Interactions of Key Charged Residues Contributing to Selective Block of Neuronal Sodium Channels by μ-Conotoxin KIIIA

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ABSTRACT

Voltage-gated sodium channels are important in initiating and propagating nerve impulses in various tissues, including cardiac muscle, skeletal muscle, the brain, and the peripheral nerves. Hyperexcitability of these channels leads to such disorders as cardiac arrhythmias (Nav1.5), myotonia (Nav1.4), epilepsies (Nav1.2), and pain (Nav1.7). Thus, there is strong motivation to identify isoform-specific blockers and the molecular determinants underlying their selectivity among these channels. μ-Conotoxin KIIIA blocks rNav1.2 (IC_{50}, 5 nM), rNav1.4 (37 nM), and hNav1.7 (97 nM), expressed in mammalian cells, with high affinity and a maximal block at saturating concentrations of 90 to 95%. Mutations of charged residues on both the toxin and channel modulate the maximal block and/or affinity of KIIIA. Two toxin substitutions, K7A and R10A, modulate the maximal block (52–70%). KIIIA-H12A and R14A were the only derivatives tested that altered Na_{i} isoform specificity. KIIIA-R14A showed the highest affinity for Nav1.7, a channel involved in pain signaling. Wild-type KIIIA has a 2-fold higher affinity for Nav1.4 than for Nav1.7, which can be attributed to a missing outer vestibule charge in domain III of Nav1.7. Reciprocal mutations Na_{i}1.4 D1241I and Nav1.7 I1410D remove the affinity differences between these two channels for wild-type KIIIA without affecting their affinities for KIIIA-R14A. KIIIA is the first μ-conotoxin to show enhanced activity as pH is lowered, apparently resulting from titration of the free N terminus. Removal of this free amino group reduced the pH sensitivity by 10-fold. Recognition of these molecular determinants of KIIIA block may facilitate further development of subtype-specific, sodium channel blockers to treat hyperexcitability disorders.

Introduction

Voltage-gated sodium (Nav) channels are important in the initiation and propagation of nerve impulses in neurons and muscle (Hille, 2001). To date, nine mammalian Na_{i} channels have been described (Nav1.1–1.9) (Catterall et al., 2005; Al-Sabi et al., 2006); these have differing distributions throughout the body. Gain-of-function mutations in Na_{i} channels causing hyperexcitability, have been linked to such disease states as cardiac arrhythmias (Wang et al., 1995), epilepsy (Escayg et al., 2000), myotonia (Cannon, 1997), and pain (Waxman et al., 1999). Thus, there is much interest in producing subtype-selective blockers of specific Na_{i} channel isoforms.

Venoms from fish-hunting cone snails contain many different toxins, which represent possible therapeutic compounds targeting various ion channels. μ-Conotoxins (μCTXs) make up one such group of toxins. As a group, μCTXs are identified by their conserved disulfide backbone structure and because they all target toxin site 1, in the outer pore vestibule, of Nav channels (Catterall et al., 2005). μCTXs from different species target various Na_{i} channels and show differing selectivity profiles. μCTX GIHIA from Conus geographus specifically targets skeletal muscle channels (Nav1.4) (Cruz et al., 1985), whereas the very similar μCTX, PIIFIA, from Conus purpurascens most strongly inhibits skeletal muscle channels but also blocks some neuronal channels (Nav1.2 and Nav1.7) with lower affinity (Shon et al., 1998). μCTX KIIIA, from Conus kinositai, points to the potential importance of μCTXs as possible therapeutic compounds by showing analgesic activity (Zhang et al., 2007). KIIIA is the shortest known μCTX, at only 16 amino acids in length, but retains the typical μCTX disulfide bond pattern (Bulaj et al., 2005) (Fig. 1). KIIIA has a nominal net charge of +6, lower than both GIHIA (+6) and PIIFIA (+7). It is noteworthy that KIIIA was the first μCTX to show higher affinity for the
neuronal channel, Na\textsubscript{a,1.2}, than for skeletal muscle channels (Zhang et al., 2007). Indeed, KIIIA block of Na\textsubscript{a,1.2} is almost irreversible on a normal experimental time scale. KIIIA also shows a high affinity (IC\textsubscript{50} in the nanomolar range) for Na\textsubscript{a,1.7}, a channel involved in pain perception (Yang et al., 2004).

Unlike \(\mu\text{CTXs}\) studied previously, KIIIA does not block 100\% of the single-channel current (Zhang et al., 2007). This residual single-channel current may be permitted by the absence of arginine and lysine residues in its N-terminal segment (residues 1–6), given that the PIIIA R12A derivative shows a small residual current similar to that for KIIIA (McArthur et al., 2011a). Even though KIIIA binds to the sodium-channel pore site 1, either tetrodotoxin or saxitoxin can bind simultaneously (Zhang et al., 2009), increasing the range of possible pharmacological actions of KIIIA by its use in combination with the smaller pore blockers.

Here we examine differences in binding, resulting from charge-neutralizing substitutions in KIIIA, for Na\textsubscript{a,1.2} (central nervous system), Na\textsubscript{a,1.4} (skeletal muscle), and Na\textsubscript{a,1.7} (peripheral nervous system). Channel mutants were selected based on sequence comparisons (Fig. 1B) and previous docking simulations of \(\mu\text{CTXs}\) GIIIA and PIIIA (Choudhary et al., 2007; McArthur et al., 2011a). We focused on two positions: 1) the outer ring charge in domain III, which is absent in Na\textsubscript{a,1.7} but present in Na\textsubscript{a,1.4} and Na\textsubscript{a,1.2}; and 2) the aromatic residue in domain I, adjacent to the DEKA locus, which has important implications for tetrodotoxin and saxitoxin block (Santarelli et al., 2007) and thus could be important in KIIIA block. These residues distinguish the canonical \(\mu\text{CTX}\) target, Na\textsubscript{a,1.4}, from the neuronal channels Na\textsubscript{a,1.2} and Na\textsubscript{a,1.7}. We tested the effects of changes at these loci to find how they might contribute to the observed selectivity of KIIIA among the different channel isoforms.

We show that, by substitution of individual KIIIA residues, or by altering the extracellular pH, we can change key features of KIIIA block, including maximal block, affinity and targeting selectivity. Two residues, Lys7 and Arg10, are key determinants of KIIIA’s fractional block of single-channel currents, or of maximal conductance in whole-cell experiments. KIIIA’s affinity, for all three Na\textsubscript{a} channels studied, increased with a decrease in extracellular pH based on increases in the toxin association rates. The increased affinity at low pH can be mostly attributed to titration of KIIIA’s free N terminus. By comparison, PIIIA, with a neutral, cyclized pyroglutamate as its N-terminal residue, shows no substantial pH dependence. Changes in channel isoform targeting were seen for the KIIIA-R14A derivative, which showed 10-fold selectivity for Na\textsubscript{a,1.7}, a channel involved in the pain pathway (Yang et al., 2004; Cox et al., 2006), over Na\textsubscript{a,1.2} and Na\textsubscript{a,1.4}. These molecular determinants of \(\mu\text{CTX}\)-selective targeting, offer clues to the design of more selective blockers of tissue-specific Na\textsubscript{a} channel isoforms.

