Molecular Requirements for Recognition of Brain Voltage-gated Sodium Channels by Scorpion α-Toxins

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The scorpion α-toxin Lqh2 (from Leiurus quinquestriatus hebraeus) is active at various mammalian voltage-gated sodium channels (Na s) and is inactive at insect Na s. To resolve the molecular basis of this preference we used the following strategy: 1) Lqh2 was expressed in recombinant form and key residues important for activity at the rat brain channel rNa 1.2a were identified by mutagenesis. These residues form a bipartite functional surface made of a conserved "core domain" (residues of the loops connecting the secondary structure elements of the molecule core), and a variable "NC domain" (five-residue turn and the C-tail) as was reported for other scorpion α-toxins. 2) The functional role of the two domains was validated by their stepwise construction on the similar scaffold of the anti-insect toxin Lqh01T. Analysis of the activity of the intermediate constructs highlighted the critical role of Phe 15 of the core domain in toxin potency at rNa 1.2a, and has suggested that the shape of the NC-domain is important for toxin efficacy. 3) Based on these findings and by comparison with other scorpion α-toxins we were able to eliminate the activity of Lqh2 at rNa 1.4 (skeletal muscle), hNa 1.5 (cardiac), and rNa 1.6 channels, with no hindrance of its activity at Na s 1.1–1.3. These results suggest that by employing a similar approach the design of further target-selective sodium channel modifiers is imminent.

The pivotal role of voltage-gated sodium channels (Na s) in excitability mark them as major targets for a large variety of toxins that bind at distinct receptor sites and modify their gating (1). These channels are large membrane proteins made of a pore-forming α-subunit of ~260 kDa and auxiliary β-subunits of ~30 kDa. The α-subunit is composed of four homologous domains (D1–D4), each consisting of six α-helical transmembrane segments (S1–S6) connected by intracellular and extra-cellular loops. A key feature in Na s function is their ability to rapidly activate and inactivate, leading to transient increase in Na + conductance through the cell membrane. This mechanism is attributed to the ability of the positively charged S4 voltage sensors to move across the membrane in response to changes in membrane potential (1, 2).

In mammals, at least nine genes encode a variety of Na s subtypes (1, 3), whose expression varies greatly in different tissues (Na s 1.1–1.3 mainly in the central nervous system; Na s 1.6 in both central and peripheral neurons; Na s 1.7 in the peripheral nervous system; Na s 1.8 and Na s 1.9 in sensory neurons; Na s 1.4 and Na s 1.5 in skeletal and cardiac muscles, respectively). Na s subtypes are distributed heterogeneously in the human brain and their expression is regulated under developmental and pathological conditions (1, 3–5). In addition, many disorders in humans result from abnormal function due to mutations in various Na s genes (6–8). Thus, ligands that show specificity for Na s subtypes may be used for their identification at various tissues and as leads for design of specific drugs. This requires that the bioactive surfaces of these ligands be resolved along with molecular details that determine their specificity.

Among the wide range of Na s modifiers, those derived from scorpion venoms play an important role in studying channel activation (β-toxins) and inactivation (α-toxins) (9–11). The channel site of interaction with scorpion α-toxins, named neurotoxin receptor site-3 (12), is shared also by structurally unrelated toxins from sea anemone and spider venoms (13, 14), which raises questions as to its architecture and boundaries. Based on the findings that site-3 toxins eliminate a gating charge component associated with the movement of D4/S4 (15, 16), and that this segment plays a critical role in coupling channel inactivation by hindering the outward movement of this segment during depolarization (9).

Scorpion α-toxins constitute a class of structurally and functionally related 61–67-residue long polypeptides reticulated by four conserved disulfide bridges. Despite a common βαββ core (10, 18, 19) these toxins are highly diverse in sequence and preference for insect and mammalian Na s. Indeed, the α-toxin class is divided to pharmacological groups according to their toxicity in insects and mice brain and ability to compete on binding to insect and mammalian Na s (10) (supplemental Fig.

