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Critical Molecular Determinants of Voltage-Gated Sodium Channel Sensitivity to μ-Conotoxins GIIIA/B

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ABSTRACT

GIIIA/B μ-conotoxins block the rat skeletal muscle sodium channel (rNa$_v$1.4) with high affinity by binding to specific residues in the pore. However, human Na$_v$1.4 (hNa$_v$1.4) channels, which are resistant to block by GIIIA/B, have these same pore residues. We used chimera constructs, site-directed mutagenesis, and electrophysiological techniques to investigate which residues determine GIIIA/B selectivity. Exchange of serine 729 in the D2/S5-S6 linker of rat Na$_v$1.4 with leucine (S729L), the corresponding residue in hNa$_v$1.4, reduces the sensitivity of rNa$_v$1.4 by ~20-fold and largely accounts for the differential sensitivity of rNa$_v$1.4 and hNa$_v$1.4 to both GIIIA and GIIIB. To determine whether D2/S5-S6 linker residues might contribute to the resistance of neuronal channels to GIIIA/B, we exchanged residues in this linker that differed between rNa$_v$1.4 and neuronal channels. Substitution of aspargine 732 with lysine (N732K), the corresponding residue in rNa$_v$1.1a and rNa$_v$1.7, reduced the GIIIB sensitivity of rNa$_v$1.4 by ~20-fold. The N732K substitution, however, only reduced GIIIA sensitivity of rNa$_v$1.4 by ~4-fold, demonstrating that GIIIA and GIIIB have distinct interactions with the D2/S5-S6 linker. Our data indicate that naturally occurring variants in the extra-pore region of the D2/S5-S6 linker contribute to the isoform-specific sensitivity of sodium channels to GIIIA/B. Because S729 and N732 are not part of the high-affinity binding site for μ-conotoxins, these extra-pore residues probably influence the accessibility of the toxin to the binding site within the pore and/or the stability of the toxin-channel complex. Our results should aid the development of toxins that block specific neuronal sodium channel isoforms.

The mollusk genus *Conus* consists of a large number of carnivorous marine snails that produce a cocktail of small-peptide toxins, conotoxins, that are used to paralyze their prey and that can also be fatal to mammals (Gray et al., 1988). The conotoxin peptide is generally characterized by a compact structure of hypervariable amino acid residues superimposed over a scaffold of two to three disulfide bridges (Olivera et al., 1990). Members of a conotoxin family have been used as pharmacological reagents that distinguish between individual members of the respective target ion channel family. For example, GIIIA/B μ-conotoxins target rat skeletal muscle voltage-gated sodium channels (rNa$_v$1.4) but not neuronal sodium channels (Shon et al., 1998; McIntosh et al., 1999; Safo et al., 2000). GIIIA and GIIIB μ-conotoxins, which are often considered indistinguishable, block rNa$_v$1.4 with an IC$_{50}$ of 50 nM (Cruz et al., 1985; Moczydlowski et al., 1986; Yanagawa et al., 1987; Trimmer et al., 1989; Chen et al., 1992). However, cardiac and neuronal Na^+ channels show much reduced affinity to the GIIIA/B toxins (Cruz et al., 1985; Moczydlowski et al., 1986; White et al., 1991; Chen et al., 1992; Gellens et al., 1992; Shon et al., 1998), and Chahine et al. (1994a) reported that human skeletal muscle (hNa$_v$1.4) channels expressed in human embryonic kidney (HEK) 293 cells are less sensitive to GIIIA (IC$_{50}$ of ~1500 nM) than rNa$_v$1.4.

Although it is not clear what determines the relative insensitivity of specific sodium channels to μ-conotoxins, several studies have identified specific toxin-channel interactions that are critical determinants of the toxin block of rNa$_v$1.4. Alanine-scanning mutagenesis revealed that the basicity of GIIIA (+6 at neutral pH) is crucial for the activity of the toxin, with the arginine 13 (R13) residue having the biggest effect on blocking the Na^+ current (Sato et al., 1991; Becker et al., 1992). The GIIIA and GIIIB μ-conotoxins have a similar asymmetrical three-dimensional structure (Lancelin et al., 1991; Hill et al., 1996) and recent studies have shown that these μ-conotoxins might have a specific docking orientation (Dudley et al., 2000; Li et al., 2001a). Mutations

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ABBREVIATIONS: Na$_v$1.4, voltage-gated sodium channel α-subunit from skeletal muscle; Na$_v$1.1, brain type I; Na$_v$1.2, brain type II; Na$_v$1.7, peripheral nerve I; Na$_v$1.5, cardiac sodium channel; HEK, human embryonic kidney; PCR, polymerase chain reaction; WT, wild-type; TTX, tetrodotoxin; TTX-S, tetrodotoxin-sensitive; r, rat; h, human.

of pore-lining residues close to the selectivity filter reduced the binding affinity of the toxin, with the largest changes reported for substitutions of the negatively charged residues, glutamic acid (E) at positions 758 and 765, and aspartic acid (D) at position 762 (Dudley et al., 1995; Chang et al., 1998; Li et al., 2000). These results are consistent with the suggestion that electrostatic interaction of the R13 residue of GIIIB with the negatively charged groups of the pore is important for the toxin block of rNa$_v$1.4 (Becker et al., 1992; Dudley et al., 1995; Chang et al., 1998; Li et al., 2000). Although electrostatic interactions within the pore are clearly important to the high-affinity block of rNa$_v$1.4 by GIIIA/B, the pore residues reported to interact with GIIIA/B are conserved at the comparable positions in nearly all members of the voltage-gated sodium channel family; hence, residues at these positions cannot determine the selectivity of GIIIA/B to rat Na$_v$1.4.

We report in this study the identification of naturally occurring amino acid variants at comparable positions in the S5-S6 linker of domain 2 of multiple channels (S729 in rNa$_v$1.4 to L735 in hNa$_v$1.4, and N732 in rNa$_v$1.4 to K928 and K903 in neuronal rNa$_v$1.1a and rNa$_v$1.7 channels, respectively), which are individually sufficient to confer resistance to GIIIB on recombinant rNa$_v$1.4 channels. We also demonstrate different kinetic properties of binding of GIIIA and GIIIB with wild-type rNa$_v$1.4 and a differential effect of the two toxins on mutant channels, demonstrating distinct features of these toxins. These results support a model of complex channel-toxin interaction whereby specific residues N-terminal to the SS1 segment of the P loop are critical to determine the toxin selectivity to rat skeletal muscle sodium channel.

Materials and Methods

Construction of Human-Rat Na$_v$1.4 Channel Chimera. The insert encoding hNa$_v$1.4 in the vector pRc/CMV (Chahine et al., 1994b) was subcloned in two steps into the vector RBG4 to facilitate linker swapping between human and rat channels and to use the same promoter to control the expression of the two inserts. The plasmid μ1-RBG4 (Ukomadu et al., 1992) carrying the rNa$_v$1.4 insert was digested with EcoICRI in the vector and *Sac*II in the insert to remove the sequence encoding domains 1 to 3, and the remainder of the construct (vector plus domain 4) was band isolated. The plasmid hNa$_v$1.4-pRC-CMV was digested by *Not*I and the ends polished using T4 DNA polymerase (Roche Applied Science, Indianapolis, IN), and the fragment (4467 base pairs) encoding the respective human cognate of domains 1 to 3 was subsequently released by digestion with *Sac*II. This partial hNa$_v$1.4 insert was band isolated and subcloned into the partial rNa$_v$1.4 construct described above to produce a chimera channel of human domains 1 to 3 and rat domain 4. Chimera inserts were confirmed by sequencing the junctions. The chimera channel showed toxin sensitivity (data not shown) identical to that of hNa$_v$1.4, in general agreement with published results (Chahine et al., 1998a) that domain 2 determines GIIIA/B sensitivity. The sequence encoding hNa$_v$1.4 domain 4 was isolated as a *Sac*II/*Eco*RI fragment and used to replace the remaining rNa$_v$1.4 domain 4 in the chimera.

Exchange of Domain 2 S5-S6 Linker between Human and Rat Na$_v$1.4 Channels. A 244-base pair fragment that encodes D2/S5-S6 linker was amplified from both human and rat Na$_v$1.4 constructs using forward primer F1 (5′-GGCCATCATCGTCTTCATCTTCG-3′, corresponding to nucleotides 2552 to 2574 and 2198 to 2220 of rat and human Na$_v$1.4, respectively) and reverse primer R1 (5′-CAATGACCATGACCATGAGGAAG-3′, corresponding to nucleotides 2796 to 2774 and 2442 to 2420 of rat and human Na$_v$1.4, respectively). F1 has two nucleotide changes compared with the respective human sequence (C to T at position 4, and C to G at position 13), whereas the reverse primer sequence is identical in both channels. These changes do not affect the amplification of the product under the conditions that were used in this study and are eliminated from the respective constructs because they are distal to the restriction enzymes *Sph*I and *Fse*I (see below) used in swapping fragments encoding the linkers.

