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Mammalian Skeletal Muscle Voltage-Gated Sodium Channels Are Affected by Scorpion Depressant "Insect-Selective" Toxins when Preconditioned

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ABSTRACT

Among scorpion β- and α-toxins that modify the activation and inactivation of voltage-gated sodium channels (Na_vs), depressant β-toxins have traditionally been classified as anti-insect selective on the basis of toxicity assays and lack of binding and effect on mammalian Na_vs. Here we show that the depressant β-toxins LqhIT2 and Lqh-dprIT3 from *Leiurus quinquestriatus hebraeus* (Lqh) bind with nanomolar affinity to receptor site 4 on rat skeletal muscle Na_vs, but their effect on the gating properties can be viewed only after channel preconditioning, such as that rendered by a long depolarizing prepulse. This observation explains the lack of toxicity when depressant toxins are injected in mice. However, when the muscle channel $rNa_v1.4$, expressed in *Xenopus laevis* oocytes, was modulated by the site 3 α-toxin LqhαIT, LqhIT2 was capable of inducing a negative shift in the voltage-dependence of activation after a short prepulse, as was shown for other β-toxins. These unprecedented results suggest that depressant toxins may have a toxic impact on mammals in the context of the complete scorpion venom. To assess whether LqhIT2 and Lqh-dprIT3 interact with the insect and rat muscle channels in a similar manner, we examined the role of Glu24, a conserved "hot spot" at the bioactive surface of β-toxins. Whereas substitutions E24A/N abolished the activity of both LqhIT2 and Lqh-dprIT3 at insect Na_vs, they increased the affinity of the toxins for rat skeletal muscle channels. This result implies that depressant toxins interact differently with the two channel types and that substitution of Glu24 is essential for converting toxin selectivity.

Voltage-gated sodium channels (Na_vs) are critical in generation and propagation of action potentials in excitable cells and are targeted by a large variety of chemically distinct compounds that bind at several receptor sites on the pore-forming α-subunit (Gordon, 1997; Catterall, 2000). Most lipid-soluble Na_v activators, including pyrethroid insecticides, toxic alkaloids (e.g., veratridine and batrachotoxin), and marine cyclic polyether toxins (e.g., brevetoxins), affect Na_vs of both insects and mammals. Yet, despite the general conservation of Na_v structure, certain scorpion toxins show preference for Na_v subtypes in mammals or insects (Gordon et al., 1998, 2007; Cestèle and Catterall, 2000; Gurevitz et al., 2007), which raised the idea of using some representatives for insect pest control (reviewed in Gurevitz et al., 2007).

Scorpion toxins that modulate Na_v gating are divided between the α and β classes according to their mode of action and binding features to distinct receptor sites (Catterall, 2000). α-Toxins prolong the action potential by inhibiting the fast inactivation of Na_vs upon binding to receptor site 3 (e.g., LqhαIT from *Leiurus quinquestriatus hebraeus*; Eitan et al., 1990; Martin-Eauclaire and Couraud, 1995; Gordon et al., 1996) assigned mainly to extracellular loops in domains 1 and 4 (Catterall, 2000). β-Toxins shift the voltage dependence of channel activation to more hyperpolarized membrane potentials upon binding to receptor site 4, assigned mainly to external loops in domains 2 and 3 (Marcotte et al.,

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ABBREVIATIONS: Na_vs, voltage-gated sodium channels; Css4, *Centruroides suffusus suffusus* toxin 4; LqhIT2 and Lqh-dprIT3, *Leiurus quinquestriatus hebraeus* anti-insect depressant toxins; PP, preconditioning depolarizing prepulse.

1997; Cestèle et al., 1998, 2006; Shichor et al., 2002; Leipold et al., 2006). The β-toxins are further classified to 1) anti-mammalian β-toxins (e.g., Css2 and Css4 from *Centruroides suffusus suffusus*; Martin-Eauclaire and Couraud, 1995; Gordon et al., 1998; Gurevitz et al., 2007); 2) β-toxins that affect both insect and mammalian Na$_v$s (e.g., Ts1 from *Tityus serrulatus* and Lqhβ1; Possani et al., 1999; Gordon et al., 2003); 3) Anti-insect selective excitatory β-toxins (e.g., AahIT from *Androctonus australis hector* and Bj-xtrIT from *Buthotus judaicus*), typified by the symptoms of contraction paralysis they produce in blowfly larvae (Zlotkin et al., 1978; Froy et al., 1999), and by their unique structures (Oren et al., 1998; Li et al., 2005; Gurevitz et al., 2007); and 4) anti-insect depressant toxins, which upon injection to blowfly larvae induce flaccid paralysis as a result of sustained depolarization of the axonal membrane, leading to block in the evoked action potentials and loss of muscle tonus (Lester et al., 1982; Zlotkin et al., 1991; Ben Kalifa et al., 1997; Strugatsky et al., 2005). These ~61-residue polypeptides were classified as β-toxins because of their ability to modulate Na$_v$ activation and to compete with excitatory toxins on binding to receptor-site 4 in insect neuronal membranes (Gordon et al., 1984, 1992). Yet depressant toxins did not compete with anti-mammalian β-toxins on binding to rat brain membranes and were harmless when injected to mice (Lester et al., 1982; Herrmann et al., 1995; Strugatsky et al., 2005).

The bioactive surfaces of the anti-insect excitatory β-toxin Bj-xtrIT, the anti-mammalian β-toxin Css4, and the anti-insect depressant β-toxin LqhIT2 have been described previously (Cohen et al., 2004, 2005; Karbat et al., 2007). These studies highlighted a conserved "pharmacophore" composed of a key negatively charged Glu in the α-helix (Fig. 1), flanked by hydrophobic residues that may isolate the point of interaction with a counterpart channel residue from the bulk solvent. An additional hydrophobic cluster of bioactive residue, at the C-tail of Bj-xtrIT and on the loop connecting the second and third β-strands of Css4, was suggested to determine toxin selectivity (Cohen et al., 2004, 2005). Although the key residues involved in Css4 and Bj-xtrIT activity form two topologically distinct domains, the bioactive surface of LqhIT2 is continuous but involves the conserved pharmacophore (Glu24) and its vicinity (Karbat et al., 2007). The subset of residues common to the bioactive surfaces of Css4, Bj-xtrIT, and LqhIT2 raised the possibility that these toxins might compete in binding for receptor site 4 on different Na$_v$s.