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S1): (i) classical anti-mammalian toxins, such as Aah2 (from Androctonus australis hector) and Lqh2 (from Leiurus quinquestriatus hebraeus), which bind with high affinity to Naₙ,s at rat brain synaptosomes and are practically non-toxic to insects; (ii) α-toxins, such as Lqh2αT, which strongly affect insect Naₙ,s and are weak in mammalian brain; and (iii) α-like toxins, such as Lqh3 and BmKM1 (from Buthus martensii Karsch), which are active in both mammalian brain and insects.

Efforts to identify α-toxin residues involved in the interaction with the Naₙ, receptor site-3 revealed a generally common bioactive surface divided to two topologically distinct domains: a conserved “core domain” formed by residues of the loops connecting the secondary structure elements of the molecule core, and a variable “NC domain” formed by the five-residue turn (residues 8–12) and the C-tail (20–23). These analyses raised the hypothesis that a protruding conformation of the NC domain correlates with high activity at insect Naₙ,s whereas a flat conformation of this domain appears in α-toxins active at the brain channel rNa₁,2a (21). The correlation of this structural difference with toxin preference for Naₙ, subtypes was corroborated by constructing the bioactive surface of Lqh2αT on the scaffold of the anti-mammalian α-toxin Aah2 ending up with a chimera (Aah2Lqh2αT(face)) active on insects, whose NC domain is in the protruding conformation (21). Despite this result, the molecular requirements that enable high affinity binding of classical α-toxins to mammalian Naₙ,s have not been clarified, and only initial data about the channel region that constitutes receptor site-3 is available (Refs. 24–26; also see Ref. 10 for review).

Lqh2 is a 64-residue long toxin from L. quinquestriatus hebraeus (Israeli yellow scorpion) (27) that is almost identical in sequence (96% identity) to the most active anti-mammalian toxin, Aah2, whose structure and action are documented (18, 28, 29). By functional expression and mutagenesis we uncovered residues on the Lqh2 exterior that are putatively involved in bioactivity. By construction of these residues on the scaffold of the anti-insect toxin Lqh2αT we confirmed their bioactive role and differentiated those that determine toxin potency from those contributing to toxin efficacy. Comparison to other α-toxins was then instrumental for the design of an Lqh2 mutant that exhibits high specificity for the neuronal channels hNa₁,1, rNa₁,2a, and rNa₁,3.

**EXPERIMENTAL PROCEDURES**

**Materials, Bacterial Strains, and Animals**—Native Lqh2 was purchased from Latoxan (Valence, France). Escherichia coli strain DH5α was used for plasmid constructions, and the BL21 strain (DE3, pLys) was used for toxin expression using the vector pET-14b (Novagen) in a protocol similar to that described previously (30). cDNAs encoding hNa₁,1 (human, Ref. 31), rNa₁,2 (rat, Ref. 32), and rNa₁,3 (33) were subcloned into the pCDM8 vector, the cDNA sequence was determined, and any amino acid sequence errors were corrected to the sequences recorded in GenBank. The pAlter vectors encoding rNa₁,4 and hNa₁,5 were a gift from Dr. R. G. Kallen (University of Pennsylvania, Philadelphia, PA). The pNa200 vector encoding for rNa₁,6 was a gift from Dr. A. Goldin (University of California, Irvine, CA). Wistar rats for the preparation of brain synaptosomes were purchased from the Animal Housing at Tel Aviv University.

**Expression and Production of Recombinant Lqh2—Lqh2-cDNA was isolated from a cDNA library of the Israeli yellow scorpion (34), cloned at the NdeI-BamHI restriction sites of pET-14B expression vector, and expressed in fusion behind a His₁₀ tag and a thrombin cleavage sequence. Overexpression, in vitro folding, and purification by reverse phase-high pressure liquid chromatography followed a previously described protocol (30). Mutagenesis was performed via PCR and each toxin mutant derivative was produced and purified as described for the unmodified toxin. Quantification of the purified recombinant toxins was performed by amino acid analysis (21). The activity of ¹²⁵I-Lqh2 was comparable with that of the native toxin in binding and electrophysiological assays and therefore it was used throughout this study (supplemental Fig. S2).