PCR amplification was performed in 50-μl final volume using 1 ng of respective plasmid DNA template, 0.5 μM primers, and 3.5 U Expand Long Template enzyme mixture in buffer 3 (Roche Applied Science). Amplification was via 1) 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and elongation at 72°C for 30 s; or 2) elongation at 72°C for 10 min. The respective PCR products were digested with the *Sph*I and *Fse*I enzymes (New England Biolabs, Beverly, MA) that are unique in both the rat and human inserts, and the remaining 163-base pair fragment encoding D2/S5-S6 linker was band isolated and exchanged between the two plasmids to produce the hRhh and rHrr chimera channels. The reciprocal chimeras were verified by sequencing the respective inserts. Sequencing was carried out at the Howard Hughes Medical Institute/Keck Biotechnology Resource Laboratory at Yale University (New Haven, CT).

Point Mutation of rNa$_v$1.4. PCR-based mutagenesis (Horton et al., 1993) was used to introduce amino acid substitutions to D2/S5-S6 linker of Na$_v$1.4. Wild-type primers F1 and R1 (described above) were used together with multiple mutagenic primers (Table 1) to introduce the respective substitutions.

Separate PCR reactions were performed using 1 ng of the μ1-RGB4 plasmid as a template with the F1/MR1 and MF1/R1 primer pairs, where MR1 and MF1 are the reverse and forward mutagenic primers, respectively. The two PCR products were band isolated and used as a mixed template for PCR with the F1/R1 primer pair. The final PCR product (244 base pairs) was digested with *Sph*I and *Fse*I and the 163-base pair fragment, containing the respective mutation, was used to replace the WT fragment in μ1-RGB4 as described above.

TABLE 1

Mutagenic primers used to introduce amino acid substitutions in the D2/S5-S6 linker of rNav1.4

Forward and reverse mutagenic primers 1 to 3 correspond to nucleotides 2622 to 2648; forward and mutagenic primers 4 correspond to nucleotides 2652 to 2678; forward and reverse mutagenic primers 5 to 9 correspond to nucleotides 2631 to 2660. Substituted codons are underlined.

	Mutagenic Forward Primer	Mutagenic Reverse Primer	Substitution
1	TGCAAGATCGCCTTGGACTGCAACCTG	CAGGTTGCAGTCCAAGGCGATCTTGCA	S729L
2	TGCAAGATCGCCACTGACTGCAACCTG	CAGGTTGCAGTCAGTGGCGATCTTGCA	S729T
3	TGCAAGATCGCCGCGGACTGCAACCTG	CAGGTTGCAGTCCGCGGCGATCTTGCA	S729A
4	CGCTGGCACATGCATGACTTCTTCCAC	GTGGAAGAAGTCATGCATGTGCCAGCG	N739H
5	GCCTCAGACTGCAAGCTGCCTCGCTGGCAC	GTGCCAGCGAGGCAGCTTGCAGTCTGAGGC	N732K
6	GCCTCAGACTGCGAGCTGCCTCGCTGGCAC	GTGCCAGCGAGGCAGCTCGCAGTCTGAGGC	N732E
7	GCCTCAGACTGCCAGCTGCCTCGCTGGCAC	GTGCCAGCGAGGCAGCTGGCAGTCTGAGGC	N732Q
8	GCCACTGACTGCAAGCTGCCTCGCTGGCAC	GTGCCAGCGAGGCAGCTTGCAGTCAGTGGC	S729T, N732K
9	GCCTTGGACTGCAAGCTGCCTCGCTGGCAC	GTGCCAGCGAGGCAGCTTGCAGTCCAAGGC	S729L, N732K

Transfection of HEK 293 Cells. Transfections were carried out using the calcium phosphate precipitation technique, as described previously (Cummins et al., 1998). HEK 293 cells are grown under standard tissue culture conditions (5% CO_2, 37°C) in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. The calcium phosphate-DNA mixture (channel constructs and a green fluorescent protein reporter plasmid) was added to the cell culture medium and left for 1 to 2 h, after which time the cells were washed with fresh medium. Cells with green fluorescent protein fluorescence were selected for whole-cell patch-clamp recordings after 1 to 2 days in culture.

Whole-Cell Patch-Clamp Recordings. Whole-cell patch-clamp recordings were conducted at room temperature (~21°C) using an EPC-9 amplifier (HEKA, Lambrecht, Germany). Data were acquired on a Pentium III computer using the Pulse program (version 8.31; HEKA). Fire-polished electrodes (0.8–1.5 MΩ) were fabricated from 1.7-mm capillary glass (VWR, West Chester, PA) using a P-97 puller (Sutter, Novato, CA). Cells were not considered for analysis if the initial seal resistance was less than 5 GΩ or if they had high leakage currents (holding current >0.2 nA at −100 mV), membrane blebs, or an access resistance greater than 4 MΩ. The average access resistance was 1.5 ± 0.1 MΩ (mean ± S.E., n = 208). Voltage errors were minimized using 80 to 85% series resistance compensation and the capacitance artifact was canceled using the computer-controlled circuitry of the patch-clamp amplifier. The average current amplitude was 11.6 ± 0.8 nA (n = 208) and the average maximum theoretical voltage-clamp error was 3.5 ± 0.2 mV. Linear leak subtraction, based on resistance estimates from four to five hyperpolarizing pulses applied before the depolarizing test potential, was used for all voltage-clamp recordings. Membrane currents were usually filtered at 2.5 kHz and sampled at 10 kHz. The pipette solution contained 140 mM CsF, 1 mM EGTA, 10 mM NaCl, and 10 mM HEPES, pH 7.3. The standard bathing solution was 140 mM NaCl, 3 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 10 mM HEPES, pH 7.3. The liquid junction potential for these solutions was <8 mV; data were not corrected to account for this offset. The osmolarity of all solutions was adjusted to 310 mOsM with sucrose (5500 osmometer; Wescor, Logan, UT).

Toxin Solutions and Bath Application. GIIIB was obtained from Alamone Laboratories (Jerusalem, Israel) and Sigma-Aldrich (St. Louis, MO). Similar results were obtained for $rNa_v1.4$ currents with GIIIB from both sources. GIIIA was obtained from Sigma-Aldrich. Stock solutions (20 μM for GIIIA and GIIIB) were made using extracellular recording solution and aliquots were stored at −20°C. Toxins were diluted into the recording chamber (volume of 500 μl) and mixed by repeatedly pipetting 50 μl to achieve the specified final concentration. The chamber was not perfused during toxin application. The mixing procedure typically took ~5 s. Toxin was applied for 5 to 25 min to allow the peak current amplitude to reach a steady-state level. Toxins were washed off in specific experiments using a gravity-fed perfusion system, with the delivery tube (0.5 mm i.d., 0.5 ml/min flow rate) placed 1 mm from the cell. This directly bathed the cell in toxin-free solution and allowed a rapid solution change (<1 s) for measuring off rates. Toxin wash-off was continued for 10 to 60 min to allow peak current amplitude to reach a steady-state level. Toxin application and wash-off protocols were validated using tetrodotoxin (TTX) and $rNa_v1.4$ channels. A τ_{on} of 8 ± 0.2 s and a τ_{off} of 26.7 ± 4.1 s was measured using 25 nM TTX, which results in a calculated K_D of 9.1 nM (n = 3). The K_D closely matches the IC_{50} measured using 25 nM TTX (8.0 ± 0.2 nM, n = 3), and demonstrates that this system is sufficient for measuring the "on" and "off" kinetics of μ-conotoxins.

