INTERACTIONS OF KEY CHARGED RESIDUES CONTRIBUTING TO SELECTIVE BLOCK OF NEURONAL SODIUM CHANNELS BY $\mu$-CONOTOXIN KIIIA

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List of nonstandard abbreviations:
μCTX, μ-Conotoxin; GIIIA, μ-Conotoxin GIIIA; PIIIA, μ-Conotoxin PIIIA; Na+, Voltage-gated sodium channel;
TTX, Tetrodotoxin; STX, Saxitoxin; BTX, Batrachotoxin; $K_d$, Dissociation constant; $k_{on}$, Toxin binding kinetics; $k_{off}$, Toxin unbinding kinetics; $IC_{50}$, Half inhibitory concentration.
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 (Na,1.5), myotonsias (Na,1.4), epilepsies (Na,1.2), and pain (Na,1.7). Thus, there is strong motivation to identify isoform-specific blockers and the molecular determinants underlying their selectivity among these channels. μConotoxin KIIIA blocks rNa,1.2 (IC$_{50}$=5nM), rNa,1.4 (37nM) and hNa,1.7 (97nM), expressed in mammalian cells, with high affinity and a maximal block at saturating concentrations of 90-95%. 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 which altered Na, isoform specificity. KIIIA-R14A showed the highest affinity for Na,1.7, a channel involved in pain signaling. Wildtype KIIIA has a 2-fold higher affinity for Na,1.4 than for Na,1.7, which can be attributed to a missing outer vestibule charge in domain III of Na,1.7. Reciprocal mutations Na,1.4 D1241I and Na,1.7 I1410D remove the affinity differences between these two channels for wildtype KIIIA, without affecting their affinities for KIIIA-R14A. KIIIA is the first μCTX 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 (Na_v) channels are important in the initiation and propagation of nerve impulses in neurons and muscle (Hille, 2001). To date, nine mammalian Na_v channels have been described (Na_v1.1-1.9) (Catterall et al., 2005; Al-Sabi et al., 2006); these have differing distributions throughout the body. Gain of function mutations in Na_v channels causing hyperexcitability, have been linked to such disease states as, cardiac arrhythmia (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_v channel isoforms.

Venoms from fish-hunting cone snails contain many different toxins, which represent possible therapeutic compounds targeting various ion channels. μConotoxins (μCTXs) make up one such group of toxins. As a group μCTXs are identified by their conserved disulphide backbone structure and because they all target toxin site-1, the outer pore vestibule, of Na_v channels (Catterall et al., 2005). μCTXs from different species target various Na_v channels, and show differing selectivity profiles. μCTX GIIIA from Conus geographus specifically targets skeletal muscle channels (Na_v1.4) (Cruz et al., 1985), while the very similar μCTX, PIIIA, from Conus purpurascens most strongly inhibits skeletal muscle channels, but also blocks some neuronal channels (Na_v1.2 and Na_v1.7) with lower affinity (Shon et al., 1998).

μCTX KIIIA, from Conus kinoshitai, 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 disulphide bond pattern (Bulaj et al., 2005) (Fig. 1). KIIIA has a nominal net charge of +4, lower than both GIIIA (+6) and PIIIA (+7). Interestingly, KIIIA was the first μCTX to show higher affinity for the neuronal channel, Na_v1.2, than for skeletal muscle channels (Zhang et al., 2007). Indeed, KIIIA block of Na_v1.2 is almost irreversible on a normal experimental time scale. KIIIA also shows a high affinity (IC_{50} in the nanomolar range) for Na_v1.7, a channel involved in pain perception (Yang et al., 2004).

Unlike previously studied μCTXs, 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 PIII A 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 (TTX) or saxitoxin (STX) 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,1.2 (central nervous system), Na,1.4 (skeletal muscle), and Na,1.7 (peripheral nervous system). Channel mutants were selected based on sequence comparisons (Fig. 1B), and previous docking simulations of μCTXs GIIIA and PIIIA (McArthur et al., 2011a; Choudhary et al., 2007). We focused on two positions: (a) the outer ring charge in domain III, which is absent in Na,1.7, but present in Na,1.4 and Na,1.2, and (b) the aromatic residue in domain I, adjacent to the DEKA locus which has important implications for TTX and STX block (Ahern et al., 2008; Santarelli et al., 2007) and thus could be important in KIIIA block. These residues distinguish the canonical μCTX target, Na,1.4, from the neuronal channels, Na,1.2 and Na,1.7. We tested the effects of changes at these loci in order 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 efficacy of block, affinity and targeting selectivity. Two residues, K7 and R10, are key determinants of KIIIA’s fractional block of single-channel currents, or maximal conductance in whole-cell experiments. KIIIA’s affinity, for all three Na, channels studied, increased with a decrease in extracellular pH based 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 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,1.7, a channel involved in the pain pathway (Yang et al., 2004; Cox et al., 2006), over Na,1.2 and Na,1.4. These molecular determinants of μCTX selective targeting, offer clues to the design of more selective blockers of tissue-specific Na, channel isoforms.
Materials and Methods

Toxin Synthesis and Preparation.

Conotoxin synthesis, purification and disulphide bond formation were performed as previously described in detail (Hui et al., 2002). Briefly, linear peptides were synthesized by solid-phase synthesis using 9-fluorenylmethoxycarbonyl (Fmoc) chemistry. Coupling of Fmoc amino acids was performed using the HBTU/HOBT/DIPEA method on a Quartet Synthesizer (Protein Technologies Inc.).