**Binding Experiments**—Preparation of rat brain synaptosomes, membrane protein quantification, and Lqh2 radioiodination were carried out as described (29). Purification of the monoiodotoxin and determination of its concentration, as well as the composition of media used in the binding assays and termination of the reactions have been previously described (29, 35). Nonspecific toxin binding was determined in the presence of excess (1 μM) unlabeled toxin. Equilibrium competition assays were performed using increasing concentrations of the unlabeled toxin in the presence of a constant low concentration of the labeled toxin (30–50 pmol). The median concentration values for inhibition of toxin binding (IC₅₀) were determined by a non-linear regression analysis using the Hill equation, employing a Hill coefficient of 1. Mathematical curve fitting for IC₅₀ determination was accomplished using KaleidaGraph (version 3.08, Synergy Software). Kᵣ values were calculated by the equation $K_r = IC_{50}/1 + [L^*/K_d]$, in which $L^*$ is the concentration of the radioactive ligand and $K_d$ is its dissociation constant (29, 35).

**Expression of Naₙ,s in Oocytes and Two-electrode Voltage Clamp Experiments**—cRNAs encoding the α-subunit of each channel and the auxiliary β1 subunit were transcribed in vitro using T7 RNA polymerase and the mMMESSAGE mMACHINE™ system (Ambion, Austin, TX) and injected into Xenopus laevis oocytes as was previously described (36). Currents were measured 2–3 days after injection using a two-electrode voltage clamp and a Gene Clamp 500 amplifier (Axon Instruments, Union City, CA). Data were sampled at 10 kHz and filtered at 5 kHz. Data acquisition was controlled by a Macintosh PPC 7100/80 computer, equipped with an ITC-16 analog/digital converter (Instrutech Corp., Port Washington, NY), utilizing Synapse (Synergistic Systems, Sweden). Capacitance transients and leak currents were removed by subtracting a scaled control trace utilizing a P/6 protocol (37). Bath solution contained (in mM): 96 NaCl, 2 KCl, 1 MgCl₂, 2 CaCl₂, 5 HEPES, pH 7.85. Toxins were diluted with bath solution containing 1 mg/ml bovine serum albumin, and applied directly to the bath to the final desired concentration. To avoid application artifacts, 1 mg/ml bovine serum albumin solution was applied prior to toxin addition.
Dose-Response Curves of α-Toxin Effect on Fast Inactivation—

Currents were elicited by a 50 ms depolarization to −20 mV from a −80 mV holding potential in the presence of increasing toxin concentrations. At each toxin concentration, the currents were allowed to reach a steady-state level prior to the final measurement. The dose dependence for toxin-induced removal of fast inactivation is calculated by plotting the ratio of the steady-state current remaining 50 ms after depolarization ($I_{ss}$) to the peak current ($I_{peak}$) as a function of toxin concentration and fitting with the Hill equation,

$$\frac{I_{ss}}{I_{peak}} = a_0 + \frac{a_1 - a_0}{1 + \left(\frac{[\text{Toxin}]}{EC_{50}}\right)^H} \quad \text{(Eq. 1)}$$

where $H$ is the Hill coefficient, [Toxin] is the toxin concentration, and $a_0$ is the offset measured prior to toxin application. The $a_1 - a_0$ amplitude provides the maximal effect obtained at saturating toxin concentrations. $EC_{50}$ is the toxin concentration at which half-maximal inhibition of fast inactivation is obtained. To reduce variability, $H$ was set to 1 in all calculations. Mutant toxin efficacy was determined as the ratio between its maximal effect ($I_{ss}/I_{peak}$) under saturating concentrations relative to that of Lqh2, which was normalized to 1.