Data Analysis. Data were analyzed using the Pulsefit (HEKA) and Origin (MicroCal Software, Northampton, MA) software programs. Unless otherwise noted, statistical significance was determined at $p < 0.05$ using an unpaired t test. The half-blocking concentration (IC_{50}) was calculated based on the single-site Langmuir inhibition isotherm using the following function: $IC_{50} = (I_{toxin} / I_0) \times [toxin] / (1 - I_{toxin} / I_0)$, where I_0 and I_{toxin} are the peak sodium currents measured before and after application of toxin, respectively, and [toxin] is the concentration of toxin. Unless otherwise noted, the IC_{50} was calculated using a toxin concentration of 300 nM. Time course data for toxin block and wash-off were fitted with single exponential functions to estimate the time constants τ_{on} and τ_{off}. The "on" and "off" rate constants for block (k_{on} and k_{off}) were calculated using the following equations: $k_{off} = 1 / \tau_{off}$ and $k_{on} = [(1 / \tau_{on}) - (1 / \tau_{off})] / [toxin]$. The kinetically derived toxin equilibrium constant (K_D) was calculated using the equation $K_D = k_{off} + k_{on}$. Results are presented as mean ± S.E.M. and error bars in the figures represent S.E..

Results

Human $Na_v1.4$ Sodium Channel Is Resistant to GIIIB μ-Conotoxin. The skeletal muscle sodium channel cloned from rat ($rNa_v1.4$) is blocked by GIIIA/B μ-conotoxins at nanomolar concentrations (Cruz et al., 1985; Moczydlowski et al., 1986; Yanagawa et al., 1987; Trimmer et al., 1989; Chen et al., 1992). As Fig. 1A illustrates, most of the current through $rNa_v1.4$ expressed in HEK 293 cells is blocked by 300 nM GIIIB. Although rat and human $Na_v1.4$ channels exhibit more than 90% identity at the amino acid level, the current through $hNa_v1.4$ expressed in HEK 293 cells is relatively insensitive to GIIIB (Fig. 1B). Based on fits to the relationship between percentage of current inhibition versus toxin concentration, the IC_{50} for GIIIB block of $hNa_v1.4$ channels is 1065 nM (18 measurements from 12 cells), compared with 49 nM (14 measurements from 8 cells) for $rNa_v1.4$ channels (Fig. 1C). Based on the percentage of current inhibition measured with just a single concentration (300 nM) of GIIIB, the estimated IC_{50} for $hNa_v1.4$ channels is 1357 ± 314 nM (n = 6), compared with 49 ± 5 nM (n = 12) for $rNa_v1.4$ channels. The sensitivity of $hNa_v1.4$ channels to GIIIB is similar to that previously reported for μ-conotoxin GIIIA (Chahine et al., 1994a).

Sensitivity to GIIIB Is Determined by Residues in S5-S6 Linker of Domain 2. Chahine et al. (1999b) reported that mutations in the S5-S6 linker of domain 2 (D2/S5-S6) altered $rNa_v1.4$ sensitivity to GIIIA by ~5- to 6-fold. Therefore, we compared the sequences of $rNa_v1.4$ and $hNa_v1.4$ in this region to identify residues that might contribute to the differential sensitivity of the two channels to μ-conotoxins. The residues that Chahine et al. (1998b) mutated in $rNa_v1.4$ (A728 and D730) are conserved in $hNa_v1.4$; however, the D2/S5-S6 linker in these two channels differs by two amino acid residues (Fig. 2). A serine at position 729 (S729) in $rNa_v1.4$ is replaced by leucine in $hNa_v1.4$; and an asparagine at position 739 (N739) of $rNa_v1.4$ is replaced by histidine (H) in $hNa_v1.4$ (numbers are based on $rNa_v1.4$ sequence). We reasoned that one or both of these substitutions might be critical for the selectivity of GIIIA/B to $rNa_v1.4$. To examine the effect of these residues on GIIIB sensitivity, we exchanged the D2/S5-S6 linker between $rNa_v1.4$ and $hNa_v1.4$. The effect of swapping this linker on the toxin block was studied in transiently transfected HEK 293 cells. Cells transfected with parental and chimera constructs produced currents with indistinguishable kinetic and voltage-dependent properties. We used a toxin concentration of 300 nM (unless otherwise noted) to estimate the sensitivity of the channels to the toxin. As can be seen in Fig. 3A, $hNa_v1.4$ channels with the rat D2/S5-S6 linker (hRhh) had a GIIIB sensitivity (IC_{50} = 80 ± 7, n = 6) close to that of $rNa_v1.4$ channels. In

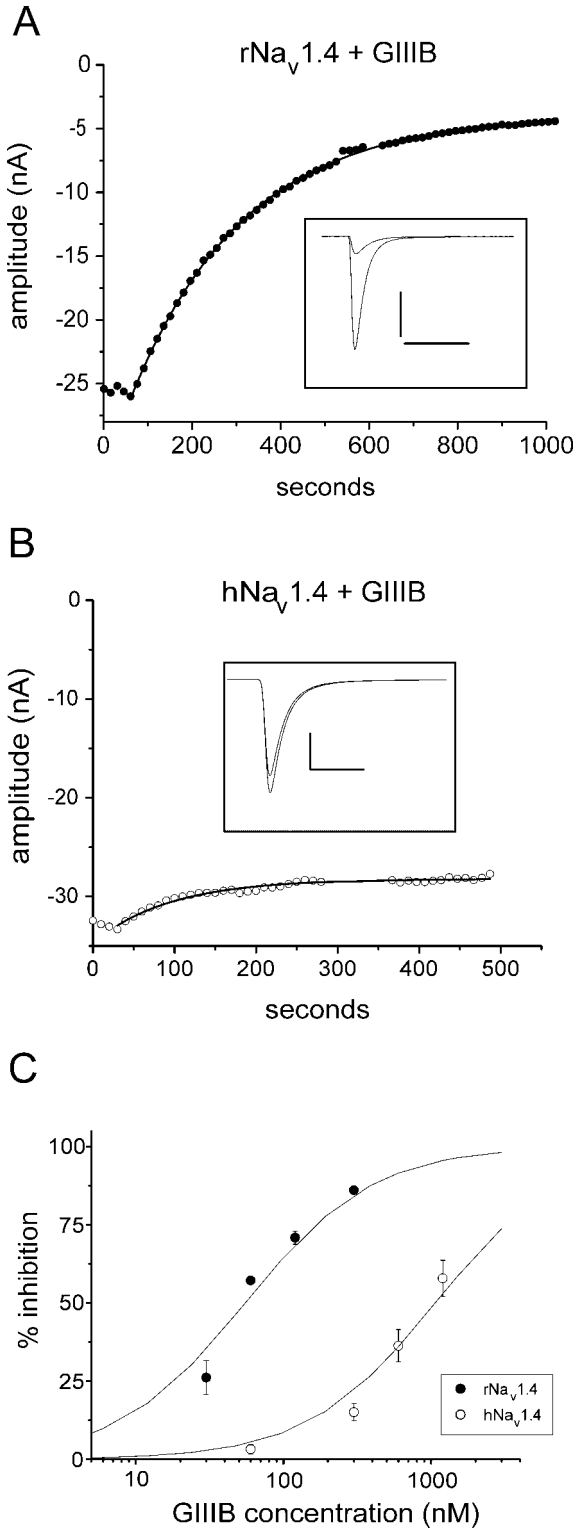

Fig. 1. GIIIB differentially blocks rat and human Na$_v$1.4 sodium channels. Cells expressing rNa$_v$1.4 (A) or hNa$_v$1.4 (B) sodium channels were exposed to 300 nM μ-conotoxin GIIIB. The cells were held at −120 mV and stepped to −10 mV for 50 ms every 10 s. GIIIB was applied in the bath solution after the fourth pulse. Data were fitted with single exponential functions to estimate the time constants for toxin binding (τ_{on} is 257 s in A and 93 s in B). Insets show representative current traces before and after application of 300 nM GIIIB. Horizontal scale bars, 2 ms. Vertical scale bars, 10 nA. C, dose-response relationship for μ-conotoxin GIIIB inhibition of rNa$_v$1.4 (●) and hNa$_v$1.4 (○) channels expressed in HEK 293 cells. The rNa$_v$1.4 channels are ~20-fold more sensitive to GIIIB inhibition than hNa$_v$1.4 channels.

contrast, rNa$_v$1.4 channels with the human D2/S5-S6 linker (rHrr) had a GIIIB sensitivity (IC_{50} = 1304 ± 287, n = 5) similar to that of hNa$_v$1.4 channels (Fig. 3B). Thus, the D2/S5-S6 linker of rNa$_v$1.4 channels is crucial in determining sensitivity to GIIIB conotoxins.