**Fig. 1.** Model of KIIIA structure. A, structure of KIIIA (coordinates kindly provided by Drs. Brian Smith and Ray Norton) with important residues highlighted (Lys7, cyan; Arg10/Arg14, blue; His12, purple). B, sodium-channel sequence alignment of the domains I and III p-loop regions, with inner ring (blue) and outer ring (red) labeled. Sequences of channel mutants rNa\textsubscript{a,1.4} Y401F, rNa\textsubscript{a,1.4} D1241I, and hNa\textsubscript{a,1.7} D1241I are shown.
Materials and Methods

Toxin Synthesis and Preparation. Conotoxin synthesis, purification, and disulfide bond formation were performed as described previously in detail (Hui et al., 2002). In brief, linear peptides were synthesized by solid-phase synthesis using 9-fluorenemethoxycarbonyl chemistry. Coupling of 9-fluorenemethoxycarbonyl amino acids was performed using the 1-hydroxybenzotriazole/2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethylurea-hexafluorophosphate/N,N-disopropylethylamine method on a Quarto 1 Synthesizer (Protein Technologies Inc., Tucson, AZ).

Crude linear peptide was subjected to oxidative folding under equilibrating condition (i.e., air oxidation in ammonium acetate buffer, pH 7.6, in the presence of a small amount of mercaptoethanol (10 μl in 150 ml)) to form the most stable disulfide bonds. During oxidation, the cyclization of the peptide was monitored by analytical HPLC, which was completed in 2 to 4 days at 4°C. The crude cyclized peptide showed a single major peak on analytical HPLC, with some minor peaks being seen in each case (the number and size of minor peaks varied with the derivative being cyclized). The crude cyclized peptide was then isolated from the acidified reaction mixture by reversed-phase extraction, was purified to near homogeneity by HPLC, and the identity of the purified peptide was confirmed by matrix-assisted laser desorption ionization mass spectrometric molecular weight determination. Some derivatives produced two separate peaks, and thus the peak identified as active by Zhang et al. (2007) was used.

Lyophilized conotoxin derivatives were then dissolved in the bath solution to an appropriate stock concentration. Toxin solutions used in the experiments were further diluted in the bath solution to the required concentration.

Sodium Channel Expression in HEK293 Cells. Mammalian expression plasmids encoding rNa,1.2 (pCDMA8, a gift from W. A. Catterall) (Linford et al., 1998), rNa,1.4 (pCDNA3.1) (Trimmer et al., 1989), and hNa,1.7 (pCMV6) (Hildebrand et al., 2011) were used. Channel constructs were created using rNa,1.4 or hNa,1.7 as a template. Three channel mutants (rNa,1.4 Y401F, rNa,1.4 D1241I, and hNa,1.7 I1410D) were constructed. In brief, sense and antisense primers encoding point mutations for the desired amino acid substitution were synthesized and used in high-fidelity PCRs (Phusion polymerase; Thermo Fisher Scientific, Waltham, MA) with primers located either 3’ or 5’, respectively, from unique flanking restriction sites in the wild-type construct. Amplified fragments (upstream and downstream) were run on agarose gels, and the resulting products were isolated, mixed, and subjected to another round of high-fidelity PCR using only the 3’ and 5’ primers. The resulting product was purified, digested with the appropriate restriction endonucleases, and cloned into like-digested wild-type channel clones. All fragments cloned from PCR products were completely sequenced to ensure no misincorporations had occurred during cloning.

HEK293 cells were transiently cotransfected with the plasmid encoding the particular sodium channel ω-subunit (2 μg) and a plasmid encoding green fluorescent protein (0.5 μg), allowing transfected cells to be identified by their green fluorescence. Twenty-four hours after transfection, cells were plated on coverslips and used for voltage-clamp studies at least 2 h after plating.

Electrophysiology. Sodium channel currents were recorded by the patch-clamp technique in the whole-cell configuration at room temperature (23–25°C). The bath solution contained 140 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES, and 10 mM glucose, adjusted to pH 7.4, or with HCl or NaOH (−305 mOsm/kg). The pipette electrodes had a final tip resistance of 1 to 3 MΩ with an internal solution composed of 35 mM NaCl, 100 mM CsF, 1 mM MgCl₂, 10 mM HEPES, and 1 mM EGTA, with pH adjusted to 7.2 with CsOH (−295 mOsm/kg).

Whole-cell patch clamp was performed with an EPC7 Amplifier (HEKA, Lambrecht/Pfalz, Germany). Current traces were filtered at 3 kHz (low-pass, three-pole Bessel filter, EPC7) and sampled at 200 kHz using pClamp9.2 software (Molecular Devices, Sunnyvale, CA), with series resistance compensated typically at 40 to 60%. Cells showing peak currents between 0.5 and 5 nA were used to ensure adequate voltage control while maintaining good current resolution. Toxins were then locally superfused over the cell at a rate of 10 to 20 μl/min (bath volume of 5 ml). Currents were elicited with a 2-s prepulse to −140 mV to remove inactivation followed by a test pulse to −10 mV for 10 ms repeated every 5 s during toxin application and washout to record kinetics. Single-channel bilayer experiments using batrachotoxin-modified skeletal muscle Na₉ channel at steady state were carried out as described previously (McArthur et al., 2011a).

Data Analysis. Each point data on a dose-response curve was from data for one toxin-containing solution applied to a single cell, with control data taken before and after toxin application on the same cell. Overall, 27 dose-response curves were generated using 6 different channel constructs, and 6 toxin derivatives, with 18 ± 4 experiments contributing to each dose-response curve. Typically three to four determinations were done at each concentration (range, 1–9). Dose-response curves are plotted as the estimated fraction of channels blocked Fb = (1 − I/Iₘ) versus concentration of toxin, where Iₘ is the residual current in the presence of the toxin at steady state and I₀ is the current level before toxin application. Data were fit with a rectangular hyperbola, assuming a Hill coefficient of 1, using the following expression, where Iₜ₀ is the toxin concentration for half-maximal inhibition. Here, Fb is the fraction of current blocked by the toxin at steady state, at a particular concentration, and Fb₉₉ represents the maximal fraction of current blocked at saturating concentrations of the toxin (and the fractional block of the single-channel current). The parameters Fb₉₉ and IC₅₀ were varied to obtain the best fit.

\[
Fb = \frac{Fb₉₉}{1 + IC₅₀ / [tx]}
\]

Toxin blocking kinetics (k₉₉ and k₉₉⁻¹) were measured by fitting the peak currents for successive depolarizations during toxin wash-in or wash-out test with a single exponential to determine τ₉₉ and τ₉₉⁻¹. The rates constants, k₉₉ and k₉₉⁻¹, and the equilibrium dissociation constant, K₉₉ (Fig. 2, A and B) were calculated using the following equations:

\[
k₉₉ = \frac{Fb₉₉}{Fb₉₉ - Fb₀} \quad \text{and} \quad \frac{1}{k₈₉⁻¹} = \frac{1}{τ₉₉} = \frac{k₈₉⁻¹}{k₉₉}
\]

For ease of reading in the text, group data are represented as mean values, with full statistics provided in the tables. Differences between group data sets were considered significant if p < 0.05 in an unpaired t test, unless otherwise stated.