By analyzing the ability of depressant toxins to bind and affect various mammalian Na$_v$s, we found that despite the lack of effect on subcutaneously injected mice, depressant toxins bound to receptor site 4 on rat muscle membranes with nanomolar affinity. We show that LhqIT2 and Lqh-dprIT3 are effective on rNa$_v$1.4 when the channel is excited before toxin application by either a long depolarizing prepulse or by modulation with an α-toxin. These results suggest that the reported selectivity of depressant toxins to insect Na$_v$s rests on the way they were tested and that depressant toxins may have a toxic impact on mammals in the context of the complete venom.

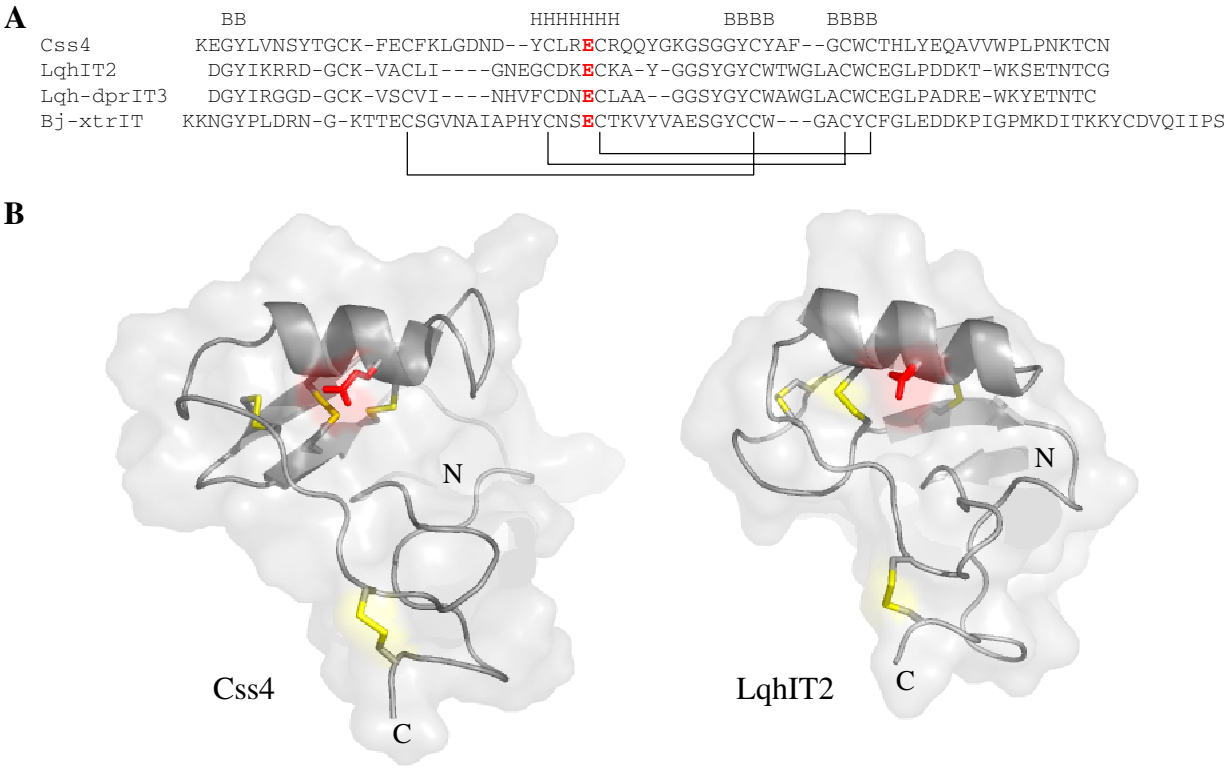

Fig. 1. Sequence alignment and three-dimensional structure of β-toxin representatives. A, sequences were aligned according to the conserved cysteine residues. The disulfide bonds formed between cysteine pairs in all "long-chain" scorpion toxins are designated by lines. Dashes indicate gaps. Secondary structure motifs (B, β-strand; H, α-helix) in Css4 follow the published structure of the β-toxin Cn2 (from *Centruroides noxius*; Pintar et al., 1999). B, the Cα structure of Css4 and LqhIT2 (in gray) covered by a semitransparent molecular surface of the toxins. LqhIT2 is derived from Protein Data Bank accession 2I61. The Css4 model is from Cohen et al. (2005) and is spatially aligned with that of LqhIT2. The sulfur atoms in the disulfide bonds are highlighted in yellow, and the conserved glutamate is in red (see also A). The figure was prepared using PyMOL (http://www.pymol.org).

Materials and Methods

Toxins and Their Mutagenesis

Production of Bj-xtrIT, LqhIT2, Css4, and Lqh-dprIT3 variant c in recombinant forms, polymerase chain reaction-driven mutagenesis, expression in *Escherichia coli*, in vitro folding, and purification of toxin derivatives have been described in detail (Turkov et al., 1997; Froy et al., 1999; Cohen et al., 2005; Strugatsky et al., 2005)

Binding Experiments

Neuronal membranes from cockroach were prepared from whole heads of adult *Periplaneta americana* according to a previously described method (Froy et al., 1999). Rat skeletal muscle membranes were prepared from adult albino Wistar strain (~300 g, laboratory bred) as described previously (Gordon et al., 1988). Mammalian brain synaptosomes were prepared from the same rats as described previously (Gilles et al., 2001). Membrane protein concentration was determined by a Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA), using bovine serum albumin as standard. Bj-xtrIT and Css4 (with a His-tag attached; His-Css4) were radioiodinated by lactoperoxidase (Sigma, St. Louis, MO; 7 units per 60 μl of reaction mix) using 10 μg of toxin and 0.5 mCi of carrier-free $Na^{125}I$ (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK), and the monoiodotoxin was purified as described previously (Cohen et al., 2004, 2005). The media composition used in the binding assays and termination of the reactions were described elsewhere (Gilles et al., 2000, 2001). Nonspecific toxin binding was determined in the presence of 1 to 10 μM unlabeled toxin and consisted typically of 10 to 30% of total binding. Equilibrium competition binding assays were performed and analyzed as described previously (Cohen et al., 2005). Each experiment was performed in duplicate and repeated at least three times as indicated (n). Data are presented as mean ± S.D. of the number of independent experiments.