RESULTS

The scorpion α-toxins Lqh2 and LqhαIT are very similar in structure and yet they differ greatly in preference for the mammalian brain versus insect sodium channels (rNav1.2a and DmNav1, respectively) (10, 21, 27). To identify the Lqh2 face of interaction with the rat brain Nav, we produced the toxin in recombinant form and analyzed the contribution of surface amino acids to bioactivity using mutagenesis. Because the activity of the recombinant Lqh2 fused to a His₆ tag and a thrombin cleavage site was similar to that of the native toxin (supplemental Fig. S2), we used in all further experiments the toxin and mutants in their fused forms.

### Mutagenic Dissection of Lqh2—Substitution of residues at the Lqh2 surface relied primarily on the results of mutagenic analyses of α-toxins LqhαIT, Lqh3, and BmKM1, which highlighted the bioactive role of residues at the core and NC domains (21–23). Each recombinant toxin derivative was assayed at rNav1.2a expressed in *Xenopus* oocytes in the presence of the β1 auxiliary subunit. CD spectroscopy was used to discern effects that were due to structural perturbations from those directly associated with bioactivity. From a total of 29 mutants, the CD spectrum of only Y21A changed, and because a conservative substitution, Y21F, had negligible effect on Lqh2 activity (Fig. 1 and supplemental Table S1), it is conceivable that Tyr21 has a structural role. Substitution to Ala of Lys2, Phe15, Arg18, Trp38, Asn44, Thr57, and Lys58 decreased the potency of Lqh2 more than 5-fold (in $EC_{50}$ values; Fig. 1; supplemental Table S1 and Fig. S3), whereas substitution of all other residues had little or no effect (Fig. 1 and supplemental Table S1). Because residues equivalent to all these amino acids but Lys2 have been implicated in activity of α-toxins, we analyzed in parallel how
substitution of Arg2 in LqhαIT affects the activity at DmNa.1. We found that substitution R2A decreased LqhαIT potency at DmNa.1 by 8-fold (not shown) with no change in the CD spectrum, and therefore conclude that the second amino acid in scorpion α-toxins is an integral part of the NC domain as was also suggested for BmKM1 (23).

As residues of the five-residue turn in the α-toxins LqhαIT and BmKM1 have previously been shown to be involved in bioactivity (20, 21, 38), we have substituted one by one their equivalents (Asp8, Asp9, and Val10) in Lqh2. Surprisingly, these substitutions had no effect on activity at rNa.1.2a (Fig. 1 and supplemental Table S1), which led us to examine the possibility that it is the general fold of the five-residue turn that affects the spatial arrangement of the entire NC domain and hence the activity. We constructed a triple mutant, in which these three residues were simultaneously exchanged by their Lqh2 equivalents (Asp8, Asp9, and Val10) in Lqh2. Surprisingly, these substitutions had no effect on activity at rNa.1.2a (Fig. 1 and supplemental Table S1), suggesting that by itself the five-residue turn of Lqh2 contributes only slightly to toxin activity.

A conservative substitution of Phe15 by Trp had no effect, whereas substitution to Ala decreased the potency of Lqh2 for rNa.1.2a by 5.5-fold (Fig. 1 and supplemental Table S1 and Fig. 3). However, substitution of Phe15 to Glu, which appears at this position in LqhαIT, decreased the toxin potency for rNa.1.2a by 60-fold. These results have suggested that Phe15 is involved in hydrophobic interactions with the sodium channel.

Unlike in BmKM1 (23, 39), LqhαIT (21), and Lqh3 (22), only two residues at the C-tail of Lqh2 were important for activity. Although substitution of Lys58 by Ala decreased the activity nearly 100-fold, a conservative substitution to Thr57 to Ser strongly reduced the activity (Fig. 1 and supplemental Table S1 and Fig. 3).