To investigate the individual roles of S729 and N739 in determining the sensitivity of rNa$_v$1.4 channels to GIIIB, we introduced single amino acid substitutions at these positions. When N739 of rNa$_v$1.4 was replaced by histidine (N739H[1]), the corresponding residue in hNa$_v$1.4, the GIIIB sensitivity was still similar to that of wild-type rNa$_v$1.4 channels (Fig. 3C; IC_{50} = 67 ± 13, n = 5). However, when S729 was replaced by leucine (S729L), the GIIIB sensitivity was similar to that of wild-type hNa$_v$1.4 channels (Fig. 3D; IC_{50} = 1138 ± 157, n = 6). Thus, the leucine residue in hNa$_v$1.4 at the position corresponding to the serine (S729) in rNa$_v$1.4 plays a critical role in conferring the relative insensitivity of hNa$_v$1.4 to the GIIIB μ-conotoxin.

Neuronal sodium channels are thought to be insensitive to GIIIB. Much of the D2/S5-S6 linker is conserved between rNa$_v$1.4 and neuronal sodium channels, but there are some differences (Fig. 2). Rat brain type I (rNa$_v$1.1a) channels have a threonine (T) at the position corresponding to S729 in rNa$_v$1.4. Therefore, we replaced S729 by threonine (S729T). The S729T channels were ~2.5-fold less sensitive to GIIIB (IC_{50} = 121 ± 15, n = 6) compared with wild-type rNa$_v$1.4 channels (Fig. 3E). This suggests that this threonine residue is not a major determinant of the insensitivity of rNa$_v$1.1a to GIIIB μ-conotoxin. Our data showing that the S729L mutation reduced GIIIB sensitivity much more than the S729T mutation raises the possibility that a hydroxyl group in the side chain of the amino acid at this position could be an important determinant of GIIIB sensitivity. To examine the role of the hydroxyl group of the side chain of S729 in GIIIB inhibition of rNa$_v$1.4, we replaced S729 with alanine (S729A). The GIIIB sensitivity of S729A channels (IC_{50} = 68 ± 8, n = 6) was similar to that of wild-type rNa$_v$1.4 channels (Fig. 3F). The S729A replacement clearly suggests that the hydroxyl group of this residue is not crucial for toxin binding. However, the sensitivity of rNa$_v$1.4 channels to GIIIB seems to be inversely correlated with the size of the amino acid side chain at the 729 position.

We asked whether other differences in the D2/S5-S6 linker between rNa$_v$1.1a and rNa$_v$1.4 might affect GIIIB sensitivity. Rat Na$_v$1.1a has a lysine (K) at the position corresponding to N732 in rNa$_v$1.4 (Fig. 2). Therefore, we also introduced the N732K and the S729T/N732K replacements to examine their effect on GIIIB inhibition of rNa$_v$1.4. The sensitivity of rNa$_v$1.4 to GIIIB was significantly reduced by the N732K replacement (Fig. 4A; IC_{50} = 972 ± 137, n = 7). The double mutant S729T/N732K had a similar reduced sensitivity to GIIIB (IC_{50} = 995 ± 159, n = 6), suggesting no additive effect of the S729T mutation in this assay. The reduced sensitivity of rNa$_v$1.4-N732K channels to GIIIB shows that specific residues in the D2/S5-S6 linker of neuronal sodium channels are also likely to play a significant role in determining the sensitivity of these channels to the GIIIB toxin.

Interestingly, the human Na$_v$1.1 sodium channel has a

[1]The notation X#Z represents the replacement of WT residue X at that position of rNa$_v$1.4 with residue Z. The notation $X_1\#Z_1/X_2\#Z_2$ represents a double mutation.

glutamine (Q) at the position corresponding to N732 in rNa$_v$1.4 (Fig. 2). Therefore, we also introduced the N732Q replacement into rNa$_v$1.4. The sensitivity of rNa$_v$1.4 to GIIIB was not altered by the N732Q replacement (Fig. 4B; IC_{50} = 56 ± 8, n = 8). To the best of our knowledge, the sensitivity of hNa$_v$1.1 sodium channels to μ-conotoxins has not been determined. Therefore, these data raise the intriguing possibility that although hNa$_v$1.4s are resistant to GIIIB, hNa$_v$1.1 sodium channels may be sensitive to this toxin. Because both of the N732K and N732Q replacements increase the size of the amino acid side chain at this position, but only the N732K replacement alters GIIIB sensitivity, the size of the side chain at this position does not seem to be a critical factor in determining GIIIB sensitivity. However, because the N732K replacement alters the charge at this position, the basicity of the residue at this position may be important.

Although rNa$_v$1.7 also has a lysine (K) at the position corresponding to N732 in rNa$_v$1.4, which is likely to contribute to the insensitivity of rNa$_v$1.7 channels to GIIIB (Safo et al., 2000), rat Na$_v$1.2a channels have a glutamate (E) at the position corresponding to N732 in rNa$_v$1.4 (Fig. 2). Therefore, we tested the N732E replacement to examine the effect of a negative charge at this position on GIIIB inhibition of rNa$_v$1.4. The sensitivity of rNa$_v$1.4 to GIIIB was not significantly affected by the N732E mutation (Fig. 4C; IC_{50} = 41.2 ± 4.8, n = 5). This indicates that a positive charge, but not a negative charge, at position 732 can reduce rNa$_v$1.4 sensitivity to GIIIB.

The S729L and the N732K replacements individually altered the sensitivity of rNa$_v$1.4 channels for GIIIB by ~20-fold. To determine whether these mutations might have additive effects on GIIIB sensitivity, we tested the double mutant rNa$_v$1.4-S729L/N732K. This double mutant was significantly less sensitive to GIIIB (Fig. 4D; IC_{50} = 2554 ± 320, n = 6, estimated with 1200 nM GIIIB) than either of the single mutants. This indicates that the region between D2/S5 and D2/SS1 (residues 717–738) contains major molecular determinants of the differential sensitivity of various sodium channels to GIIIB μ-conotoxin.

Sensitivity of Mutant Na$^+$ Channels to GIIIA μ-Conotoxin. Rat Na$_v$1.4 channels are also more sensitive to μ-conotoxin GIIIA (IC_{50} = 58 ± 5, n = 14; Fig. 5A) than are hNa$_v$1.4 channels (IC_{50} = 1228 ± 139 nM, n = 11; Fig. 5B) (Chahine et al., 1994a). Although these GIIIA and GIIIB are closely related and often considered indistinguishable, they do have slightly different sequences (Sato et al., 1983). Therefore, we investigated whether the residues that altered μ-conotoxin GIIIB sensitivity also altered μ-conotoxin GIIIA sensitivity. Replacing the D2/S5-S6 linker of hNa$_v$1.4 channels with the D2/S5-S6 linker of rNa$_v$1.4 channels caused a significant increase ($p < 0.002$) in the sensitivity of hNa$_v$1.4 to μ-conotoxin GIIIA (IC_{50} = 139 ± 16 nM, n = 5; Fig. 5C). Similar to its effect on sensitivity to GIIIB, the S729L substitution significantly decreased ($p < 0.002$) rNa$_v$1.4 sensitivity to μ-conotoxin GIIIA (IC_{50} = 778 ± 74 nM, n = 9; Fig. 5D). However, the GIIIA sensitivity of rNa$_v$1.4 channels was differentially affected by replacements of N732. Both the rNa$_v$1.4-N732E and rNa$_v$1.4-N732Q replacements, which had no effect on GIIIB sensitivity, slightly increased the sensitivity of rNa$_v$1.4 channels to GIIIA (IC_{50} = 32 ± 4 nM, n = 6 and IC_{50} = 37 ± 5 nM, n = 6; $p < 0.01$). In addition, although GIIIB sensitivity was reduced 20-fold by the N732K