Molecular Dynamics Simulations. Molecular dynamics (MD) simulations of the docking of μCTX KIIIA (Bulaj et al., 2005) to a sodium channel model (Choudhary et al., 2007) were performed as described previously (McArthur et al., 2011b). In brief, MD simulations were carried out using the GROMACS set of programs (Berendsen et al., 1984; Lindahl et al., 2001) using the AMBER 99 force field. At first, KIIIA was superposed onto the docked structure of GIIA. Ten simulations of 20 ns were run, and the resulting structures were aligned with respect to the channel backbone using a least-squares fit and clustered using the g.cluster program (Daun et al., 1999), with a root mean square deviation cutoff of 0.4 nm. The top cluster incorporated >60% (2818) of all the structures (4501), and the center of the cluster was chosen to represent the toxin-bound conformation. All visualization of molecules was carried out using visual MD (Humphrey et al., 1996).

Results

Charged residues play critical roles in defining how μCTXs target and block Na₉ channels. Despite KIIIA being shorter than both PIIIA and GIIIA, the charged residues in the C-terminal segment are highly conserved across the three toxins despite their differences in Na₉ channel selectivity (Fig. 1). To identify residues that might be involved in iso-
form selectivity, we neutralized all basic residues in KIIIA by replacing them with alanines (KIIIA-K7A, R10A, H12A, and R14A) and synthesized a KIIIA derivative, which lacks the N-terminal charge (KIIIA-DA, des-amino). Whole-cell voltage-clamp recordings were used to determine the kinetics of toxin block of Na_1.2, 1.4, and 1.7. The toxin substitutions altered toxin binding and unbinding kinetics, as well as the maximal block at saturating toxin concentrations, to varying

**Fig. 2.** KIIIA-R14A selectivity profile is altered compared with KIIIA-wt. A, example experiment of KIIIA-wt (1 μM) wash-in in Na_1.4. B, example washout experiment of KIIIA-wt (1 μM) from same cell as A. C and D, dose-response curves for KIIIA-wt (data from 96 cells) and KIIIA-R14A (data from 96 cells) in Na_1.2, Na_1.4, Na_1.7, Na_1.4 D1241I, and Na_1.7 I1410D, respectively. Note that the curve for Na_1.2 and KIIIA-wt in part C, was forced through the kinetically determined $K_{d}$ (○), because the time constant block was too long near this concentration to reliably attain steady-state block. In other cases, both IC$_{50}$ and the maximal saturating block, $F_{m}$, were determined directly from fits to the steady-state dose-response data. E, comparison of IC$_{50}$ values for KIIIA-wt and KIIIA-R14A in each channel.
degrees of differences among the three channel subtypes. To assess the importance of differences among the channels, we used the following constructs: Na1.4 Y401F, Na1.4 D1241I, and Na1.7 I1410D. These represented the major differences, among the three channels, located near the inner and outer ring charges (Fig. 1B). Unlike previous studies with the larger μCTX GIIIA (Li et al., 2001, 2003; Cummins et al., 2002), which found residues in the domain II turret region to be important in toxin selectivity, we focused on residue differences around the inner and outer ring residues, in part because of KIIIA’s smaller size and lower net charge.

Block of Na1.2, 1.4, and 1.7 by Wild-Type KIIIA. Channel isoforms Na1.2, 1.4, and 1.7 were expressed heterologously in HEK293 cells, and currents were recorded using the whole-cell patch-clamp technique at pH 7.4. Kinetics of block by KIIIA-wt were determined from whole-cell currents elicited by repeated depolarizing steps (Fig. 2, A and B). KIIIA blocked Na1.2 with the highest affinity (Ka = 5 nM) compared with Na1.4 (Ka = 37 nM) and Na1.7 (Ka = 97 nM) (Table 1). Dose-response curves are plotted in Fig. 2C. As shown in the dose-response curves, all three channels showed similar maximal block levels (Na1.2, 90%; Na1.4, 95%; and Na1.7, 94%) at saturating concentrations of wild-type KIIIA. Kinetics are very slow in Na1.2, making it impractical to collect truly steady-state data. Thus, this dose-response curve was fit using an IC50 of 5 nM, equal to the kinetically determined Ka. Other cases provided steady-state data, which allowed direct determination of IC50 values from the dose-response relation, and these values correspond well with Ka values determined from the kinetics. Overall, wild-type KIIIA (KIIIA-wt) shows a range of binding affinities of ~30-fold in the following order: Na1.2 > Na1.4 > Na1.7 (IC50/Ka values: 5, 54, and 147 nM, respectively).

The underlying differences in binding and unbinding kinetics are shown in Table 1. Na1.2 and Na1.7 had slow association rates, k(on) (0.28 and 0.18 μM⁻¹·min⁻¹, respectively) relative to that of Na1.4 (1.6 μM⁻¹·min⁻¹). The dissociation rate constants, k(off), also differed among channel isoforms with Na1.2 being the slowest (0.002 min⁻¹) followed by Na1.7 (0.017 min⁻¹) with Na1.4 being the fastest (0.060 min⁻¹). These indicate that the toxin-channel complex has a longer mean lifetime for the neuronal channels, Na1.2 and Na1.7, than for the skeletal muscle isoform, Na1.4. These relationships are reminiscent of previously reported data from studies in Xenopus laevis oocytes (Zhang et al., 2007). Despite the skeletal muscle targeting for which the μCTX family is named, KIIIA shows its highest affinity for the brain channel Na1.2.

Substitution R14A in KIIIA Changes Its Target Specificity. Replacement of KIIIA’s arginine-14 with alanine removed a basic side chain resulting in the derivative KIIIA-R14A, which is selective for Na1.7 over both Na1.2 and Na1.4 (IC50 values 0.36, 3.7, and 6.5 μM, respectively; see Fig. 2D). KIIIA-R14A showed a slightly decreased k(on) but unchanged k(off) values (Table 1) in its interaction with Na1.7 (0.038 μM⁻¹·min⁻¹ and 0.020 min⁻¹, respectively). In contrast, for Na1.2 and Na1.7, there were changes in both k(on) and k(off), with k(on) getting slower (0.024 and 0.21 μM⁻¹·min⁻¹ for Na1.2 and 1.4, respectively), and k(off) (0.026 and 1.2 min⁻¹ for Na1.2 and 1.4, respectively) getting faster for both. Despite the changes in affinity there were no significant changes in maximal block compared with KIIIA-wt (range, 87–91%; see Table 1). Here, the dramatic result is that the peripheral nerve channel isoform, Na1.7, becomes the preferred target for KIIIA R14A by a factor of 10- to 20-fold over Na1.2 and Na1.4.