To correlate these differences in effect with structural features at the bioactive surface of the two toxins, and on the basis of a previous successful construction of LqhαIT bioactive surface on the scaffold of Aah2 (21), we substituted in a stepwise manner residues on the exterior of LqhαIT by their Lqh2 equivalents. The activity of the resulting constructs was examined at rNa.1.2a expressed in Xenopus oocytes. This stepwise construction was useful in determining the contribution of each of the bioactive domains to Lqh2 function. The core domain of the two toxins is nearly identical in amino acid composition, except for Phe17 in LqhαIT whose Lqh2 equivalent is Gly, and Glu15 in LqhαIT whose Lqh2 equivalent is Phe (Fig. 2). Substitution of Gly17 to Phe had only a minute effect on the potency of Lqh2, whereas substitution of Phe15 by non-aromatic residues had a significant effect at rNa.1.2a (Fig. 1 and supplemental Table S1). This result led us to examine whether substitution of Glu15 to Phe in LqhαIT would increase its potency for rNa.1.2a. Indeed, the potency of mutant LqhαITLqh2(8–10,56–64), in which the core domain was aligned in principle with that of Lqh2, increased 30-fold with no change in efficacy, which was similar to that of LqhαIT (Fig. 2). In sharp contrast was the result obtained when the NC domain (five-residue turn and C-tail) of Lqh2 was constructed on the scaffold of LqhαIT. Whereas the potency of mutant LqhαITLqh2(8–10,56–64) was similar to that of the unmodified LqhαIT, its efficacy increased substantially and was comparable with that of Lqh2 (Fig. 2). When both constructs were combined, the potency and efficacy of the chimera, LqhαITLqh2(8–10,15,56–64), at rNa.1.2a were almost indistinguishable from those of Lqh2, and therefore this chimera was named LqhαITLqh2(face) (Fig. 2). These results suggest that the full effect at rNa.1.2a requires the cooperative interaction of the two functional domains at the toxin exterior.

Abolishment of Lqh2 Activity at rNa.1.4, hNa.1.5, and rNa.1.6—Because the bioactive surfaces of scorpion α-toxins show general resemblance, their diverse activities at various Na+ subtypes are likely conferred by subtle differences either in structure or amino acid composition at their face of interaction with receptor site-3 (40). Any attempt to design a toxin specific for a particular Na+ requires the identification of these fine differences. Although Lqh2 is highly active at a
variety of mammalian Navs such as Nav1.2a (EC$_{50}$ = 13.4 ± 1.5 nM; n = 6), Nav1.4 (EC$_{50}$ = 42 ± 1.2 nM; n = 3), Nav1.5, Nav1.6, and Na1.7 (Refs. 35, 41, and 42 and insets in Fig. 3), the scorpion α-toxin Amm8 (from Androctonus mauretanicus mauretanicus), which exhibits 90% sequence identity with Lqh2 (supplemental Fig. S1), shows clear preference (14.3-fold) for rNav1.2a over rNav1.4 (43). To identify the amino acid residues that determine this differential effect we mutagenized Lqh2 in a sequential manner focusing on residues that vary in Amm8, particularly at the NC domain, and examined the activity of the mutants at rNav1.2a versus rNav1.4. Interestingly, substitutions D8/A/K/N (where D8 was replaced by A, K, or N), V10/A/Y/I (where V10 was replaced by A, Y, or I), or Arg$_{64}$ by Asn and addition of Asp (R64N-D) (a single substitution where two residues replace one), as appears in Amm8, did not alter the activity at rNav1.4 (not shown). Therefore, guided by the idea that the general shape of the NC domain is important, we analyzed how combined substitutions at Lqh2 would affect its activity at rNav1.4. Complete exchange of the NC domain in Lqh2 by its Amm8 counterpart, achieved through substitutions D8N, V10I, R64N-D, resulted in a toxin mutant with >10-fold preference for rNav1.2a over rNav1.4 similar to that of Amm8 (EC$_{50}$ = 42 ± 6.5 nM, n = 3, and 438 ± 19 nM, n = 3, respectively). To correlate this change in selectivity with the two entities that constitute the NC domain, we mutagenized separately the C-tail and the five-residue turn. The activity of mutant Lqh2$_{R64N-D}$ was similar to that of Lqh2. However, mutant Lqh2$_{D8N,V10I}$ in concentrations up to 5 μM hardly slowed the decay of the sodium current at rNav1.4, hNav1.5, and rNav1.6, although the sodium peak current increased especially at hNav1.5. It has been suggested that the increase in peak current in the presence of an α-toxin varies with sodium channel subtype and the details of experimental conditions and likely results from increased channel open probability due to prevention of inactivation from channel states other than the open state (41, 44). Therefore the most direct measure of the effect of an α-toxin is on the steady-state current ($I_{ss}$) normalized to the peak current ($I_{peak}$). In contrast to these results, Lqh2$_{D8N,V10I}$ was highly active at rNav1.2a (only a 3-fold decrease; EC$_{50}$ = 37 ± 2.9, n = 4; Figs. 1 and 3), as well as at hNav1.1 and rNav1.3 (Fig. 3).