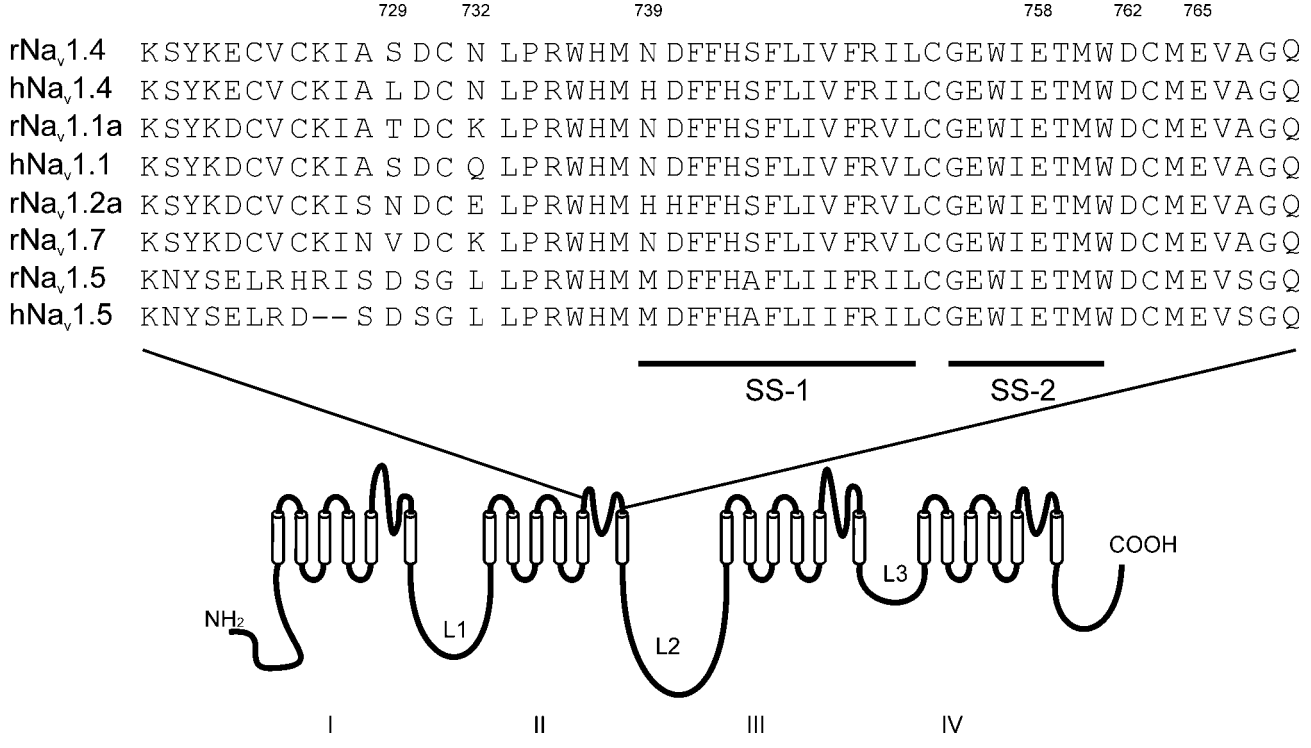

Fig. 2. Alignment of the S5-S6 linker of domain 2 containing pore-lining and extra-pore residues of select mammalian sodium channels. Residues that are predicted to line the pore are underlined and designated SS-1 and SS-2. The 21-amino acid residues that are N-terminal to SS-1 are designated extra-pore residues. The amino acid numbers are those of rNa$_v$1.4. Compared with hNa$_v$1.4, rNa$_v$1.1a, rNa$_v$1.2a, and rNa$_v$1.7a, rNa$_v$1.4 is different at one, three, and four extra-pore residues, respectively. The E722D replacement in rNa$_v$1.1a, rNa$_v$1.2a, and rNa$_v$1.7a may not have a functional consequence. The GenBank accession numbers of these sodium channel α-subunits are as follows: rNa$_v$1.4, NM_013178; hNa$_v$1.4, NM_000334; rNa$_v$1.1a, X03638; hNa$_v$1.1 AY043484; rNa$_v$1.2a, NM_012647; rNa$_v$1.7, U79568; rNa$_v$1.5, NM_013125; and hNa$_v$1.5, NM_000335.

mutation, rNa$_v$1.4-N732K channels were only 4-fold less sensitive to GIIIA (IC_{50} = 228 ± 37 nM, n = 5; Fig. 5E) compared with wild-type rNa$_v$1.4 channels. The double mutant S729L/N732K also had less of an effect on GIIIA sensitivity than on GIIIB sensitivity. The GIIIA sensitivity of rNa$_v$1.4-S729L/N732K channels (IC_{50} = 906 ± 110 nM, n = 5; Fig. 5F) was only reduced by 16-fold compared with wild-type rNa$_v$1.4 channels, and was similar to that of the single substitution S729L. Therefore, although rNa$_v$1.4 channels and hNa$_v$1.4 channels show similar IC_{50} values for GIIIA and GIIIB and these μ-conotoxins have often been considered indistinguishable, inhibition of rNa$_v$1.4 channels by these two closely related μ-conotoxins was differentially affected by a single amino acid substitution in the D2/S5-S6 linker (Fig. 6).

Effect of rNa$_v$1.4 Mutations on k_{on} and k_{off}. The S729L and the N732K mutations individually altered the GIIIB IC_{50} of rNa$_v$1.4 channels by ~20-fold, and the S729L/N732K double mutation altered the IC_{50} by ~50-fold (Fig. 7). To further characterize the mechanisms by which these mutations alter toxin sensitivity, we examined the on (k_{on}) and off (k_{off}) rates for GIIIB block of rNa$_v$1.4 and hNa$_v$1.4 channels using 600 nM μ-conotoxin (unless otherwise noted). GIIIB was difficult to wash off rNa$_v$1.4 channels, and for some cells little or no recovery was seen after 30 min (data not shown). In those cells in which wash-off did seem to occur, the time constant for wash-off (τ_{off}) was 1326 ± 311 s (n = 4), which is similar to that reported by Li et al. (2000). In contrast, GIIIB washed off hNa$_v$1.4 channels relatively easily (τ_{off} = 47.6 ± 8 s, n = 5). Although the k_{on} for GIIIB was slightly increased for hNa$_v$1.4 channels compared with rNa$_v$1.4 channels, the k_{off} was increased by at least 20-fold for hNa$_v$1.4 channels (Table 2). GIIIB also washed-off rNa$_v$1.4-S729L mutant channels relatively easily (τ_{off} = 46.6 ± 2.8 s, n = 4). The S729L mutation increased the k_{off} of GIIIB by ~20-fold but only increased k_{on} by ~2-fold (Table 2), suggesting that this mutation destabilizes the toxin-channel complex.

Although GIIIB did not wash off appreciably from rNa$_v$1.4-N732K channels, the on time constant (τ_{on}) was much slower

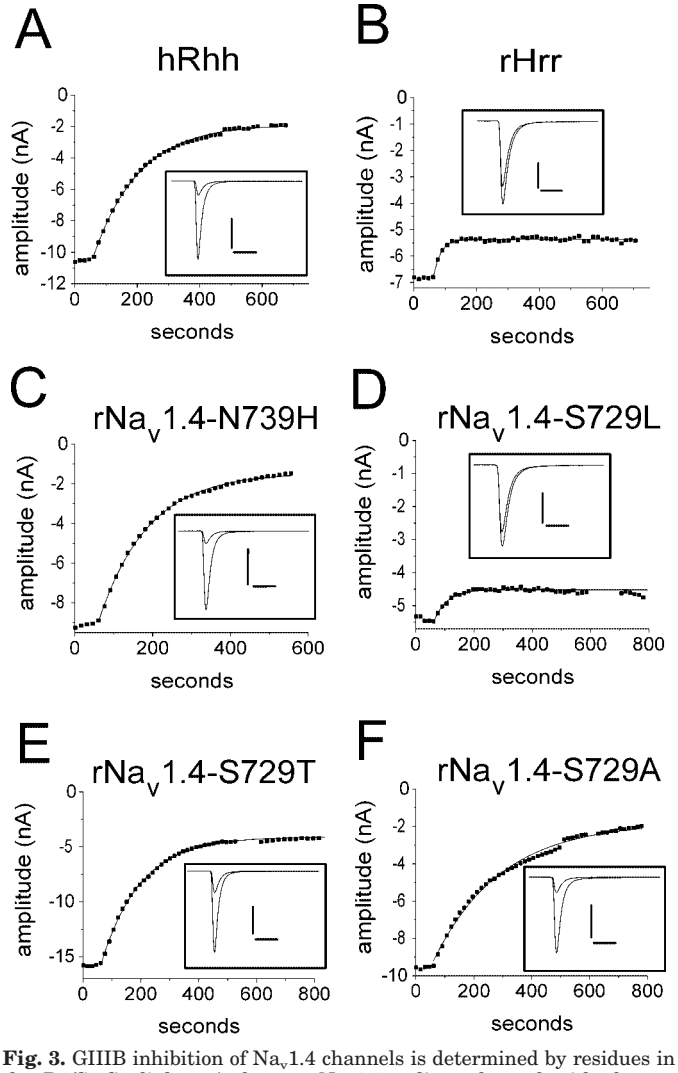

Fig. 3. GIIIB inhibition of Na$_v$1.4 channels is determined by residues in the D2/S5-S6 linker. A, human Na$_v$1.4 sodium channel with the rat D2/S5-S6 linker (hRhh) is sensitive to GIIIB. B, rat Na$_v$1.4 sodium channel with the human D2/S5-S6 linker (rHrr) is relatively insensitive to GIIIB. C, N739H mutation does not alter the sensitivity of rNa$_v$1.4 channel to GIIIB. D, S729L mutation decreases the sensitivity of rNa$_v$1.4 channels to GIIIB by 20-fold. E, S729T mutation has little effect on the GIIIB sensitivity of rNa$_v$1.4 channels. F, S729A mutation also has little effect on the GIIIB sensitivity of rNa$_v$1.4 channels. Cells expressing mutant sodium channels were exposed to 300 nM GIIIB. The cells were held at −120 mV and stepped to −10 mV for 50 ms every 10 s. GIIIB was applied in the bath solution after the fourth pulse. Data were fitted with single exponential functions to estimate the time constants for toxin binding (τ_{on} is 146 s in A, 24 s in B, 126 s in C, 44 s in D, 135 s in E, and 202 s in F). Insets show representative current traces before and after application of GIIIB. Horizontal scale bars, 2 ms. Vertical scale bars, 4 nA (A, E, and F) and 2 nA (B, C, and D).