Channel Residues in the Domain III Outer Ring Interact with KIIIA-R14A. Two channel mutants were constructed to look at the effects of the domain III outer ring charge on KIIIA-R14A’s binding. Based on previous studies of other μ-conotoxins (Choudhary et al., 2007; McArthur et al., 2011a), it seemed likely that KIIIA’s Arg14 should interact with residues in domain III and IV near the outer ring charges. To explore the basis for the observed differences we scanned for sequence differences among Na1.2/1.4 and Na1.7 in these domains (Fig. 1B). In human Na1.7, an isoleucine (Ile1410) is present at the “outer ring” position of domain III, taking the place of the aspartate found in all other Na channels (Asp1241 in rat Na1.4). Thus, we constructed two “exchange” mutants, rNa1.4 D1241I and hNa1.7 I1410D, to test for possible interactions with Arg14 in KIIIA.

First, we tested KIIIA-wt on both channels to determine whether this exchange could explain the differences in affinity between Na1.4 and Na1.7 for KIIIA-wt (Fig. 2C, Table 1). The Na1.4 D1241I mutation increased the Ka of wild-type KIIIA to 102 nM (close to that of Na1.7, 97 nM), whereas the Na1.7 I1410D mutation decreased the Ka to 56 nM (similar to that of Na1.4, 37 nM). The mutations had opposite effects on the k(on) and k(off) rates. The Na1.4 D1241I mutation slowed the association rate and sped up the disso-

### Table 1

<table>
<thead>
<tr>
<th>Toxic (KIIIA) and Channel</th>
<th>k(on) μM⁻¹·min⁻¹</th>
<th>S.E.M.</th>
<th>k(off) μM⁻¹·min⁻¹</th>
<th>S.E.M.</th>
<th>Kd μM</th>
<th>Fb wt</th>
<th>S.E.M.</th>
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<td>Na1.2</td>
<td>0.280</td>
<td>0.026</td>
<td>0.002</td>
<td>5.0 × 10⁻⁵</td>
<td>0.005</td>
<td>0.897</td>
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<td>Na1.4</td>
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<td>0.202</td>
<td>0.060</td>
<td>0.006</td>
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<td>0.014</td>
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<td>0.017</td>
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<td>0.937</td>
<td>0.059</td>
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<td>0.940</td>
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<td>0.014</td>
<td>0.782</td>
<td>0.643</td>
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</table>

Fractional block at saturating toxin concentrations (Fb wt) are determined from the dose-response data in Fig. 2.
cation rate, compared with KIIIA-wt block of Na,1.4, accounting for the observed lower affinity of the mutant channel. In turn, Na,1.7 I1410D showed an increased association rate, with little change in the dissociation rate, leading to the observed higher affinity of the mutant channel.

Next, we looked at block of the two channel mutants by KIIIA-R14A (Fig. 2D). In both cases compared with KIIIA-R14A's block of native Na,1.4 and Na,1.7, there was little change in the parameters $k_{on}$, $k_{off}$, and $K_d$. The on and off rates for Na,1.4 D1241I were not significantly different from native Na,1.4. The Na,1.7 I1410D mutant showed slightly increased values of both $k_{on}$ and $k_{off}$. These changes yielded the observed small differences in $K_d$ (3.7 and 5.7 μM between Na,1.4 and Na,1.4 D1241I, and 0.51 and 0.78 μM for Na,1.7 and Na,1.7 I1410D, respectively; see Table 1).

The Na,1.4 D1241I mutation had no effect on the maximal block by KIIIA-wt (98%), but the Na,1.7 I1410D mutation decreased KIIIA-wt's maximal block to 59% (Fig. 2C). With KIIIA-R14A, the Na,1.4 D1241I mutation induced no significant change in maximal block (94%) compared with Na,1.4, whereas the Na,1.7 I1410D decreased the maximal block level to 64% compared with native Na,1.7 (Fig. 2D).

General implications for binding affinity that emerge of these experiments are that 1) the differences of binding of KIIIA-wt among Na,1.4, Na,1.7, and their reciprocal mutants depend on the presence or absence of an interaction between toxin Arg14 and the domain III outer ring aspartate; and 2) the substitution R14A removes this interaction, so subsequent replacement of the outer ring aspartate has little influence (affinities for each parent channel and its respective mutant are essentially equal; see Fig. 2E).

Substitution K7A Affects Both the Affinity and Maximal Block. Neutralization of lysine-7 in KIIIA lowers the toxin's affinity for all three channel isoforms, but the K7A derivative has the same selectivity profile as KIIIA-wt (Na,1.2 > Na,1.4 > Na,1.7) (Fig. 3A). KIIIA-K7A has a similar effect on IC$_{50}$ in all three channels in that it decreases affinity ~30-fold for all three channel isoforms (Tables 1 and 2). The effects on affinity are mostly due to increases in $k_{off}$ (11- to 20-fold), with small decreases in $k_{on}$ (1.5- to 3-fold). Removal of the Lys7 charge decreased the maximal block of the toxin compared with KIIIA-wt. In Na,1.2 the maximal block was 68%, which was larger than both Na,1.4 and Na,1.7 (60 and 58%, respectively) (Fig. 3B). Examining a similar μCTX, PIIIA, a similar decrease in residual current is seen in Na,1.2 compared with Na,1.4 and 1.7, at the homologous position (PIIIA-R14A; see Fig. 3, C and D), suggesting a similar bound orientation of this residue.

A Tyrosine/Phenylalanine Substitution in Domain I Modulates the Maximal Block by KIIIA-K7A. From previous studies of μ-conotoxins, we expected that Lys7 would face down into the pore and would interact predominantly with residues in domain I and II. We scanned the pore regions of Na,1.2, 1.4, and 1.7 in these two domains and found that Na,1.4 and Na,1.7 have a conserved tyrosine one residue external to DEKA locus in domain I, whereas Na,1.2 has a phenylalanine at this position. To test for a role for this residue in determining maximal block by KIIIA-K7A in Na,1.2 versus Na,1.4 and 1.7, we generated a Na,1.4 Y401F mutant. The Na,1.4 Y401F mutant showed a maximal block of 69%, significantly larger than Na,1.4 and 1.7 and the same as Na,1.2 (Fig. 3, A and B). When PIIIA-R14A was tested in the Na,1.4 Y401F construct, The
residual current was reduced to the same level as the native Na_{1.2} residual current level (Fig. 3, C and D). This mutation had little effect on either the IC_{50} values or the kinetics compared with native Na_{1.4}. In contrast, the Na_{1.4} D1241I outer ring mutation did not affect the maximal block by KIIIA-K7A (Supplementary Fig. 1).