Expression of Sodium Channels in Oocytes and Two-Electrode Voltage Clamp Experiments

The genes encoding the *Drosophila melanogaster* sodium channel α-subunit ($DmNa_v1$) and the auxiliary TipE subunit were kindly provided by J. Warmke (Merck, Whitehouse Station, NJ) and M. S. Williamson (IACR-Rothamsted, Harpenden, Hertfordshire, UK), respectively. The gene encoding the rat skeletal muscle sodium channel, $rNa_v1.4$, in the pAlter vector was a gift from Dr. R. G. Kallen (University of Pennsylvania, Philadelphia, PA). These genes and that for the auxiliary subunit hβ1 were transcribed in vitro using T7 RNA-polymerase and the mMESSAGE mMACHINE system (Ambion, Austin, TX) and were injected into *Xenopus laevis* oocytes as described previously (Shichor et al., 2002).

Two-Electrode Voltage-Clamp Recording

Currents were measured 1 to 2 days after injection using a two-electrode voltage clamp and a Gene Clamp 500 amplifier (Molecular Devices, Sunnyvale, CA). Data were sampled at 10 kHz and filtered at 5 kHz. Data acquisition was controlled by a Macintosh PPC 7100/80 computer (Apple Corp., Cupertino, CA), equipped with ITC-16 analog/digital converter (Instrutech Corp., Port Washington, NY), using Synapse (Synergistic Systems, Stockholm, Sweden). The bath solution contained 96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, and 5 mM HEPES, pH 7.85. Oocytes were washed with bath solution flowing from a BPS-8 perfusion system (ALA Scientific Instruments, Westbury, NY) with a positive pressure of 4 psi. Toxins were diluted with bath solution and applied directly to the bath at their final desired concentration.

Data Analysis

Leak Subtraction. Capacitance transients and leak currents were removed by subtracting a scaled control trace using a P/6 protocol (Armstrong and Bezanilla, 1974).

GV Analysis. Mean conductance (G) was calculated from peak current/voltage relations using the equation $G = I/(V - V_{rev})$, where I is the peak current elicited upon depolarization, V is the membrane potential, and V_{rev} is the reversal potential. Normalized conductance voltage relationship were fit with either a one or two component Boltzmann distribution according to the equation $G/G_{max} = (1 - A)/(1 + \exp[(V1_{1/2} - V)/k_1]) + A/(1 + \exp[(V2_{1/2} - V)/k_2])$, where $V1_{1/2}$ and $V2_{1/2}$ are the respective membrane potentials for two populations of channels for which the mean conductance is half-maximal; k_1 and k_2 are their respective slopes, and A defines the proportion of the second population (amplitude) with respect to the total. For fits in which only one population of channels was apparent, A was set to zero.

Steady-State Fast Inactivation. The voltage dependence of steady-state fast inactivation is described by a single Boltzmann distribution: $I/I_{max} = \alpha_0 + \alpha_1/(1 + \exp[(V - V_{1/2})/k])$, where I is the peak current measured during the test depolarization step, I_{max} is the current obtained without a preceding conditioning step; V is the membrane potential of the conditioning step; $V_{1/2}$ is the membrane potential at which half-maximal inactivation is achieved, k is the slope factor, α_0 is the remaining normalized peak current at very high depolarizing conditioning potentials, and α_1 is the normalized amplitude (Chen and Heinemann, 2001).

Results

Binding of Depressant Toxins to Rat Skeletal Muscle Na_vs. Scorpion depressant toxins have traditionally been considered insect-selective on the basis of their exclusive toxicity to insects and high binding affinity for insect Na_vs (Lester et al., 1982; Zlotkin et al., 1991; Gordon et al., 1992; Strugatsky et al., 2005). Still, the elucidation of a pharmacophore common to the bioactive surface of scorpion β-toxins active on insects and mammals (Cohen et al., 2005; Karbat et al., 2007) prompted us to analyze whether the toxins that show selectivity for insects would compete with the anti-mammalian β-toxin Css4 on binding to receptor site 4 on rat muscle and brain Na_vs. Although the excitatory toxin Bj-xtrIT did not displace ^{125}I-Css4 in concentrations up to 10 μM, the depressant toxins LqhIT2 and Lqh-dprIT3 inhibited in a dose-dependent manner the binding of ^{125}I-Css4 to rat muscle membranes with Ki values of 45 ± 7 and 30 ± 3.2 nM, respectively (Fig. 2, Table 1). In contrast, the excitatory and