**DISCUSSION**

**Commonalities and Variability in the Bioactive Surfaces of Scorpion α-Toxins**—Comparison of the bioactive surfaces of Lqh2 with those of the insecticidal α-toxin LqhαIT (20, 21) and the α-like toxin Lqh3 (22) highlights considerable commonality. In the three toxins this surface is divided into two distinct domains (Fig. 4). The conserved core domain, composed in Lqh2 of Phe$_{15}$, Arg$_{18}$, Trp$_{38}$, and Asn$_{44}$, resembles that of LqhαIT (Phe$_{17}$, Arg$_{19}$, Trp$_{39}$, and Asn$_{45}$), and Lqh3 (His$_{15}$, Phe$_{17}$, Pro$_{18}$, Phe$_{39}$, and Leu$_{49}$) (Fig. 4). Although the spatial orientation of amino acid side chains involved in this domain relative to the βαββ toxin core is not identical, its general similarity may explain the ability of scorpion α-toxins to compete in binding at various Navs (reviewed in Ref. 10). Of particular interest in α-toxins that show high activity at mammalian Navs is residue 15 (supplemental Fig. S1). It is evident from substitution of Phe$_{15}$ in Lqh2 (Fig. 1 and supplemental Table S1) and
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His\textsuperscript{15} in Lqh3 (22), as well as the 30-fold increase in potency of LqhαIT\textsuperscript{\textalpha1:15\textalpha\textbeta} for rNa\textsubscript{\textalpha}1.2a, that position 15 is critical for the potency at mammalian Na\textsubscript{\textalpha}s (Fig. 2) and not at the insect Na\textsubscript{\textalpha} (21).

In the NC domain of Lqh2, substitution of three residues (Lys\textsuperscript{2}, Thr\textsuperscript{57}, and Lys\textsuperscript{58}) decreased the activity compared with (21).

The close resemblance of the pharmacological properties of LqhαIT\textsuperscript{\textalpha1:15\textalpha\textbeta} (Fig. 2) was intriguing, suggesting that the general shape of the NC domain did not change. Close inspection of the x-ray structure of Aah2 (PDB accession 1aho; almost identical with Lqh2 except for the N and C termini; supplemental Fig. S1) reveals that besides a disulfide bond, backbone atoms of the five-residue turn form a network of chemical interactions with C-tail residues, thereby stabilizing the structure of the NC domain. This may explain how substitution of side chains of residues at the five-residue turn probably did not change the overall shape of the NC domain in Lqh\textsuperscript{\textalpha2\textalpha8K,D9N,V10\textalpha\textbeta} and Lqh\textsuperscript{\textalpha2\textalpha9N,V10\textalpha\textbeta}.