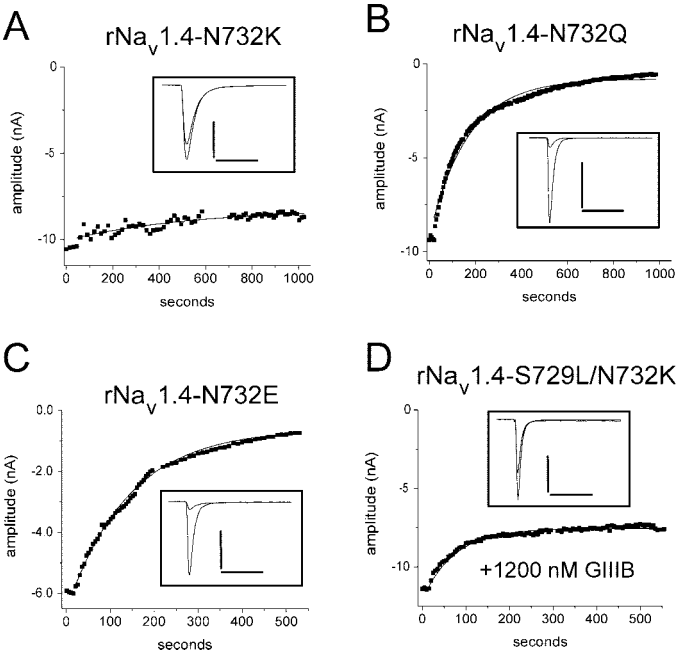

Fig. 4. GIIIB inhibition of rNa$_v$1.4 channels is significantly reduced by replacing another residue in the external pore region of the D2/S5-S6 linker. A, rat Na$_v$1.4 sodium channel with the N732K mutation exhibits reduced sensitivity to GIIIB inhibition. B, N732Q mutation does not reduce GIIIB sensitivity. C, rat Na$_v$1.4 sodium channels with the N732E mutation also exhibit a GIIIB sensitivity similar to that of rNa$_v$1.4. D, S729L/N732K mutation reduces the GIIIB sensitivity of rNa$_v$1.4 channels by more than 50-fold. Cells expressing mutant sodium channels were exposed to 300 nM (A–C) or 1200 nM (D) μ-conotoxin GIIIB. The cells were held at −120 mV and stepped to −10 mV for 50 ms every 10 s. GIIIB was applied in the bath solution after the fourth pulse. Data were fitted with single exponential functions to estimate the time constants for toxin binding (τ_{on} is 533 s in A, 168 s in B, 142 s in C, and 74 s in D). Insets show representative current traces before and after application of GIIIB. Horizontal scale bars, 2 ms. Vertical scale bars, 5 nA (A, B, and D) and 3 nA (C).

for these channels than for wild-type rNa$_v$1.4 (383S for N732K channels versus 71S for WT rNa$_v$1.4 channels with 1200 nM GIIIB). Based on this and the very slow wash-off, we estimate that k_{on} is at least 5-fold smaller for rNa$_v$1.4-N732K channels. To further investigate the effect of the N732K substitution we examined the kinetics of GIIIB block of rNa$_v$1.4-S729L/N732K channels using 1200 nM GIIIB. In this double mutant the k_{off} was significantly greater compared with wild-type rNa$_v$1.4 channels ($p < 0.005$) (Table 2). However, k_{off} was 2-fold smaller for rNa$_v$1.4-S729L/N732K channels than for rNa$_v$1.4-S729L channels, and k_{on} was almost 5-fold smaller for rNa$_v$1.4-S729L/N732K channels than for rNa$_v$1.4-S729L channels. Thus, although the S729L substitution considerably increases the off rate of GIIIB, the N732K substitution seems to significantly decrease the on rate (Table 2). This suggests that changes in the D2/S5-S6 linker might affect both the accessibility of the pore to GIIIB and the stabilization of the toxin-channel complex.

In contrast to GIIIB, GIIIA (300 nM) washed off both rNa$_v$1.4 (τ_{off} = 586.2 ± 61.4 s, n = 6) and hNa$_v$1.4 (τ_{off} = 18.5 ± 2.0 s, n = 3) channels more readily. Furthermore, both

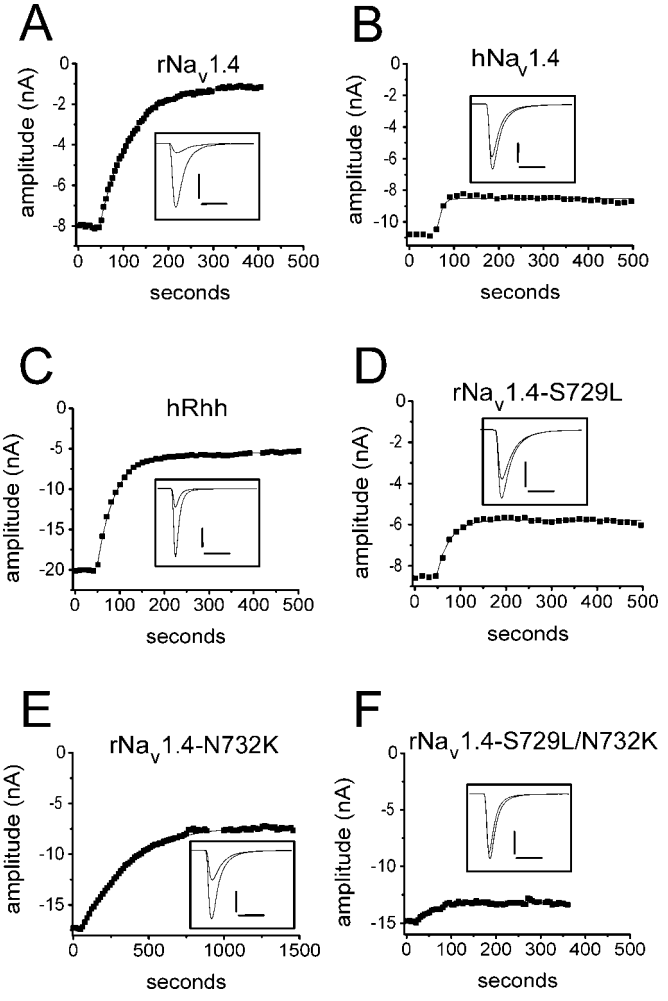

Fig. 5. GIIIA differentially blocks rat and human Na$_v$1.4 sodium channels, and this block is sensitive to changes in the D2/S5-S6 linker. Cells expressing rNa$_v$1.4 (A) or hNa$_v$1.4 (B) sodium channels were exposed to 300 nM μ-conotoxin GIIIA. C, hNa$_v$1.4 sodium channel with the rat D2/S5-S6 linker (hRhh) is sensitive to GIIIA. D, S729L mutation decreases the sensitivity of rNa$_v$1.4 to GIIIA by ~15-fold. E, N732K mutation, on the other hand, only reduces the sensitivity of rNa$_v$1.4 to GIIIA inhibition by ~4-fold. F, S729L/N732K double mutation reduces the GIIIA sensitivity of rNa$_v$1.4 by slightly more than 15-fold. The cells were held at −120 mV and stepped to −10 mV for 50 ms every 10 s. GIIIA (300 nM) was applied in the bath solution after the fourth pulse. Data were fitted with single exponential functions to estimate the time constants for toxin binding (τ_{on} is 67 s in A, 9 s in B, 40 s in C, 28 s in D, 293 s in E, and 35 s in F). Insets show representative current traces before and after application of GIIIA. Horizontal scale bars, 2 ms. Vertical scale bars, 3 nA (A, B, and D) and 5 nA (C, E, and F).