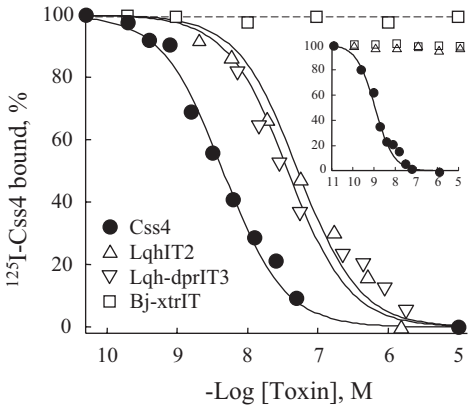

Fig. 2. Competition of β-toxins with Css4 on binding to rat muscle membranes. Membranes were incubated 60 min at 22°C with 0.1 nM ^{125}I-Css4 and increasing concentrations of the various β-toxins. Nonspecific binding, determined in the presence of 1 μM Css4, was subtracted. The K_i values are (in nanomolar, $n \geq 3$): Css4, 3.9 ± 1.17; Lqh-dprIT3, 30 ± 3.2; LqhIT2, 45 ± 7; Bj-xtrIT, ≫10,000. The binding of Css4, LqhIT2, and Bj-xtrIT to rat brain synaptosomes under the same conditions is shown in the inset. The K_i values are (in nanomolar, $n \geq 3$): Css4, 0.98 ± 0.1; LqhIT2 and Bj-xtrIT, ≫10,000. Representative experiments are shown.

the depressant toxins did not displace ^{125}I-Css4 from rat brain synaptosomes at concentrations up to 10 μM (inset in Fig. 2). The ability of depressant toxins to bind with an apparent high affinity to rat skeletal muscle Na$_v$s raised the inevitable question of why these toxins were inactive when injected subcutaneously into mice (Lester et al., 1982; Zlotkin et al., 1991, 1993). Therefore, we analyzed the effects of LqhIT2 and Lqh-dprIT3 on the rat muscle channel rNa$_v$1.4 and compared them with those obtained on the *Drosophila melanogaster* channel DmNa$_v$1, expressed in *X. laevis* oocytes.

TABLE 1

Changes in apparent binding affinity of depressant β-toxins and selected mutants to rat muscle and cockroach neuronal membranes

The K_i values obtained from competition binding studies using ^{125}I-Bj-xtrIT (cockroach neuronal membranes) and ^{125}I-Css4 (rat muscle membrane preparation). See Fig. 4 for details.

Toxin	K_i		
	Muscle	Cockroach	Ratio Muscle/ Cockroach
	nM		
Lqh-dprIT3	30 ± 3.2	0.2 ± 0.04	150
LqhIT2	45 ± 7	0.7 ± 0.14	81
LqhIT2^{K23A}	900 ± 122	11.1 ± 1.2	86
LqhIT2^{E24A}	11 ± 0.5	87 ± 5	0.13
LqhIT2^{E24N}	8 ± 1.2	91.5 ± 1.5	0.09
Css4	3.9 ± 1.17	>5000	
Bj-xtrIT	>10,000	0.16 ± 0.03	

The Effects of LqhIT2 and Lqh-dprIT3 on the Activation of DmNa$_v$1 and rNa$_v$1.4. The voltage-dependent activation of DmNa$_v$1 and rNa$_v$1.4 expressed in *X. laevis* oocytes was monitored by two-electrode voltage-clamp in the absence and presence of LqhIT2 or Lqh-dprIT3 (Fig. 3, Table 2). Because the negative shift of voltage-dependent activation induced by β-toxins is better observed after a preconditioning depolarizing prepulse (PP) (Cestèle et al., 1998; Tsushima et al., 1999), we first examined the PP duration required to observe such a shift in the insect Na$_v$ in the presence of LqhIT2. In the absence of toxin, DmNa$_v$1 was not activated by a 50-ms test pulse to −50 mV independent of whether or not a depolarizing PP was provided, whereas in the presence of 1 μM LqhIT2, a hyperpolarizing shift in current-voltage relations at DmNa$_v$1 was observed only after a preconditioning depolarizing PP (Fig. 3, A1). This shift was highly dependent on the PP length (Fig. 3, A). A 100-ms PP to +60 mV provided the maximal effect measured by