Neutral Replacement of Arg10 Decreases Affinity and Maximal Block of KIIIA. Replacement of Arg10 with an alanine in KIIIA increases the IC_{50} of the toxin for all three channel isoforms (~14- to 97-fold) yet retains the KIIIA-wt isoform specificity profile of Na_{1.2} > Na_{1.4} > Na_{1.7} (Fig. 4A). The decreases in affinity result from decreases in $k_{on}$ (2- to 4-fold), and increases in $k_{off}$ (3- to 11-fold; see Table 3).

The maximal block of KIIIA-R10A is reduced compared with KIIIA-wt in all three channel isoforms. The largest reduction was seen for Na_{1.7}, to 52%, whereas reduction of Na_{1.2} to 66% and Na_{1.4} to 70% showed similar maximal block levels.

We looked at the effects of KIIIA-R10A in the domain III channel mutants, Na_{1.4} D1241I and Na_{1.7} I1410D. The Na_{1.4} D1241I mutant did not significantly affect the IC_{50} of KIIIA-R10A, whereas the Na_{1.7} I1410D only slightly increased the IC_{50} (<4-fold). For Na_{1.4} D1241I and Na_{1.7} I1410D, there were small decreases in both the $k_{on}$ (~2.5-fold) and $k_{off}$ (6-fold), respectively, compared with their respective native channels (Tables 1 and 3). Both channel mutants showed increased maximal block with KIIIA-R10A, to 93% for Na_{1.4} D1241I and 77% for Na_{1.7} I1410D.

Substitution H12A Decreases KIIIA Affinity by Increasing Dissociation Rate and Modifies Specificity. Substitution of His12 with an alanine severely reduced the affinity of the toxin for all native channel isoforms (Fig. 4B). This substitution increased the IC_{50} values for Na_{1.2} and Na_{1.4} by >2000-fold, whereas in contrast, the increase in IC_{50} for Na_{1.7} was only 133-fold (Fig. 4B). The resulting sequence of affinities for KIIIA-H12A was Na_{1.2} > Na_{1.7} > Na_{1.4}. These drastic changes in affinity were not due to changes in $k_{on}$ (2- –6-fold decreases) but rather to changes in $k_{off}$, 1.4 (700-fold increases), 30.4 (500-fold increase), and 2.0 min \(^{-1}\) (120-fold increase), for Na_{1.2}, 1.4, and 1.7, respectively). Maximal block by KIIIA-H12A was slightly reduced in all three channels (to

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**TABLE 2**

Kinetic analysis of KIIIA K7A binding ($k_{on}$) and unbinding ($k_{off}$).

<table>
<thead>
<tr>
<th>Channel</th>
<th>$k_{on}$</th>
<th>S.E.M.</th>
<th>$k_{off}$</th>
<th>S.E.M.</th>
<th>$K_d$</th>
<th>$F_{bs}$</th>
<th>S.E.M.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na_{1.2}</td>
<td>0.180</td>
<td>0.013</td>
<td>0.004</td>
<td>0.016</td>
<td>0.678</td>
<td>0.017</td>
<td></td>
</tr>
<tr>
<td>Na_{1.4}</td>
<td>0.512</td>
<td>0.035</td>
<td>0.680</td>
<td>0.045</td>
<td>1.327</td>
<td>0.603</td>
<td>0.021</td>
</tr>
<tr>
<td>Na_{1.7}</td>
<td>0.117</td>
<td>0.019</td>
<td>0.369</td>
<td>0.022</td>
<td>3.170</td>
<td>0.581</td>
<td>0.033</td>
</tr>
<tr>
<td>Na_{1.4} Y401F</td>
<td>0.441</td>
<td>0.027</td>
<td>0.445</td>
<td>0.042</td>
<td>1.011</td>
<td>0.687</td>
<td>0.017</td>
</tr>
<tr>
<td>Na_{1.4} D1241I</td>
<td>0.455</td>
<td>0.056</td>
<td>0.603</td>
<td>0.046</td>
<td>1.326</td>
<td>0.625</td>
<td>0.029</td>
</tr>
</tbody>
</table>

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**Fig. 4.** Substitutions in KIIIA decrease maximal block (R10A), and binding affinity (H12A). A, dose-response curves for KIIIA-R10A (data from 89 cells) block of Na_{1.2}, Na_{1.4}, Na_{1.7}, Na_{1.4} D1241I, and Na_{1.7} I1410D. Maximal block ranges from ~0.5 to ~0.9. B, dose-response curves of KIIIA-H12A (data from 89 cells) for Na_{1.2}, Na_{1.4}, and Na_{1.7}. IC_{50} values are ~1000-fold larger than for KIIIA wt for the same channel isoforms. C, batrachotoxin-modified, single-channel currents showing KIIIA-H12A blocked levels (red arrow; black dotted line denotes fully closed level) of rat skeletal muscle sodium channels (Na_{1.4}). Given the low affinity of H12A, it was not possible to use high enough concentrations to determine maximal block from whole-cell dose-response data; in the single-channel recordings, the blocked fraction, with H12A bound, was ~0.8. This value was used to constrain the fit of the dose-response curve for Na_{1.4} in B.
81% for Na,1.2, 78% for Na,1.4, and 88% for Na,1.7). For Na,1.4, given the very low affinity, the maximal block was estimated from single-channel recordings using lipid bilayers (Fig. 4C).

**Binding of KIIIA Is Enhanced at Low pH Based on Increased Association Rates.** To examine the effects protonation on KIIIA-wt block, we measured the kinetics of toxin binding and unbinding at pH 6.0, 7.4, and 9.0. An example of the effects of pH on KIIIA-wt block of Na,1.4 is shown in Fig. 5A. Increasing the pH caused a decrease in \( k_{on} \) in Na,1.2, 1.4, and 1.7 (Fig. 5C). For Na,1.2, the \( k_{on} \) decreased from 2.2 \( \mu M^{-1} min^{-1} \) at pH 6.0 to 0.28 \( \mu M^{-1} min^{-1} \) at pH 7.4, and to 0.053 \( \mu M^{-1} min^{-1} \) at pH 9.0. Similar to Na,1.2, the channels Na,1.4 and Na,1.7 also showed decreases in \( k_{on} \) as the pH was increased (Fig. 5C). On average, across the three channel isoforms, \( k_{on} \) decreased 13-fold per pH unit over the pH range from 6.0 to 9.0.

The effects of pH on \( k_{on} \) were less pronounced (Fig. 5D). In Na,1.2, there was no significant difference in \( k_{on} \) between pH 6.0 and 7.4 (0.004 and 0.002 \( min^{-1} \), respectively), whereas Na,1.4 showed a slight decrease in \( k_{on} \) from pH 6.0 to 7.4 (0.11 and 0.060 \( min^{-1} \), respectively). However, from pH 6.0 to 7.4 to 9.0, both Na,1.2 and Na,1.4 showed an increase in \( k_{on} \) (to 0.010 and 0.43 \( min^{-1} \), respectively). For Na,1.7 there was no significant changes in \( k_{on} \) across all three pH values (0.012, 0.017, and 0.015 \( min^{-1} \), respectively).