The close resemblance of the pharmacological properties of LqhαIT\textsuperscript{\textalpha1:15\textalpha\textbeta} to those of Lqh2 suggest similar orientation of the functional surfaces with contribution of both the core domain and the NC domain to the activity at rNa\textsubscript{\textalpha}1.2a. Although the efficacy is mainly determined by the configuration of the NC domain (see LqhαIT\textsuperscript{\textalpha1:15\textalpha\textbeta} in Fig. 2), the core domain contributes to potency as indicated by (i) the 30-fold increase at rNa\textsubscript{\textalpha}1.2a when just Glu\textsuperscript{15} of LqhαIT was substituted with Phe; (ii) the 1000-fold increase when Glu\textsuperscript{15} was substituted by Phe in Lqh\textsuperscript{\textalpha1:15\textalpha\textbeta} providing the fully active chimera Lqh\textsuperscript{\textalpha1:15\textalpha\textbeta}.

FIGURE 4. Comparison of the bioactive surface of three pharmacologically distinct scorpion α-toxins. The structures of Lqh\textsubscript{\textalpha} and Lqh\textsubscript{\textalpha}T have been determined (PDB codes 1fh3 and 2asc, respectively). Modeling of the Lqh\textsubscript{\textalpha}2 structure was based on the known structure of Aah\textsubscript{\textalpha}2 (PDB code 1aho) employing the SWISS-MODEL protein homology-modeling server (EXPASY). The ribbons indicate the backbone structures covered by a semi-transparent molecular surface of the toxins. Residues composing the bioactive surfaces are space-filled and colored according to their chemical nature (aliphatic, green; aromatic, magenta; polar, yellow; and positive, blue). The bioactive surfaces of Lqh\textsubscript{\textalpha}T and Lqh\textsubscript{\textalpha}3 have been determined (21, 22, 51). Note the division of the bioactive surface in the three toxins to a core domain and an NC domain.
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itivity would be identified, and then examination of the variety of toxin mutants against different Na$_x$ subtypes may uncover a toxin derivative with altered selectivity. Whereas the first part in this approach was achieved with relative ease, none of the mutations introduced at the bioactive surface of Lqh2 changed the selectivity toward rNa$_x$1.2a brain and rNa$_x$1.4 skeletal muscle channels. Still, comparative analysis of scorpion α-toxins demonstrates natural variability in preference for Na$_x$ subtypes. In this respect, the high sequence similarity between Amm8 and Lqh2 (supplemental Fig. S1), on the one hand, and the 14.3-fold lower potency of Amm8 at rNa$_x$1.4 over rNa$_x$1.2a (43), on the other hand, suggested that minor differences at the toxin exterior might dictate its selectivity. This implication served as the basis of further mutagenesis of Lqh2 with focus on residues associated with the NC domain. By using this approach, the combination of two substitutions at the five-residue turn resulted in a toxin mutant, Lqh2D8N,V10I, which hardly affected rNa$_x$1.4, whereas the activity at rNa$_x$1.2a decreased only to a small extent (Fig. 3). It seems that the double mutation changed the toxin exterior in such a way that had little effect on binding to Na$_x$1.1, Na$_x$1.2a, and Na$_x$1.3, but a strong effect on the binding to Na$_x$1.4. This suggestion is supported by the inability of high Lqh2D8N,V10I concentrations (5 μM) to inhibit Lqh2 (50 nM) activity at rNa$_x$1.4 (n = 4; not shown). A possible explanation to the substantial difference in Lqh2D8N,V10I interaction with these Na$_x$ subtypes is that receptor site-3 differs between Na$_x$1.1–1.3 and Na$_x$1.4–1.6. Validation of this suggestion may require comparison of the structure of the toxin-channel complexes, which currently is still an unachievable task.

Overall, Lqh2D8N,V10I seems to be a valuable toxin derivative in that it may be used as a specific probe for analysis of the distribution and function of rNa$_x$1.1–1.3 channels in various tissues, in studying their expression through embryonic development, and as a model for design of selective drugs in genetic plexes, which currently is still an unachievable task.

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