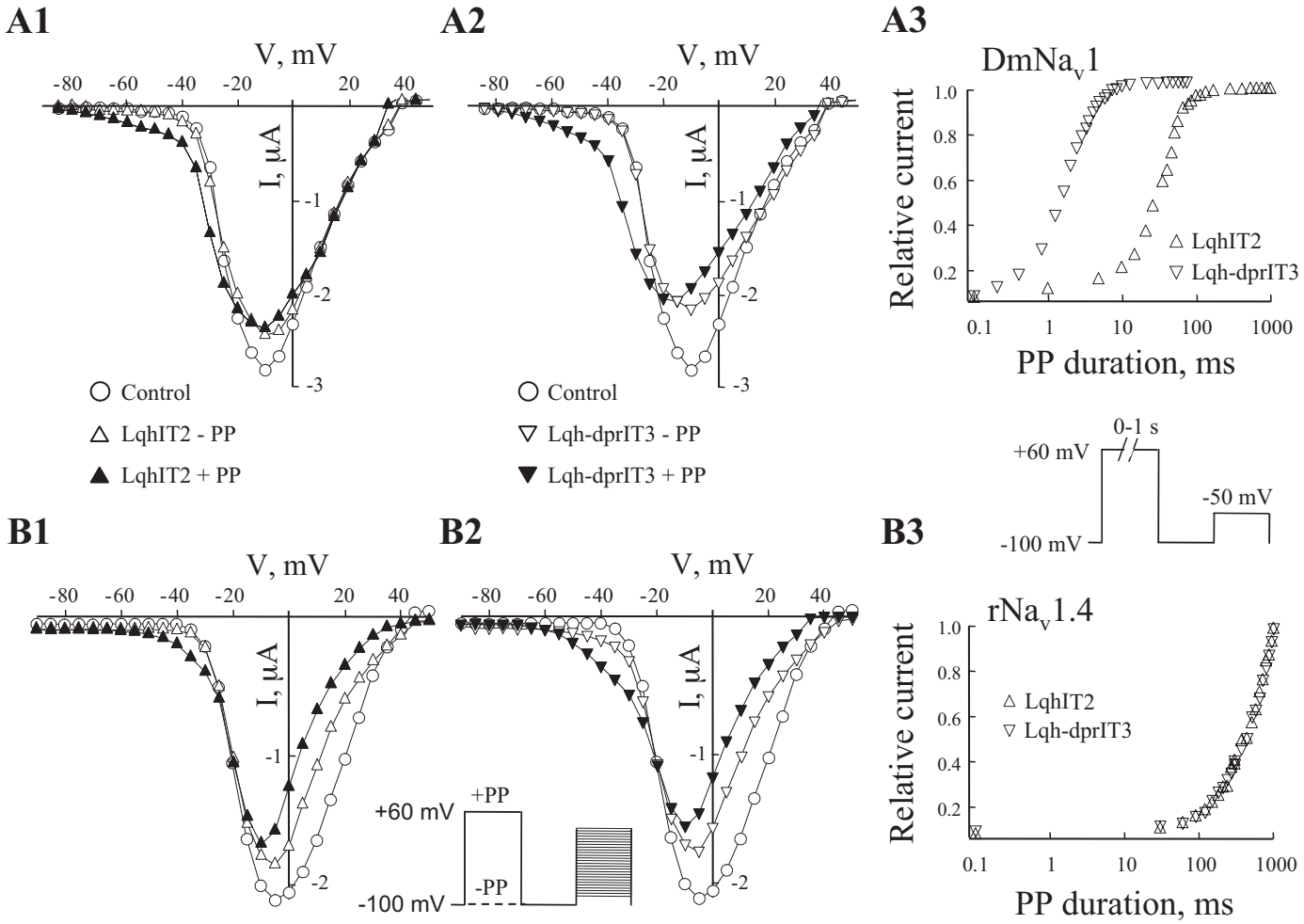

Fig. 3. LqhIT2 and Lqh-dprIT3 effect on activation of DmNa$_v$1 and rNa$_v$1.4. A1 and A2, current-voltage (I-V) relations of DmNa$_v$1 under control conditions and in the presence of 1 μM LqhIT2 (A1) or 0.2 μM Lqh-dprIT3 (A2) with or without a 50-ms PP to +60 mV from a −100-mV holding potential. B1 and B2, current-voltage relations of rNa$_v$1.4 under control conditions and in the presence of 5 μM LqhIT2 (B1), or 5 μM Lqh-dprIT3 (B2) with a 50- or 500-ms PP to +60 mV from a −100-mV holding potential. A3 and B3, analysis of the effect of depressant toxins on DmNa$_v$1 (A3) and rNa$_v$1.4 (B3) activation after various PP durations (0–1 s) to +60 mV and measuring at −50 mV. The current at each point was normalized to the maximal effect. Representative experiments are shown.

the developing currents elicited by a test pulse to −50 mV (Fig. 3, A3). Similar results were obtained with 200 nM Lqh-dprIT3 (Fig. 3, A2), a depressant toxin with higher toxicity to blowfly larvae (Strugatsky et al., 2005). It is noteworthy that Lqh-dprIT3 already induced a maximal effect on $DmNa_v1$ activation after 10 ms PP (Fig. 3, A3). In comparison, a very short preconditioning depolarizing PP (2 ms) was sufficient to induce a hyperpolarizing shift in activation of $rNa_v1.2a$ by the anti mammalian β-toxin Css4 (Cestèle et al., 1998). These results have indicated that the effects of various β-toxins on the hyperpolarizing shift in channel activation clearly depend on the PP duration.

Based on the observation that LqhIT2 requires a relatively long PP to induce a hyperpolarizing shift in $DmNa_v1$ activation, and that LqhIT2 and Lqh-dprIT3 bind with relatively high affinity to the rat muscle membranes (Fig. 2), we examined whether longer PP durations would facilitate the effect of these toxins on $rNa_v1.4$ activation. We found that LqhIT2 was indeed capable of shifting the $rNa_v1.4$ activation in the hyperpolarizing direction, but it required a longer PP to +60 mV, and even after a 2-s PP, the effect was not maximal (Fig. 3B). In the absence of toxin, a 500-ms PP had no effect on the peak current, and a 1- or 2-s PP induced a 10 or 20% decrease, respectively, in the peak current elicited at −10 mV (data not shown), probably as a result of the development of slow inactivation (Featherstone et al., 1996; Mitrovic et al., 2000). Similar results were obtained with Lqh-dprIT3 (Fig. 3, B2 and B3), suggesting that depressant toxins not only bind with high affinity to rat muscle Na_vs but also have a clear effect under the appropriate conditions. In contrast, regardless of the PP length, the depressant toxins did not shift the activation curve of the rat brain channel $rNa_v1.2a$ expressed in oocytes (data not shown), which was in concert with their inability to bind to rat brain synaptosomes (Fig. 2, inset). These data demonstrated that receptor site 4 varies in different Na_vs.

TABLE 2

Alterations of the mid-voltage of activation ($V_{0.5}$) derived from conductance-voltage (G-V) curves of $DmNa_v1$ and $rNa_v1.4$ induced by depressant toxins and their mutants

The $V_{0.5}$ values are from the G-V curves presented in Fig. 5. Depressant toxin effect on $rNa_v1.4$ exhibit two components: a minor negative shift in the $V_{0.5}$ of the entire channel population (upper number) and a stronger shift in the $V_{0.5}$ of a fraction of the toxin-modified channel population (lower number), indicated by the numbers in parentheses. The data represent the mean ± S.E.M. of at least six independent experiments.