The strong influence of pH on \( k_{on} \), combined with its minimal influence on \( k_{off} \), resulted in a decreased affinity as pH increases. As a consequence, for KIIIA-wt block of Na,1.2, the \( K_d \) increased by \~90-fold from 2 to 183 nM, from pH 6.0 to 9.0. Similar results were seen for Na,1.4 and Na,1.7 (Fig. 5E). Even in the absence of a free N-terminal amino group (see below), there is sufficient residual pH sensitivity to see a monotonic increase in \( K_d \) in the range of pH 6 to 9.

**Removal of the Charged Amine at the KIIIA N terminus Strongly Attenuates pH Dependence.** To learn the basis of the pH effects on \( k_{on} \), we constructed a toxin derivative lacking the titratable N-terminal amino group desamino KIIIA (KIIIA-DA) and tested the effects of changes in pH on its kinetics (Fig. 5B, gray). There was a reduced sensitivity of \( k_{on} \) to pH for all the native channels (Fig. 5C). These changes, averaged over the three channel isoforms, reflect a decrease in \( k_{on} \) of 1.7-fold per pH unit (approximately 13-fold for KIIIA-wt) over the pH range from 6.0 to 9.0 (see above).

Similar to the effects of pH on wild-type KIIIA’s \( k_{off} \), pH has little effect on KIIIA-DA’s \( k_{off} \). In Na,1.2, there was no significant difference in \( k_{off} \) across pH (0.025, 0.014, and 0.018 \( min^{-1} \) for pH 6.0, 7.4, and 9.0, respectively) (Fig. 5D). However, in both Na,1.4 and Na,1.7, there is a significant difference between pH 7.4 and 9.0 (0.10 and 0.48 \( min^{-1} \) for Na,1.4 and 0.016 and 0.063 \( min^{-1} \) for Na,1.7).

The reduced pH effect on KIIIA-DA’s \( k_{off} \), compared with KIIIA-wt, leads to a smaller increase in \( K_d \) as pH is increased. In Na,1.2, the \( K_d \) increases from 162 nM at pH 6.0 to 186 nM at pH 7.4 and to 635 nM at pH 9.0 (Fig. 5E). Similar to these results, Na,1.4 and Na,1.7 also increase in \( K_d \) from pH 6.0 to 7.4 to 9.0 (199 nM, 294 nM, and 3.2 \( \mu M \) for Na,1.4 and 145 \( nM \), 558 \( nM \), and 5.5 \( \mu M \) for Na,1.7, respectively).

**Double Mutant Cycle Analysis and Molecular Dynamics Simulations of \( \mu \)-CTX KIIIA-Bound Structure.** The KIIIA structure was overlaid on the GIIIA docked conformation generated previously (Choudhary et al., 2007), and MD simulations were run for 20 ns. The toxin backbone was stable within an average root mean square deviation of 1.5Å. The largest cluster contained \~60% of all the structures calculated. Its central structure is shown in Fig. 6A (green backbone), along with the starting structure (purple backbone). In this structure, Lys7 points toward the outer ring charges of domain I and II, whereas the Arg10 lies close to the domain I outer ring glutamate. Arg14 interacts with the outer ring charges in both domains III and IV, whereas the His12 lies between the outer ring charges of domains II and III.

We performed double mutant cycle analysis for toxin/channel pairs of KIIIA-Lys7 with Na,1.4 Tyr401 and Asp1241, and for both KIIIA-Arg10 and KIIIA-Arg14 with Na,1.4 Asp1241 and Na,1.7 Ile1401 (Fig. 6D; Supplementary Table 1). KIIIA-Lys7 showed no substantial coupling to Na,1.4 Tyr401 (0.27 kT), and only a small coupling (1.0 kT) to the outer ring domain III charge, Na,1.4 Asp1241. The KIIA-R10A and R14A derivatives both showed large couplings than KIIIA-K7A, of 1.5 kT or greater, to the domain III outer ring charge (Na,1.4 Asp1241). Experiments with hNa,1.7 and its reciprocal outer ring mutation (I1410D) showed increased affinity for KIIIA-wt (Fig. 2E), whereas mutant cycle analysis showed similar or slightly greater coupling energies to those obtained with hNa,1.4. Thus, these two sets of data confirm that residues Arg10 and Arg14 of KIIIA are both interacting partners with the domain III outer ring position on the channel.

**Discussion**

**Differing Kinetics of KIIIA Block among Na, Isoforms from Brain, Skeletal Muscle, and Peripheral Nerve.** Wild-type KIIIA is selective among channel isoforms,

### Table 3

**Kinetic analysis of KIIIA R10A and H12A binding (\( k_{on} \)) and unbinding (\( k_{off} \)).**

<table>
<thead>
<tr>
<th>Toxin (KIIIA) and Channel</th>
<th>( k_{on} ) ( \mu M^{-1} min^{-1} ) S.E.M.</th>
<th>( k_{off} ) ( min^{-1} ) S.E.M.</th>
<th>( K_d ) ( \mu M )</th>
<th>( F_{b_{on}} )</th>
<th>S.E.M.</th>
</tr>
</thead>
<tbody>
<tr>
<td>R10A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Na,1.2</td>
<td>0.140</td>
<td>0.031</td>
<td>0.022</td>
<td>0.011</td>
<td>0.158</td>
</tr>
<tr>
<td>Na,1.4</td>
<td>0.504</td>
<td>0.032</td>
<td>0.506</td>
<td>0.054</td>
<td>1.003</td>
</tr>
<tr>
<td>Na,1.7</td>
<td>0.039</td>
<td>0.007</td>
<td>0.053</td>
<td>0.019</td>
<td>1.366</td>
</tr>
<tr>
<td>Na,1.4 D1241</td>
<td>0.206</td>
<td>0.036</td>
<td>0.088</td>
<td>0.005</td>
<td>0.428</td>
</tr>
<tr>
<td>Na,1.7 I1410D</td>
<td>0.015</td>
<td>0.003</td>
<td>0.039</td>
<td>0.004</td>
<td>2.545</td>
</tr>
<tr>
<td>H12A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Na,1.2</td>
<td>0.129</td>
<td>0.025</td>
<td>1.392</td>
<td>0.115</td>
<td>10.791</td>
</tr>
<tr>
<td>Na,1.4</td>
<td>0.275</td>
<td>0.016</td>
<td>30.288*</td>
<td>4.114</td>
<td>110.497</td>
</tr>
<tr>
<td>Na,1.7</td>
<td>0.106</td>
<td>0.016</td>
<td>2.035</td>
<td>0.123</td>
<td>19.262</td>
</tr>
</tbody>
</table>

