope factor was 3.8 mV. The mean values were $V_{1/2} = -55 \pm 5.9$ mV and $k = 4.6 \pm 1.7$ mV (n = 3). B, binding of LqTx to cells expressing E1613R was assessed using steady-state binding with both the on-rate (Fig. 8C, ○) and off-rate (Fig. 7C, ○) protocols. Steady-state binding following dissociation was monitored at positive potentials with E1613R because of the rapid dissociation, low affinity, and large amount of toxin required for association at these potentials. A Boltzmann fit (solid line) gave values for this cell of $V_{1/2} = 50.8$ mV and $k = 4.6$ mV. The mean values were $V_{1/2} = -51.6 \pm 1.8$ mV and $k = 4.2 \pm 0.4$ mV (n = 3).

**FIG. 9.** Summary of association and dissociation time constants for binding of LqTx to wild-type and E1613R Na$^+$ channels. A, mean time constants of LqTx association to wild-type (●) and E1613R (■) channels at different recovery potentials. B, mean time constants of LqTx dissociation from wild-type (●) and E1613R (■) channels at different conditioning potentials. Data were fit with single exponential constants of 22.4 mV and 36.4 mV, respectively, and plotted on a semi-log scale.

**FIG. 10.** Voltage dependence of LqTx binding at equilibrium. A, for cells transiently expressing the wild-type IIa Na$^+$ channel, a variation of the on-rate protocol was used (Fig. 8) after incubation with 2 nM LqTx for 30 min. A 200-ms pulse to +100 mV was applied to cause complete toxin dissociation followed by repolarization for 100 ms at −120 mV to recover from fast inactivation and a 10-ms test pulse to 0 mV to assess toxin dissociation. For determination of steady-state binding at each potential, a 2000-ms pulse to the indicated binding potential was used to allow LqTx to bind the channel, followed by a 100-ms period at −120 to recover from fast inactivation and a 10-ms test pulse to 0 mV. This was repeated 11 or 16 times in order to reach steady-state at each binding potential. $k_0$ was calculated with the equation $k_0 = [LqTx] [IF_{LqTx} / F_0 - 1]$ as described in the text and plotted as a function of the voltage at which binding was monitored. The values of $k_0$ were fit with a Boltzmann equation (solid line) of the form $K_0 = 1/[1 - \exp(-V/V_{1/2})]$, where $V_{1/2}$ is the potential giving half-maximal affinity and $k$ is the slope factor. For this cell the $V_{1/2}$ was −55 mV and the slope factor was 3.8 mV. The mean values were $V_{1/2} = -55 \pm 5.9$ mV and $k = 4.6 \pm 1.7$ mV (n = 3). B, binding of LqTx to cells expressing E1613R was assessed using steady-state binding with both the on-rate (Fig. 8C, ○) and off-rate (Fig. 7C, ○) protocols. Steady-state binding following dissociation was monitored at positive potentials with E1613R because of the rapid dissociation, low affinity, and large amount of toxin required for association at these potentials. A Boltzmann fit (solid line) gave values for this cell of $V_{1/2} = -50.8$ mV and $k = 4.6$ mV. The mean values were $V_{1/2} = -51.6 \pm 1.8$ mV and $k = 4.2 \pm 0.4$ mV (n = 3).

**DISCUSSION**

High Affinity α-Scorpion Toxin Binding to Transiently Expressed Na$^+$ Channels—Voltage-gated Na$^+$ channels can be functionally expressed in a variety of nonexcitable cells. Our results show that LqTx binding with the high affinity characteristic of neurons can be observed for Na$^+$ channels expressed in tsA-201 cells if the membrane potential of the cells is hyperpolarized by incubation in Na$^+$-free medium containing gramicidin. Thus, high affinity, voltage-dependent binding of α-scorpion toxin requires only the α subunit of the Na$^+$ channel and a sufficiently negative membrane potential. Previous labeling of Na$^+$ channels with photoreactive derivatives of α-scorpion toxin have shown specific incorporation into both the α and β1 subunits of the Na$^+$ channel (24–26), and different photoreactive derivatives label the two subunits in different ratios (26). In light of our present results, photolabeling of the β1 subunit likely represents covalent attachment of photoreactive LqTx to an area of the β1 subunit which is near, but on the periphery of, the α-scorpion toxin receptor site on the α subunit.