Toxin	$DmNa_v1$	$DmNa_v1$ $V_{0.5}$	$rNa_v1.4$	$rNa_v1.4$ $V_{0.5}$
	μM	*mV*	*μM*	*mV*
Control		−19.1 ± 0.5		−26 ± 0.2
Lqh-dprIT3	0.2	−35.4 ± 0.7	5	−31 ± 0.7
				−50.7 ± 2.3 (23%)
Lqh-dprIT3^{E24N}	1	−20.2 ± 06	1	−38.5 ± 0.3
				−60.4 ± 1.5 (11%)
LqhIT2	1	−33.1 ± 0.4	5	−30 ± 0.2
				−52 ± 3.5 (8%)
LqhIT2^{K23A}	5	−20.8 ± 0.7	5	−27.2 ± 1.5
LqhIT2^{E24A}	5	−18.6 ± 0.6	5	−33.5 ± 1.5
				−54.5 ± 9 (15%)
LqhIT2^{E24N}	5	−19 ± −0.5	5	−32.8 ± 1.4
				−43.1 ± 4.1 (62%)

Effect of Substitutions at the "Hot Spot" on Selectivity of LqhIT2 and Lqh-dprIT3. We have shown that substitution of a conserved Glu residue on the bioactive surfaces of the anti-mammalian β-toxin Css4 (Glu28; Fig. 1) and the anti-insect selective excitatory β-toxin Bj-xtrIT (Glu30) abolished their binding and activity at the brain and insect Na_vs, respectively. Substitution of the adjacent Arg27 in Css4 and His25 in Bj-xtrIT also decreased the binding and activity of both toxins (Cohen et al., 2004, 2005). Therefore we analyzed whether the spatially equivalent residues in LqhIT2, Glu24 and Lys23 (Karbat et al., 2007), have a role in toxin binding and activity at $rNa_v1.4$. Substitution K23A decreased LqhIT2 affinity for both cockroach neuronal and rat muscle membranes (Fig. 4, Table 1) as well as abolished the activity at $DmNa_v1$ and $rNa_v1.4$ (Fig. 5, Table 2). Although substitutions E24A/N decreased the toxin affinity for cockroach neuronal membranes by 450-fold (Fig. 4B, Table 1) and no activity was observed at $DmNa_v1$ with up to 5 μM toxin (Fig. 5A, Table 2), LqhIT2$^{E24A/N}$ affinity for the rat muscle *increased* 5-fold (Fig. 4A, Table 1), and the effect at $rNa_v1.4$ after a 500-ms PP was higher than that of LqhIT2 (Fig. 5B, Table 2). Substitution E24N in Lqh-dprIT3 had a similar effect on toxin activity in that the effect at $DmNa_v1$ declined but increased at $rNa_v1.4$ (Fig. 5, C and D, Table 2). These results not only indicate that depressant toxins have different requirements for modifying the activation of insect versus mammalian Na_vs, but also that a single amino acid substitution is able to invert the preference of depressant toxins and make them selective to skeletal muscle Na_vs (Tables 1 and 2).

LqhαIT Modulates LqhIT2 Activity at $rNa_v1.4$. We have shown previously that the α-toxin LqhαIT and the depressant β-toxin LqhIT2 allosterically increase the binding of one another at insect Na_vs, which was manifested in a strong synergism in their toxicity to insects (Cohen et al., 2006). Considering that LqhαIT was shown to inhibit the fast inactivation of $rNa_v1.4$ expressed in HEK cells with an EC_{50} of 1.2 nM (Leipold et al., 2004), we examined whether LqhαIT would modulate LqhIT2 activity at $rNa_v1.4$. LqhαIT in a concentration of 200 nM shifted the voltage-dependence of

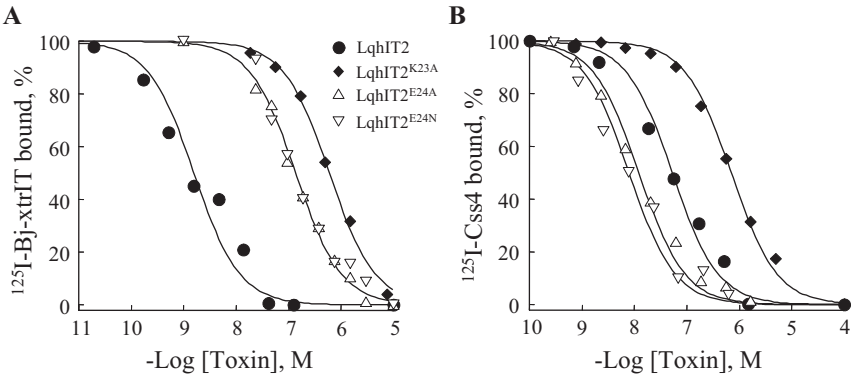

Fig. 4. Binding of LqhIT2 and its mutants to cockroach and rat muscle membrane. Competition of LqhIT2 and mutants with ^{125}I-Bj-xtrIT on binding to cockroach neuronal membranes (A) and with ^{125}I-Css4 to rat muscle membranes (B). Membranes were incubated 60 min at 22°C with 0.1 nM ^{125}I-Bj-xtrIT or ^{125}I-Css4 and increasing concentrations of the various mutants. Nonspecific binding, determined in the presence of 1 μM Bj-xtrIT or Css4, was subtracted. The K_i values in nanomolar, $n \geq 3$, are given in Table 1. Representative experiments are shown.

steady-state fast inactivation of rNa$_v$1.4 expressed in *X. laevis* oocytes by +15 mV ($V_{1/2}$ = −48 ± 0.3 mV in the control and −33.7 ± 0.3 mV in the presence of LqhαIT), thus increasing the percentage of channels available for activation at subthreshold membrane potentials (under −40 mV) with no effect on the channels conductance-voltage relations (Fig. 6). These data suggested that LqhαIT might have facilitated the activity of the depressant toxin. Upon coapplication of LqhαIT (200 nM) and LqhIT2 (5 μM), a 50-ms PP to +60 mV induced a hyperpolarizing shift in rNa$_v$1.4 conductance-voltage relations (Fig. 6A). Such an effect on the channel activation was comparable with that obtained by a 500-ms PP to +60 mV when LqhIT2 was applied alone (Fig. 5B, Table 2). This result demonstrates that LqhαIT binding to rNa$_v$1.4 and/or its influence on fast inactivation modulates the activity of LqhIT2 on this channel as indicated by the shorter PP required to observe the effect of the depressant toxin.