\( a \) Maximal block was determined from the single-channel records in Fig. 4C.
as reflected by mean IC₅₀ values for Naᵥ1.2 (5 nM, brain) over Naᵥ1.4 (37 nM, skeletal muscle) and Naᵥ1.7 (97 nM, peripheral nerve). Underlying the differences in affinity are changes in both binding and unbinding kinetics. The high affinity for Naᵥ1.2 is due predominantly to extremely slow dissociation (k⁻, 0.002 min⁻¹), making it irreversible for

**Fig. 5.** Decreases in extracellular pH increase the affinity of KIIIA-wt by speeding up the association rate, largely because of protonation of the free N-terminal amine. Examples of current traces showing toxin wash-in at pH 6 and 9. Command voltage sequences were applied at 5-s intervals to elicit successive current traces. KIIIA-wt (5 μM) (A) and KIIIA-DA (10 μM) (B). At pH 6, note the much faster decrement in peak current for KIIIA-wt than for KIIIA-DA. C, kₒₐ values for Naᵥ1.2, Naᵥ1.4, and Naᵥ1.7 at pH 6.0, 7.4, and 9.0. For 18 different experimental groups at different pHs and toxin concentrations, there was a total of 103 independent estimates of kₒₐ and 68 estimates of k₋. D, values of k₋ determined from the time courses of toxin washout. E, Kₐ values calculated as the ratios of mean values of k₋ to kₒₐ; see Materials and Methods.
most practical purposes. \( \text{Na}_\text{A,7} \), another neuronal channel, shows \( k_{\text{on}} \) values similar to \( \text{Na}_\text{A,2} \) (0.18 versus 0.28 \( \mu \text{M}^{-1} \cdot \text{min}^{-1} \), respectively) but the \( k_{\text{off}} \) for \( \text{Na}_\text{A,7} \), is approximately nine times faster (0.017 \( \text{min}^{-1} \)). \( \text{Na}_\text{A,1,4} \), a skeletal muscle channel, shows an intermediate \( IC_{50} \) but has the most divergent kinetics of the three. Its \( k_{\text{on}} \) is 6- to 9-fold faster (1.6 \( \mu \text{M}^{-1} \cdot \text{min}^{-1} \)) than those for both of the neuronal channels, but it also has the fastest \( k_{\text{off}} \) (0.060 \( \text{min}^{-1} \)). The faster kinetics in \( \text{Na}_\text{A,1,4} \) may reflect an evolutionary adaptation allowing the snail to rapidly immobilize its prey by blocking skeletal muscle action potentials (Table 1; Fig. 5C).

Compared with \( \mu \text{CTX} \) GIIIA and PIIIA, \( \mu \text{CTX-KIIIA} \)'s higher affinities for both neuronal channels examined arise from a much slower dissociation rate. KIIIA's dissociation from \( \text{Na}_\text{A,2} \) is \( \approx \)100-fold slower than that of PIIIA, whereas the association rate constants are not significantly different (McArthur et al., 2011a).

**Derivative KIIIA-R14A Selectively blocks \( \text{Na}_\text{A,1,7} \), an Important Contributor to Pain Signaling.** Unlike KIIIA-wt (affinities in the sequence \( \text{Na}_\text{A,2} > 1.4 > 1.7 \)), KIIIA-R14A is selective for \( \text{Na}_\text{A,1,7} \) over both \( \text{Na}_\text{A,2} \) and \( \text{Na}_\text{A,1,4} \). The R14A substitution yielded a much less pronounced decrease in \( K_d \) for \( \text{Na}_\text{A,1,7} \) (5-fold) than for \( \text{Na}_\text{A,2} \) (200-fold) and \( \text{Na}_\text{A,1,4} \) (150-fold), suggesting that 14 lacks the strong interaction with \( \text{Na}_\text{A,1,7} \) that occurs with both \( \text{Na}_\text{A,2} \) and \( \text{Na}_\text{A,1,4} \). Sequence alignment (Fig. 1A) shows this residue to be analogous to Arg19 in GIIIA and Arg20 in PIIIA. From MD docking simulations, we expected KIIIA-Arg14 to interact with the outer ring charges of domains III/IV for \( \text{Na}_\text{A,2} \) and \( \text{Na}_\text{A,1,4} \). However, the sequence alignment (Fig. 1B) shows that in h\( \text{Na}_\text{A,1,7} \), isoleucine replaces the outer ring aspartate that is present in domain III for both \( \text{Na}_\text{A,2} \) and \( \text{Na}_\text{A,1,4} \). The lack of this aspartate seems to account for the smaller change in affinity for \( \text{Na}_\text{A,1,7} \) associated with the KIIIA-R14A substitution.

The DIII outer ring reciprocal mutants (Fig. 1B) in \( \text{Na}_\text{A,1,4} \) D1241I and \( \text{Na}_\text{A,1,7} \) I1410D reveal a clear basis for the different affinities of KIIIA-wt for these two channels (Fig. 2E). The \( \text{Na}_\text{A,1,4} \) D1241I mutation increased the \( K_d \) to 102 nM, approximating that seen for \( \text{Na}_\text{A,1,7} \) (97 nM), whereas the reciprocal mutation (\( \text{Na}_\text{A,1,7} \) I1410D) mutation decreased the \( K_d \) to 56 nM, closer to that for \( \text{Na}_\text{A,1,4} \) (37 nM). Consistent with this, KIIIA-R14A showed very similar \( K_d \) values for the wild-type and mutant channel pairs (5.7 and 3.7 \( \mu \text{M} \) for \( \text{Na}_\text{A,1,4} \) and \( \text{Na}_\text{A,1,4} \) D1241I, versus 0.51 and 0.78 \( \mu \text{M} \) for \( \text{Na}_\text{A,1,7} \) and \( \text{Na}_\text{A,1,7} \) I1410D). This suggests that the DIII outer ring charge and Arg14 are interacting partners in toxin binding (see Fig. 6D and Supplementary Table 1 for coupling energies calculated from mutant cycle analysis).

**Residues on Both Toxin and Channel Modulate Current through Toxin-Bound Channels.** KIIIA-wt was the first native \( \mu \text{CTX} \) shown to allow a residual single channel current (5–10%) when the toxin is bound (Zhang et al., 2007). This current can be eliminated when guanidinium toxins such as tetrodotoxin or saxitoxin are applied after KIIIA is bound. Simultaneous binding of a KIIIA derivative and one of such as tetrodotoxin or saxitoxin are applied after KIIIA is bound. This current can be eliminated when guanidinium toxins such as tetrodotoxin or saxitoxin are applied after KIIIA is bound. Simultaneous binding of a KIIIA derivative and one of such as tetrodotoxin or saxitoxin are applied after KIIIA is bound.