Glu$^{1613}$ and the IVS3-S4 Loop Are Critical Determinants of α-Scorpion Toxin and ATX II Binding—Three experimental results indicate that the IVS3-S4 extracellular loop of the Na$^+$ channel α subunit is a critical component of the receptor for α-scorpion and sea anemone toxins. 1) Changing Glu$^{1613}$ in this loop to Arg or His dramatically reduces LqTx and ATX II affinity. 2) Changes in either charge or size of Glu$^{1613}$ and Glu$^{1616}$ in this loop significantly affect ATX II affinity, indicat-
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...ing a tight interaction with these residues. 3) Replacement of individual residues within this loop with Ala identifies a third charged residue (Lys<sup>1617</sup>) and six uncharged residues that significantly affect LqTx and/or ATX II affinity. It is likely that Glu<sup>1613</sup> and the IVS3-S4 loop constitute an important component of neurotoxin receptor site 3, the α-scorpion toxin and sea anemone toxin receptor site.

Previous photoaffinity labeling studies led to covalent incorporation of photoreactive α-scorpion toxin derivatives into the IIS5-S6 loop of the α subunit (28), and anti-peptide antibodies directed against sequences in the IIS5-S51, IIS5-S52, and IVS5-S51 segments of the α subunit reduce α-scorpion toxin binding (29). While it was suggested that the IVS3-S4 loop may play a role in α-scorpion toxin binding (29), this extracellular loop was not experimentally identified as part of the α-scorpion toxin receptor site in previous studies, perhaps because the site-directed antibodies and the photoreactive moiety on the α-scorpion toxin derivatives could not interact with this short loop. Our present results show that negatively charged amino acid residues in the IIS5-S6, IVSS5-S51, and IVSS5-S6 loops are not required for α-scorpion toxin binding, but that both negatively charged and neutral amino acid residues in the IVS3-S4 loop are required for high affinity binding. Further mutagenesis studies will be required to identify the individual amino acid residues in the IIS5-S6, IVSS5-S51, and IVSS5-S6 loops which also participate in toxin binding.

Acidic amino acids of the Na<sup>+</sup> channel have been proposed to be important for α-scorpion toxin and anemone toxin binding based on structural information about these toxins (10–16). The identification of Glu<sup>1613</sup> as an important determinant of α-scorpion toxin binding and Glu<sup>1613</sup> and Glu<sup>1616</sup> as determinants of anemone toxin binding is consistent with an electrostatic interaction between acidic residues of the Na<sup>+</sup> channel and basic residues of α-scorpion toxins or sea anemone toxins. These mutations also demonstrate that this loop contains more determinants for binding of ATX II than LqTx, and therefore that the receptor sites for α-scorpion toxin and anemone toxins are overlapping but not identical. Previous studies with anemone toxins have implicated the conserved Arg<sup>14</sup> residue in anemone toxin binding (14). Recent mutagenesis studies of the Anthopleurin in anemone toxins found that double mutants which neutralize both Arg<sup>12</sup> and Arg<sup>48</sup> in ApB have the most dramatic reduction in affinity among the double mutants, while mutations of residues Arg<sup>12</sup> and Lys<sup>49</sup> alter the cardiac selectivity of ApA (16). Based on the residues of the Na<sup>+</sup> channel IVS3-S4 loop identified in toxin binding, residues 12 and 14 of ApB may interact with Glu<sup>1613</sup> and Glu<sup>1616</sup> of the Na<sup>+</sup> channel α subunit, whereas Lys<sup>49</sup> may interact with residues outside of this loop to contribute to cardiac selectivity. Sea anemone toxins are different from α-scorpion toxin in many respects, including smaller size, unique sequence, distinct three-dimensional structure, generally lower affinities for neuronal channels and higher affinities for cardiac Na<sup>+</sup> channels, and weaker inhibition of binding with depolarization (2, 7–9, 13, 14, 18, 22). Although we did not identify acidic binding determinants in regions previously implicated in α-scorpion toxin binding (IIS5-S6 and IVSS5-S6; Refs. 28 and 29), non-acidic residues in these other regions may contribute to α-scorpion toxin binding by providing unique determinants that are involved in interactions with LqTx but not ATX II. This is consistent with the biochemical evidence for the involvement of the IIS5-S6 and IVSS5-S6 loops in toxin binding (28, 29), and with the previously suggested concept of multiple attachment points for α-scorpion toxin binding (10, 11, 14). Our results indicate that ATX II and LqTx bind to overlapping, but not identical, determinants in the IVS3-S4 loop which form part of neurotoxin receptor site 3.