Discussion

The experiments described in this study reveal that the allegedly "insect-selective" scorpion depressant toxins are

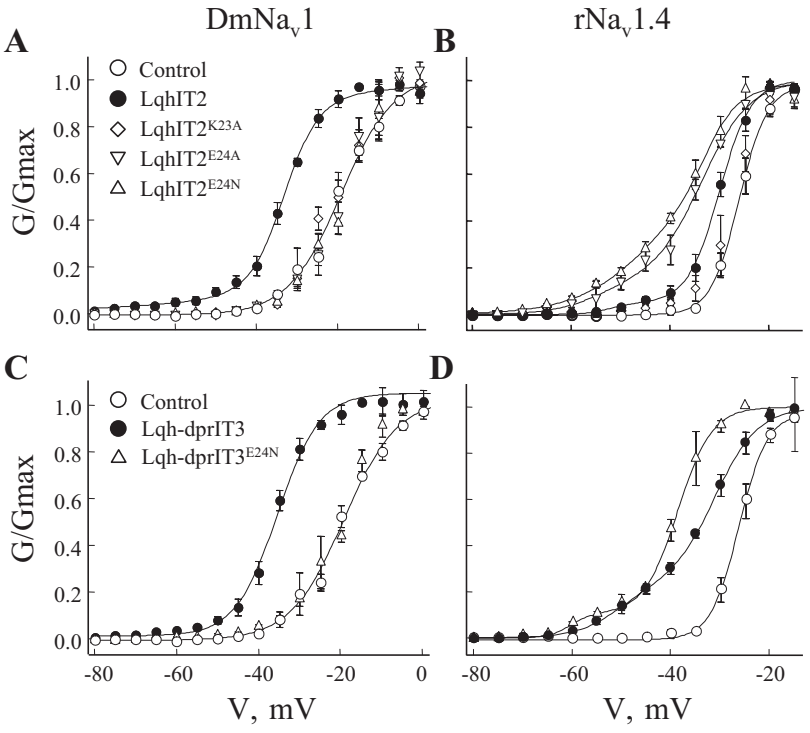

Fig. 5. Alterations in conductance-voltage (G-V) relations induced by LqhIT2 and Lqh-dprIT3 mutants at DmNa$_v$1 and rNa$_v$1.4. The effects of LqhIT2 and its mutants K23A, E24A, and E24N on DmNa$_v$1 (A) and rNa$_v$1.4 (B). The effects of Lqh-dprIT3 and its mutant E24N on DmNa$_v$1 (C) and rNa$_v$1.4 (D). The labels in A cover B and those in C cover D. Toxin concentrations and the activation parameters ($V_{0.5}$) are as described in Table 2. Conductance-voltage relations were determined as described in Fig. 3 with a 50-ms PP for DmNa$_v$1 and a 500-ms PP for rNa$_v$1.4. The data represent the mean ± S.E.M. of at least six independent experiments.

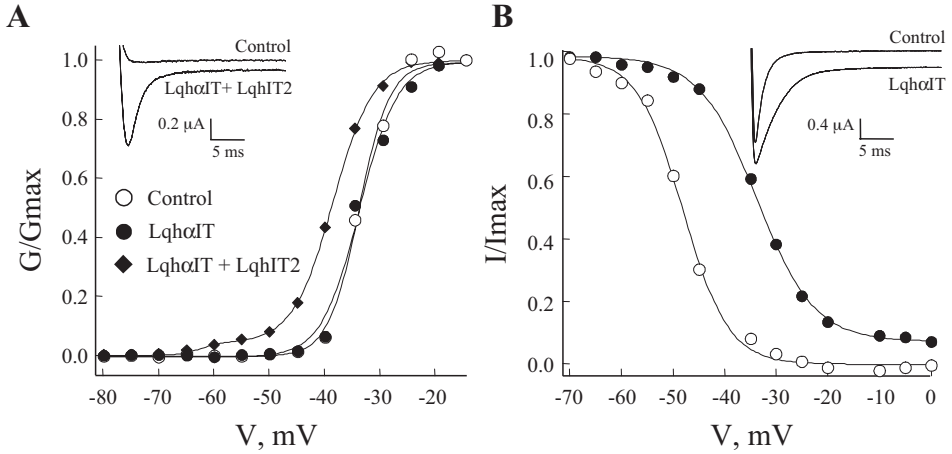

Fig. 6. Effects of combined application of LqhIT2 and LqhαIT on rNa$_v$1.4 activation. A, conductance-voltage relations of rNa$_v$1.4 in the absence of toxin ($V_{0.5}$ = −33 ± 0.3 mV, n = 3), in the presence of 200 nM LqhαIT ($V_{0.5}$ = −33 ± 0.4 mV), and in the presence of 200 nM LqhαIT and 5 μM LqhIT2 ($V_{0.5}$ = −38 ± 0.1 for all channels, and −52 ± 1.5 mV for 8% of the channel population; see *Materials and Methods* and Table 2). Conductance-voltage relations were determined as described in Fig. 2 with a 50-ms PP to +60 mV. Note that 5 μM LqhIT2 had no effect on activation (see Fig. 3B1). Inset, current traces obtained in the absence of toxins and upon coapplication of 200 nM LqhαIT and 5 μM LqhIT2 at a test pulse to −45 mV after a 50-ms PP to +60 mV. B, steady-state fast inactivation was determined from holding potential of −100 mV using a series of 50-ms PP from −80 to −20 mV in 5-mV increments before the test pulse of −20 mV. The steady-state inactivation of rNa$_v$1.4 fits a Boltzmann function with $V_{0.5}$ = −48 ± 0.3 mV, and in the presence of 200 nM LqhαIT, $V_{0.5}$ = −33.7 ± 0.3 mV. Inset, effects of 200 nM LqhαIT on the current fast inactivation elicited by a test pulse to −20 mV from a holding potential of −100 mV.