The two KIIIA residues that strongly modulate maximal block (Lys7 and Arg10) are analogous to Arg14 and Lys17 in

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**Fig. 6.** Molecular dynamics docking simulations and estimates of some toxin-channel coupling energies. A, starting (purple) and docked (green) structures of KIIIA with individually colored channel domains (domain I, ice blue; domain II, orange; domain III, yellow; domain IV, brown). B, space-filled docked structure of KIIIA, highlighting the amino acids studied. C, toxin backbone (green) with basic residues (space-filled) and with DEKA ring (licorice stick format) residues highlighted. In the experiments, each of the toxin residues labeled, was either replaced by a neutral substitution, or titrated (-NH2 terminal), to change the charge at that position. D, coupling energies, expressed as \( kT \) (where \( k \) is the Boltzmann constant and \( T \) is temperature in degrees Kelvin) and given as unsigned, absolute values, calculated from double mutant cycle analysis for interactions between toxin/channel residue pairs. The strongest couplings were found with channel domain III, pore outer ring position (see Fig. 1).
PIIIA, where neutral substitutions enable similar residual currents (McArthur et al., 2011a). Furthermore, KIIIA-wt, which is six amino acids shorter than PIIIA, lacks a charged residue analogous to PIIIA-Arg12, suggesting that the observed residual current for KIIIA-wt could result from the lack of this charge.

KIIIA-K7A, shows the lowest degree of maximal block at saturating toxin concentrations, and this level is Na$_3$, channel isoform-specific (Fig. 3A). From previous work, we expected Lys7 to interact with domain I and domain II between the inner and outer rings. Sequence alignment (Fig. 1B) shows a tyrosine adjacent to the inner ring for both Na$_{1.4}$ and 1.7, in which Na$_{1.2}$ has a phenylalanine. The mutation Na$_{1.4}$ Y401F had little effect on the $K_d$ of KIIIA-K7A but increased the maximal block to 69%, near that for Na$_{1.2}$. This suggests that with KIIIA-K7A bound, ion passage through the channel is limited by Tyr401, which is a primary determinant of tetrodotoxin and saxitoxin affinity (Favre et al., 1995).

The KIIIA-R10A substitution also modulates maximal block. With R10A, maximal block is ordered as follows: Na$_{1.7}$<Na$_{1.2}$<Na$_{1.4}$, likely as a result of the differences in domain III, where Na$_{1.7}$ has an isoleucine, whereas Na$_{1.2}$ and Na$_{1.4}$ each have an aspartate.

The key new point here is that residues from both toxin and channel help to determine the toxin efficacy by controlling the maximal block. A full analysis of the chemical and steric factors involved will require extensive substitutions in both toxin and channel, taking advantages of general approaches developed earlier (Hui et al., 2002) and could reveal additional opportunities for pharmacological approaches using KIIIA derivatives in combination with other ligands (Zhang et al., 2009; French et al., 2010).

**His12 of KIIIA Contributes Strongly to Binding to All Three Channel Isoforms.** A conserved histidine (His12 in KIIIA) is found in μCTXs, which block both neuronal and skeletal muscle channels. In PIIIA, replacement of this residue by glutamine increased the affinity of PIIIA for skeletal muscle Na$_{1.4}$ channels over neuronal Na$_{1.2}$ (McArthur et al., 2011a). The KIIIA-H12A substitution severely reduced toxin affinity, primarily based on dramatic increases in the dissociation rates (700-, 500-, and 120-fold increase in Na$_{1.2}$, 1.4, and 1.7, respectively). This contrasts strongly with PIIIA, for which H19Q caused no reduction in affinity (McArthur et al., 2011a). KIIIA’s smaller size and net charge due predominantly to titrating its free N-terminal charge, KIIIA-DA. This derivative showed a reduced pH dependence from 13-fold to 1.7-fold change per pH unit. Consequently, the observed pH dependence of KIIIA block is due to the lack of a free N-terminal residue analogous to PIIIA-Arg12, suggesting that the observed toxin kinetics. An examination of differences between the PIIIA and KIIIA structures supports this interesting possibility, because PIIIA block shows no dependence on pH (data not shown), and PIIIA lacks a free N-terminal charge as the residue is cyclized to form a pyroglutamate. Thus, we created a KIIA derivative lacking the free N-terminal charge, KIIIA-DA. This derivative showed a reduced pH dependence from 13-fold to 1.7-fold change per pH unit, consistent with the remaining dependence perhaps arising from the Asp11-His12 pair. This raises a possible complication for the development of subtype selective blockers of hNa$_{1.7}$. An interesting point is that numerous other species have a domain III outer ring aspartate instead of isoleucine, thus screening of Na$_{1.7}$ modulators for human use that should use only hNa$_{1.7}$. KIIIA has been shown previously to have analgesic behavior (Zhang et al., 2007). Further honing KIIIA targeting toward hNa$_{1.7}$ poses a challenge, in that two substitutions that were found to enhance this targeting (H12A and R14A) substantially reduce KIIIA affinity.

Changes in pH in disease states are well documented. During ischemia, a drop of almost 1 pH unit has been observed (Kraig et al., 1983), and neuroprotective action of sodium channel blockers has been demonstrated (Carter et al., 2000). Thus, blocking toxins, whose affinity increases as pH decreases, may be well suited to combat such disease states.

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**Authorship Contributions**

**Participated in research design:** McArthur, Singh, Tieleman, and French.

**Conducted experiments:** McArthur.
Contributed new reagents or analytic tools: McArthur, McMaster, Winkfein, Tieleman, and French.

Performed data analysis: McArthur, Singh, and French.

Wrote or contributed to the writing of the manuscript: McArthur, Singh, McMaster, Winkfein, Tieleman, and French.

Performed stimulations: McArthur and Singh.

References


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Supplementary Figure 1. Dose response curve for block of Na\textsubscript{v}1.4 Y401F by KIIIA-wt and Na\textsubscript{v}1.4 D1241I by KIIIA-K7A
**Supplementary Table 1.** Coupling coefficients ($\Omega$) and coupling energies ($\Delta \Delta G$, in units of $kT$) for six toxin-channel residue pairs

<table>
<thead>
<tr>
<th></th>
<th>$\Delta \Delta G$ (kT)</th>
<th>$\Omega$ (d.f)</th>
<th>$\Delta \Delta G$ (kT)</th>
<th>$\Omega$ (d.f)</th>
<th>$\Delta \Delta G$ (kT)</th>
<th>$\Omega$ (d.f)</th>
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<tr>
<td>Na$_v$1.4 Y401F</td>
<td>0.27</td>
<td>1.31±1.97 (73)</td>
<td>1.0</td>
<td>2.74±1.52 (69)</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>Na$_v$1.4 D1241I</td>
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<td>n/a</td>
<td>1.5</td>
<td>4.42±2.86 (65)</td>
<td>2.5</td>
<td>5.97±0.55 (63)</td>
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<tr>
<td>Na$_v$1.7 I1410D</td>
<td>n/a</td>
<td>n/a</td>
<td>1.8</td>
<td>12.67±1.61 (58)</td>
<td>1.7</td>
<td>5.79±0.34 (78)</td>
</tr>
</tbody>
</table>