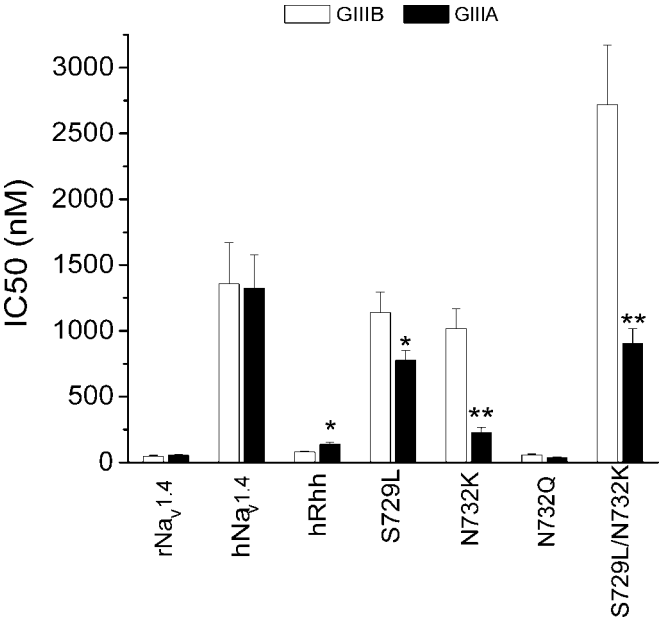

Fig. 6. Bar graph comparing the half-blocking concentration (IC_{50}) for μ-conotoxin GIIIB and GIIIA inhibition of rat, human, and mutant Na$_v$1.4 channels. *, significant difference ($p < 0.05$) between GIIIA and GIIIB IC_{50}. **, significant difference ($p < 0.005$) between GIIIA and GIIIB IC_{50}. The D2/S5-S6 mutations have a reduced effect on GIIIA sensitivity of rNa$_v$1.4.

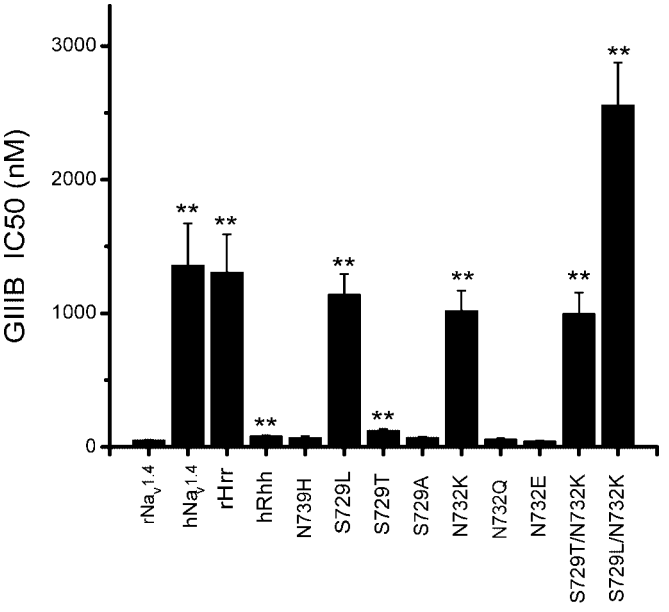

Fig. 7. Bar graph summarizing the half-blocking concentration (IC_{50}) for μ-conotoxin GIIIB inhibition of Na$_v$1.4 channels. The S729L and N732K single mutants reduced GIIIB sensitivity of rNa$_v$1.4 channels by ~20-fold. These two mutants had a partially additive effect, and the rNa$_v$1.4 S729L/N732K double mutant showed a 50-fold reduction in the GIIIB sensitivity of rNa$_v$1.4. **, significant difference from the IC_{50} of WT rNa$_v$1.4 channels ($p < 0.005$).

k_{on} and k_{off} for rNa$_v$1.4 and hNa$_v$1.4 channels are larger for GIIIA than for GIIIB (Table 2). These kinetic data, together with the reduced effect of the N732K on GIIIA sensitivity, suggest that residues in the D2/S5-S6 linker can be more of an obstacle to GIIIB than GIIIA binding.

The change in kinetic equilibrium constants (K_d) derived from the k_{on} and k_{off} rate constants paralleled that of the corresponding IC_{50} values estimated with 300 nM toxin (Table 2). However, the estimated K_d and IC_{50} values differed in some instances, with the K_d values being 3- to 4-fold smaller than the IC_{50} value for the more resistant constructs such as hNa$_v$1.4 and rNa$_v$1.4-S729L/N732K channels. Similarly, Li et al. (2000) observed 2- to 10-fold discrepancies between the estimated K_d and IC_{50} values for GIIIB block of rNa$_v$1.4-D762C and rNa$_v$1.4-E765C mutant channels, which also exhibit reduced sensitivity to GIIIB compared with wild-type rNa$_v$1.4 channels. It is not clear what accounts for this discrepancy. However, the discrepancy between the estimated K_d and IC_{50} values was smaller for GIIIA block of hNa$_v$1.4 channels when the toxin concentration was increased to 1200 nM (Table 2).

Discussion

Our data demonstrate that the part of the D2/S5–6 linker that is N-terminal to the SS-1 segment contains critical determinants of rNa$_v$1.4 sensitivity to GIIIB, and may further define contact points between the channel and the toxin. We show that exchanging the D2/S5-S6 linker between rNa$_v$1.4 channels, which are GIIIB-sensitive, and hNa$_v$1.4 channels, which are GIIIB-resistant, switches their respective sensitivity to GIIIB. We have identified a single amino acid substitution in this linker, S729L, that produces ~20-fold reduction in the sensitivity of rNa$_v$1.4 to GIIIB and accounts for the difference between rNa$_v$1.4 and hNa$_v$1.4 GIIIB sensitivity. We provide evidence that additional residues in the D2/S5-S6 linker might also be important determinants of the GIIIB resistance of neuronal channels. Both rNa$_v$1.1a and rNa$_v$1.7, which are GIIIB-resistant (Safo et al., 2000), have a lysine at the position corresponding to N732 in rNa$_v$1.4. We found that the N732K substitution also produced ~20-fold reduction in rNa$_v$1.4 GIIIB sensitivity and the double mutant S729L/N732K decreased rNa$_v$1.4 GIIIB sensitivity by ~50-fold (Fig. 6). Thus, we have identified naturally occurring polymorphic residues in the D2/S5-S6 linker of sodium channels that are critical determinants of μ-conotoxin GIIIB sensitivity.

Several studies have identified other residues in the D2/S5-S6 linker of rNa$_v$1.4 channels that can influence the affinity for GIIIA/B. Neutralization of the negatively charged residues E758, E765, and D762 decreased μ-conotoxin affinity by 10- to 50-fold (Dudley et al., 1995; Li et al., 2000). These residues are thought to line the pore, indicating that GIIIA/B block conductance by binding in the pore. However, these pore-lining residues are conserved between Na$_v$1.4 channels, TTX-sensitive (TTX-S) neuronal channels, and the cardiac (Na$_v$1.5) channel (Fig. 2), and therefore cannot be the primary determinants of the selectivity of GIIIA/B to rNa$_v$1.4. Chahine et al. (1998b) found that mutations A728L and D730Q, near S729 and N732, caused a small decrease (5- to 6-fold) in GIIIB inhibition of rNa$_v$1.4 channels. The difference in the effect of the replacement of the adjacent residues A728 and S729 by leucine (A728L and S729L cause 6- and 20-fold reduction in IC_{50}, respectively) strongly suggests a more direct role of S729 in defining the sensitivity of rNa$_v$1.4 channels to the GIIIB toxin.