capable of binding with high affinity and affecting mammalian skeletal muscle Na_vs, a fact unnoticed for almost 3 decades. The classification of depressant toxins as insect-selective relied on the lack of toxicity when injected to mice and inability to bind rat brain synaptosomes (Zlotkin et al., 1993; Gordon et al., 1998; Gurevitz et al., 2007) or affect mammalian Na_vs expressed in *X. laevis* oocytes (Gordon et al., 2003; Bosmans et al., 2005). However, a number of recent results have suggested that the issue of selectivity of depressant toxins toward insects deserves reexamination: 1) scorpion β-toxins share a common pharmacophore (Cohen et al., 2005; Karbat et al., 2007), which explains their ability to compete in binding; 2) receptor site 4 on rat skeletal muscle Na_vs was suggested to differ from those of various mammalian neuronal and cardiac Na_vs (Marcotte et al., 1997; Cestèle et al., 1998; Leipold et al., 2006; Shciavon et al., 2006; Cohen et al., 2007); and 3) scorpion α- and β-toxins exert synergistic effects as a e result of allosteric interactions between receptor sites 3 and 4 on insect Na_vs (Cohen et al., 2006).

On the basis of these considerations, we reanalyzed the activity of depressant toxins on insect and mammalian Na_vs. Although depressant toxins were unable to bind rat brain Na_vs, they exerted high affinity for the rat skeletal muscle Na_vs (Fig. 2), which motivated us to analyze their effects on channel activation. The unexpected observation that the effect of the depressant toxin LqhIT2 on the insect Na_v $DmNa_v1$ necessitated a 10-fold longer PP than that required to observe a Css4 effect on mammalian Na_vs suggested that LqhIT2 should be analyzed on $rNa_v1.4$ after a longer PP. The requirement for PP has been attributed to a putative energetic barrier that needs to be overcome before the prebound β-toxin can trap the DII/S4 voltage sensor in its outward activated position, thus leading to enhanced channel activation upon subsequent depolarizations (Cestèle et al., 1998). Thus far, a short PP (several milliseconds) was ample for inducing a noticeable effect of most anti-mammalian β-toxins on mammalian Na_vs (Cestèle et al., 1998, 2006; Tsushima et al., 1999; Cohen et al., 2005). Here we show that a very long PP (>500 ms) to +60 mV made $rNa_v1.4$ vulnerable to depressant toxins. It is likely that because depressant toxins did not modulate the gating properties of $rNa_v1.2$ (Bosmans et al., 2005) and $rNa_v1.4$ (Fig. 2; Gordon et al., 2003) after a depolarizing PP up to 50 ms, their activity on mammalian Na_vs has not been noticed thus far.

The ability of depressant toxins to influence the activation of the mammalian Na_v after a long PP was surprising and raised the question of their putative role in vivo. In light of the enhancement of LqhIT2 binding to insect Na_vs in the presence of a scorpion α-toxin from the same venom, LqhαIT (Cohen et al., 2006), and because LqhαIT is highly potent on insect as well as a variety of mammalian Na_v subtypes, including $rNa_v1.4$ (Eitan et al., 1990; Chen et al., 2000; Leipold et al., 2004; Gordon et al., 2007), it was rational to analyze the joint effect of both toxins on $rNa_v1.4$. Indeed, the synergism between site 3 and site 4 toxins observed at $rNa_v1.4$ may be explained by the reduction of at least 10-fold in PP duration required for induction of LqhIT2 effect in the presence of LqhαIT (Fig. 6). Such a mechanism may also apply to insect Na_vs, where synergism between site 3 and site-4 toxins was reported (Cohen et al., 2006). LqhαIT increases neuronal excitability and neuromuscular activity upon binding to receptor site 3 by induction of long plateau potentials in axons attributed to an increase in the probability of Na_vs to remain in open states as a result of inhibition of their fast inactivation (Eitan et al., 1990; Gilles et al., 2000; Lee et al., 2000; Benoit and Gordon, 2001). This inhibition of channel steady-state fast inactivation expands the channel population available for activation at resting membrane potential, leading to increase in the frequency of action potentials, which may act as a prepulse to facilitate β-toxin activity. This, in turn, may facilitate α-toxin action on the channels in their open states. Thus, the mutual enhancement in α-toxin effect by β-toxin interaction with receptor site 4, and vice versa, results from an indirect modification of receptor sites 3 and 4, respectively and from alteration in the voltage-dependence of channel activation (Cohen et al., 2006). We suggest, based on these considerations, that $Na_v1.4$ in a stung mammal is preconditioned upon binding of the α-toxin, thus enabling synergistic toxicity by the joint effects of α- and depressant toxins.

The venom of *L. quinquestriatus hebraeus* contains a number of α-toxins active on mice (e.g., LqhαIT, Lqh2, Lqh3, Lqh4, Lqh6, and Lqh7; for references, see Gordon et al., 2007) and β-toxins that exhibit preference for insects, including various depressant toxins (e.g., LqhIT2, LqhIT5, Lqh-dprIT3, Lqhβ1; for references, see Gurevitz et al., 2007), which together increase the impact of stinging. In their isolated form, however, the depressant toxins may be considered selective to insects, in that their affinity for insect neuronal membranes is 2 orders of magnitude higher than the affinity for rat muscle membranes (Figs. 2, 4, and 5, Table 1; Gordon et al., 1992; Strugatsky et al., 2005).

A surprising feature in the interaction of depressant toxins with the mammalian muscle Na_v is the lack of function of Glu24, found to be conserved in other scorpion β-toxins and considered a hot spot on the bioactive surface of these toxins toward insect and rat brain Na_vs (Cohen et al., 2004, 2005; Karbat et al., 2007) (Fig. 1), suggesting that receptor site 4 on the muscle channels differs from that on $DmNa_v1$. Further understanding of how scorpion β-toxins interact in a preferential manner with various receptor sites on Na_v subtypes seems to await elucidation of toxin-receptor interacting surfaces. At the moment, our results indicate that a single substitution at this position ($LqhIT2^{E24A/N}$ and $Lqh\text{-}dprIT3^{E24N}$; Figs. 4 and 5, Table 1) converts these depressant toxins from "insect-selective" to "mammal-selective."

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