Amino Acid Residues in Segment IVS3 Cause Differences in α-Scorpion Toxin Binding between Brain and Cardiac Na<sup>+</sup> Channels—Na<sup>+</sup> channels in neurons have a significantly higher affinity for LqTx than Na<sup>+</sup> channels in cardiac cells (46). Consistent with this, rat brain type IIa Na<sup>+</sup> channels have 4–10-fold higher affinity than rat cardiac H1 channels when expressed in tsA-201 cells. However, the difference in binding affinity between these two cloned and expressed channels is not as great as the difference observed between neuronal and cardiac cells in cell culture (65-fold, Ref. 46). This may reflect differences in the membrane potential in different cell populations, in the voltage dependence of toxin binding between the two Na<sup>+</sup> channel isoforms, in the assay methods used in the different studies (toxin-stimulated ion flux versus toxin binding), or in the channel processing and second messenger modulation between different cell types. Nevertheless, our results suggest that the difference in K<sub>D</sub> for α-scorpion toxin between the r11a and rh1 α subunit isoforms expressed in parallel in tsA-201 cells is due, at least in part, to amino acid sequence differences in the IVS3 transmembrane segment. These amino acid residues near the extracellular end of the IVS3 transmembrane segment may interact directly with the bound LqTx polypeptide themselves, or they may influence the position of the extracellular end of the IVS3 segment containing Glu<sup>1613</sup> or the conformation of the IVS3-S4 loop, which our results suggest are sites of direct toxin interaction.

Kinetics of α-Scorpion Toxin Binding—In our experiments, the kinetics of α-scorpion toxin binding have been determined over a wider range of voltages (–120 to +100 mV) than in previous studies in order to determine rate constants and equilibrium dissociation constants. The association rate constants determined in the present experiments at –100 mV for wild type and E1613R (1.82 × 10<sup>7</sup> and 2.20 × 10<sup>7</sup> M<sup>–1</sup> s<sup>–1</sup>, respectively) were faster than the k<sub>a</sub> value of 1.5 × 10<sup>7</sup> M<sup>–1</sup> s<sup>–1</sup> determined electrophysiologically at –100 mV with a scorpion toxin of lower affinity (αLq1a, Ref. 22), but quite similar to the k<sub>a</sub> value of the higher affinity Aahl1 scorpion toxin (k<sub>a</sub> = 1.5 × 10<sup>7</sup> M<sup>–1</sup> s<sup>–1</sup>) determined biochemically (49). By using the k<sub>a</sub> values determined at –100 mV, the corresponding calculated k<sub>−1</sub> values of 3.09 × 10<sup>–2</sup> s<sup>–1</sup> and 2.33 s<sup>–1</sup> for the wild-type and E1613R channels, respectively, are faster than the k<sub>−1</sub> values determined for αLq1a or Aahl1 (both 1.6 × 10<sup>–2</sup> s<sup>–1</sup>, Refs. 22 and 49). Thus, the association rate constants determined at negative potentials are consistent with previous work using other α-scorpion toxins, the calculated dissociation rate constants are somewhat faster than for those other toxins, and the difference in affinity of the wild-type and E1613R channels at negative potentials is entirely due to the difference in the dissociation rate.

Voltage Dependence of α-Scorpion Toxin Binding at Equilibrium—The proximity of the IVS3-S4 loop to the voltage-sensing IVS4 transmembrane segment provides a potential molecular basis for understanding the voltage dependence of toxin binding and the coupling of activation to inactivation. The voltage-dependence of equilibrium binding is well described by a Boltzmann distribution (19–21, Fig. 10), and the voltage-dependent changes in affinity are observed in the same range (–80 to –40 mV) over which voltage-dependent gating transitions occur within the channel. The midpoint for the voltage-dependent change in affinity for α-scorpion toxin is essentially identical for wild-type and mutant channels. The steepness of the change in α-scorpion toxin binding affinity (k<sub>a</sub> = 4.2–4.6 mV) is also similar between wild-type and mutant channels, but is steeper than previously reported for other α-scorpion toxins binding to Na<sup>+</sup> channels in amphibian node of Ranvier (k<sub>a</sub> = 4–8 mV) in anemone toxins (50), and for other α-scorpion toxins binding to Na<sup>+</sup> channels in neuronal cells (46, 51).
Wang and Strichartz (23) noted that both the range and steepness of voltage dependence were dependent on the toxin studied, which may explain most of the quantitative differences in voltage dependence in different studies. The similarity in voltage dependence of LqTx binding to wild-type and E1613R channels indicates that the 30- to 60-fold change in affinity as a result of the E1613R mutation does not strongly affect the voltage-dependent transition between channel states in the voltage range from −80 to −40 mV.