Does D2/S5-S6 Linker Determine GIIIB Sensitivity? Comparison of the D2/S5-S6 linker from rNa$_v$1.4 with those of hNa$_v$1.4 and TTX-S neuronal channels reveals a limited number of amino acid substitutions in the D2/S5-S6 linker. Most of these changes are located in the region N-terminal to SS-1 (Fig. 2), which includes S729 and N732 of rNa$_v$1.4. Based on our results, we propose that one or more of these naturally occurring variants play a critical role in determining the toxin sensitivity of the respective channel. The TTX-S channels rNa$_v$1.2a and rNa$_v$1.7, for example, differ from rNa$_v$1.4 at the residues corresponding to 728, 729, and 730 (Fig. 2). The partially additive effect of two mutations, S729L and N732K, on increasing the resistance to toxin block suggests that multiple variant residues in the D2/S5-S6 linker, compared with rNa$_v$1.4, may contribute to the increased resistance of some neuronal channels. Interestingly, the N732Q mutation did not decrease GIIIB binding, and, therefore it is possible that the human Na$_v$1.1 channel might be sensitive to GIIIB. It should be noted, however, that contributions by S5-S6 linkers in other domains of the sodium channels to differences in toxin binding affinity could not be ruled out at this time.

How Does D2/S5-S6 Linker Alter GIIIB Sensitivity? The S729L and N732K mutations each individually altered the GIIIB IC_{50} of rNa$_v$1.4 channels by ~20-fold. The effects of these two mutations on binding were only partially additive, suggesting that these two residues could facilitate the same aspect of the toxin-channel interaction (Mildvan et al., 1992). However, the two mutations altered different aspects of the binding of the toxin. Whereas the S729L mutation primarily increased the off rate of GIIIB,

TABLE 2
Kinetic properties of μ-conotoxin inhibition of Na$_v$1.4 channels

Toxin	Channel	k_{on} (× 10^4/ms)	k_{off} (× 10^{-3}/s)	K_d	IC_{50}	n
				nM	*nM*	
GIIIB						
600 nM	rNav1.4	2.2 ± 0.3	1.0 ± 0.4	45	49	4
600 nM	hNav1.4	5.0 ± 1.1	24.1 ± 4.8	482	1357	5
600 nM	S729L	5.1 ± 1.7	21.7 ± 1.5	425	1138	4
1200 nM	S729L/N732K	1.4 ± 0.2	9.7 ± 1.2	693	2554	4
GIIIA						
300 nM	rNav1.4	6.1 ± 0.7	1.8 ± 0.2	30	55	5
300 nM	hNav1.4	21.7 ± 3.0	44.1 ± 4.1	203	1228	6
1200 nM	hNav1.4	14.1 ± 5.4	39.6 ± 1.9	281	764	6

the N732K mutation significantly decreased the on rate. In the double mutant S729L/N732K both the on and off rates were significantly altered compared with wild-type rNa$_v$1.4, suggesting that the D2/S5-S6 linker affects both the accessibility of the pore to GIIIB and the stabilization of the toxin-channel complex. This indicates that although the effects of the S729L and N732K mutations are interdependent, S729 and N732 might facilitate related nonrate-limiting aspects of the toxin-channel interaction (Mildvan et al., 1992). Recently, Feng et al. (2001) reported that a single residue in the D3/S5-S6 linker of the N-type calcium channel α_{1B} subunit dramatically increased both the on and off rates for ω-conotoxin GVIA block, and proposed that this residue, which is N-terminal to the P loop, acts as a barrier that controls both the accessibility of GVIA to the binding site and the reversibility of GVIA block. Our data indicate that the D2/S5-S6 residues S729 and N732 may play an analogous role in μ-conotoxin GIIIB block of rNa$_v$1.4 channels. The S729L substitution, which enhanced the toxin off rate, is likely to destabilize the toxin-channel complex and enhance reversibility of toxin block due to steric hindrance. In contrast, the N732K mutation decreased the on rate of toxin binding, suggesting that it interfered with the formation of an initial toxin-channel complex, and that N732 might control access of GIIIB to the high-affinity binding site.

Are μ-Conotoxins GIIIA and GIIIB Indistinguishable? GIIIA and GIIIB are often considered indistinguishable. The two peptides share a similar folded structure and a predicted manner of interaction with their sodium channel target (Hill et al., 1996), and they seem to have a comparable effect on rNa$_v$1.4 expressed in HEK 293 cells (Fig. 6). However, GIIIA is reported to be less potent than GIIIB in vivo (Sato et al., 1983). In addition, although D762Q and E765Q substitutions rendered rNa$_v$1.4 resistant to GIIIB (Li et al., 2000), GIIIA binding was not affected by these same substitutions (Chahine et al., 1998b). Recently, Li et al. (2001b) also showed that although the GIIIB sensitivity of rNa$_v$1.4-D762K and rNa$_v$1.4-E765K mutant channels was greatly reduced (~200-fold), the GIIIA sensitivity of these channels was only slightly reduced (~3-fold) compared with wild type rNa$_v$1.4 channels. Thus, residues at various positions in the D2/S5-S6 linker differentially interact with GIIIA and GIIIB.

GIIIA differs at three positions compared with GIIIB (K8R, Q14R, and Q18M; first residue is that of GIIIA). The difference at position 14 might be the most important. R14 in GIIIB is exposed on the folded structure of the toxin such that it is predicted to interact with the binding site on the channel (Hill et al., 1996). Li et al. (2001b) demonstrated that rNa$_v$1.4-D762K and rNa$_v$1.4-E765K mutant channels, which are resistant to GIIIB but not GIIIA, were also resistant to GIIIA-Q14R. This indicates that the residue at position 14 is the major determinant of the differential affinity of mutant channels for GIIIA and GIIIB. Our data show that although the sensitivity of rNa$_v$1.4 channels to both GIIIA and GIIIB was greatly reduced by the S729L mutation, the N732K mutation had a much smaller effect on GIIIA inhibition than on GIIIB inhibition. The neuronal rNa$_v$1.1a channel has a K residue at the position corresponding to N732, and therefore could be less sensitive to GIIIB than GIIIA, a prediction that remains to be tested experimentally.

Although the D762K and E765K mutations each reduced GIIIB affinity by ~200-fold (Li et al., 2001b), the N732K mutation only reduced GIIIB affinity by ~20-fold (data from present study). This indicates that N732 is not near D762 and E765. However, if N732, D762, and E765 all interact with R14 of GIIIB, then this suggests that the stretch of residues between S5 and the P loop of D2 fold in such a way that these residues might align along an axis perpendicular to the plane of the membrane. Unfortunately, because the stretch of residues between D2/S5 and the P loop of rNa$_v$1.4 does not share homology with KcsA (Doyle et al., 1998), models of the sodium channel pore based on KcsA crystal structure have not included these residues (Lipkind and Fozzard, 2000; Hui et al., 2002). Chahine et al. (1998b) proposed that this region of rNa$_v$1.4 did not contribute to the pore of the channel because neither cadmium nor a methanethiosulfonate reagent altered the permeation properties of rNa$_v$1.4-D730C mutant channels. Based on their results they considered residues in this region to be extra-pore residues. This designation is supported by a study that examined the effects of mutations in the corresponding region of rNa$_v$1.2 on functional properties and TTX binding (Kontis and Goldin, 1993). Together, these findings suggest that the side chain of toxin residue 14 is near N732 of rNa$_v$1.4 during an initial or intermediate stage of toxin binding. Substitution of N732 with glutamine, which has a similarly sized side chain to lysine but has no charge, did not have an effect on GIIIB sensitivity, raising the possibility that the N732K mutation constitutes an electrostatic barrier that limits access of GIIIB to the high-affinity binding site. If this idea is correct then it is not entirely clear why the N732E mutation did not enhance GIIIB binding. However, if the N732E mutation altered both the on and off kinetics for the formation of an intermediate, nonrate-limiting step in a reciprocal manner then the N732E mutation might not effect the rate at which the final toxin-channel complex is formed.

GIIIA/B are thought to be rigid, flat discoidal molecules containing a flexible segment extending from K11 to R13 (Lancelin et al., 1991; Sato et al., 1991) that could change conformation to facilitate the extension of R13 into the pore. The fact that the single amino acid substitutions that we introduced to rNa$_v$1.4 are naturally occurring polymorphism in other Na^+ channels strongly argues against a structural change of the channel vestibule. The pore-lining residues E758, D762, and E765 have previously been shown to be critical for the high-affinity block of Na^+ ion flow in μ-conotoxin-sensitive sodium channels and may do so by stabilizing the toxin-channel complex. We propose that the naturally occurring resistance of various sodium channels to GIIIA and GIIIB μ-conotxins may arise from inhibition or destabilization of the slow formation of the initial toxin-channel complex and/or to enhanced reversibility of toxin block due to amino acid substitutions of specific extra-pore residues such as N732K and S729L. Our data support the conclusion that multiple contact points are involved in the formation of the toxin-channel complex (for review, see French and Dudley, 1999), and indicate that it should be possible to identify μ-conotoxin variants that target specific neuronal sodium channel isoforms.

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