In our experiments, the voltage dependence of toxin binding to transfected ril channels was significantly more negative than the voltage dependence of activation, and steeper and slightly more positive than the voltage dependence of steady-state inactivation. In comparison to our results, the voltage dependence of α-scorpion toxin binding to neuroblastoma cells and frog sartorius muscle measured in equilibrium binding experiments was much more positive than steady-state inactivation and correlated approximately with the voltage dependence of activation (17, 19), while the voltage dependence of toxin binding to Na+ channels measured electrophysiologically in frog node of Ranvier was 20 mV more positive than steady-state inactivation, and was positioned between the voltage dependence of activation and inactivation (20, 21). These differences in voltage dependence of toxin binding relative to channel gating may result from inherent isoform- or species-specific differences in gating among different Na+ channels.

The process of inactivation is thought to be intrinsically voltage-independent and to acquire its voltage dependence from coupling to activation (50). Multiple voltage-dependent transitions between closed states occur during the activation process (50). These voltage-dependent transitions among closed states along the activation pathway are likely to be responsible for voltage-dependent coupling of activation to steady-state inactivation and for voltage-dependent changes in affinity for α-scorpion toxins and sea anemone toxins. The voltage dependence of transitions among closed states leading to activation falls between that of activation and steady-state inactivation (50). The slowing of inactivation and the reduction in the steepness of voltage dependence of steady-state inactivation caused by α-scorpion toxins and sea anemone toxins (2, 17–23) suggest that the toxin receptor site undergoes a conformational change that is required for fast inactivation, that bound toxin slows this conformational change and thereby slows the inactivation process, and that toxin binding is destabilized as a result of conformational changes which lead ultimately to inactivation. Therefore, our results implicate the S3-S4 loop in domain IV of the α subunit in coupling of activation to inactivation.

Voltage Dependence of Dissociation of α-Scorpion Toxin in the Positive Potential Range—The KD for α-scorpion toxin remained constant from −40 mV up to at least +100 mV. In contrast, dissociation rates for α-scorpion toxin increased steadily between 0 and +100 mV. The rapid rates of dissociation in this voltage range may reflect the rate of change of state of the toxin-channel complex from a high affinity conformation at negative membrane potentials to a low affinity conformation at positive potentials as well as toxin unbinding itself. Voltage-dependent dissociation is likely to be driven by the voltage-dependent conformational change to the low affinity state. The voltage dependence of LqTx dissociation can be fit with a single exponential equation (21, 22, 51) and the voltage dependence of dissociation from wild-type and E1613R channels (e-fold/22–25 mV and e-fold/36 mV, respectively) is similar to previous reports (e-fold/25 mV, Ref. 21; e-fold/32 mV, Ref. 51). The significance of the apparent difference in steepness of the voltage dependence of toxin dissociation at positive membrane poten-

![Fig. 11. A model for the α-ScTx/ATX receptor.](image) A cross-sectional view of the Na+ channel depicting the juxtaposition of domains I and IV on one side of the ion pore with α-ScTx or ATX bound. Also shown are the regions previously implicated in α-ScTx binding (lightly shaded segments), sites of glycosylation (a), the intracellular II-IV loop which acts as the inactivation gate (IFM) and contains a known site of phosphorylation (P), and the IVS3-S4 loop closely interacting with bound toxin (dark shaded segment). Outward movement of the IVS4 segment with depolarization is inhibited by bound toxin and accelerates the off-rate of bound toxin. Slowing of this translocation or of a subsequent conformational change in the IVS3-S4 loop is proposed to slow the rate of fast inactivation.

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result in muscular dysfunction. Together with this previous work, our results therefore identify a short extracellular loop of the Na\textsuperscript+ channel molecule that is critical for the binding of α-scorpion and sea anemone toxins and is also important for the coupling of channel activation to fast inactivation.

Fig. 11 illustrates a model of the α-scorpion toxin receptor site based on the LoTx and ATX II binding determinants reported here, and two regions previously implicated in α-scorpion toxin binding (28, 20). In this model, α-scorpion toxins are proposed to bind across the IVS3-S4 loop through electrostatic interactions with Glu\textsuperscript{1613} and additional, unidentified contacts in the IS5-S6 and IVS5-S6 loops. The voltage dependence of α-scorpion interactions with anemone toxin binding is due to steric interactions as the IVS4 segment moves outward in response to the coupling of channel activation to fast inactivation.

The binding of sea anemone toxins differs in that they bind intimately with several residues of the IVS3-S4 loop through electrostatic, hydrogen-bonding, or van der Waals interactions. Sea anemone toxin binding is loss voltage-dependent (18, 23), perhaps because it has fewer binding contacts outside of the IVS3-S4 loop so is subjected to less steric or torsional distortion when the channel is depolarized. Both of these toxins may slow inactivation by slowing or preventing the resulting conformational changes in the IVS3-S4 loop upon translocation of the IVS4 segment.

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