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 (10μL in 150mL)) in order to promote formation of the most stable disulphide bonds. During oxidization, the cyclization of the peptide was monitored by analytical HPLC which was completed in 2-4 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 MALDI mass spectrometric molecular weight determination. Some derivatives produced two separate peaks, and thus the peak identified as active from (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 rNav1.2 (pCDM8 a gift from W.A. Catterall) (Linford et al., 1998), rt1.4 (pcDNA3.1) (Trimmer et al., 1989) and hNav1.7 (pCMV6) (Hildebrand et al., 2011) were used. Channel constructs were created using rNav1.4 or hNav1.7 as a template. Three channel mutants (rNav1.4 Y401F, rNAv1.4 D1241I and hNav1.7 I1410D were constructed. Briefly, sense and antisense primers encoding point mutations for the desired amino acid substitution were synthesized and used in high fidelity PCR reactions (Phusion polymerase, Thermo) with primers located either 3’ or 5’, respectively, from unique flanking restriction sites in the wildtype construct. Amplified fragments (upstream and downstream) were run on agarose gels and the resulting products 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 wildtype
channel clones. All fragments cloned from PCR products were completely sequenced to ensure no PCR-generated mis-incorporations had occurred during cloning.

HEK293 cells were transiently cotransfected with the plasmid encoding the particular sodium channel α-subunit (2μg) and a plasmid encoding GFP (0.5μg), allowing transfected cells to be identified by their green fluorescence. Twenty-four hours after transfection, cells were plated on to cover slips and used for voltage-clamp studies 2 hours 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 (in mM), 140 NaCl, 5 KCl, 2 CaCl2, 1 MgCl2, 10 HEPES and 10 Glucose, adjusted to pH 6, 7.4 or 9 with HCl or NaOH (~305 mOsm/kg). The pipette electrodes had a final tip resistance of 1-3MO with an internal solution composed (in mM) of, 35 NaCl, 105 CsF, 1 MgCl2, 10 HEPES, 1 EGTA, pH adjusted to 7.2 with CsOH (~295 mOsm/kg).

Whole-cell patch clamp was performed with an EPC7 Amplifier (HEKA Electronics Incorporated). Current traces were filtered at 3 kHz (low-pass, 3-pole Bessel filter, EPC7) and sampled at 200 kHz using pClamp9.2 software (Axon Instruments Inc.), with series resistance compensated typically at 40-60%. Cells showing peak currents between 0.5 and 5nA were used to ensure adequate voltage control while maintaining good current resolution. Toxins were then locally super-fused over the cell at a rate of 10-20 μl/min (bath volume of 5 mL). Currents were elicited with a 2s pre-pulse to -140mV to remove inactivation followed by a test pulse to -10mV for 10ms repeated every 5s during toxin application and wash out to record kinetics.

Single-channel bilayer experiments using batrachotoxin-modified skeletal muscle Na+ channel at steady state were carried out as described previously (McArthur et al., 2011a).

**Data Analysis.**

Each point data point 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 a 18 ± 4 experiments contributing to each dose-response curve. Typically 3-4 determinations were done at each concentration (range, 1-9). Dose-response curves are plotted as the estimated fraction of channels bound $F_{bound} = (I_{res}/I_{ac})$ versus concentration of toxin, where $I_{res}$ is the residual current in the presence of the toxin at steady state and $I_{ac}$ is the
current level prior to toxin application. Data were fit with a rectangular hyperbola, assuming a Hill coefficient of 1, using the following expression, where $IC_{50}$ is the toxin concentration for half-maximal inhibition. Here, $Fb_{ss}$ is the fraction of current blocked by the toxin at steady state, at a particular concentration, and $Fb_{sc}$ represents the maximal fraction of current blocked at saturating concentrations of the toxin (and the fractional block of the single-channel current). The parameters $Fb_{sc}$ and $IC_{50}$ were varied to obtain the best fit.

$$Fb_{ss} = \frac{Fb_{sc}}{1 + \frac{IC_{50}}{[tx]}}$$

Toxin blocking kinetics ($k_{on}$ and $k_{off}$) were measured by fitting the peak currents for successive depolarizations during toxin wash in or wash out test with a single exponential to determine $tau_{on}$ and $tau_{off}$. The rates constants, $k_{on}$, and $k_{off}$, and the equilibrium dissociation constant, $K_d$ (Fig. 2A, B) were calculated using the following equations:

$$k_{on} = \frac{Fb_{sc}}{tau_{on} \cdot [tx]}$$

$$k_{off} = \frac{1}{tau_{off}}$$

$$K_d = \frac{k_{off}}{k_{on}}$$

For ease of reading in the text, group data are represented as mean values, with full statistics provided in the tables. Differences between group datasets 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 previously described (McArthur et al., 2011b). Briefly, 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. KIIIA was superposed on to the docked structure of GIIIA. 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 (Daura et al., 1999), with an RMSD cut-off 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 VMD (Humphrey et al., 1996).
RESULTS

Charged residues play critical roles in defining how μCTXs target and block Na, 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 Na, channel selectivity (Fig. 1). To identify residues that might be involved in isoform selectivity we neutralized all charged 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 degrees in the three channel subtypes. To assess the importance of differences among the channels, we used the following constructs: Na,1.4 Y401F, Na,1.4 D1241D, and Na,1.7 D1410I. 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 (Cummins et al., 2002; Li et al., 2001; Li et al., 2003) 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 Na,1.2, 1.4 and 1.7 by Wildtype KIIIA.

Channel isoforms Na,1.2, 1.4 and 1.7 were expressed heterologously in HEK293 cells and currents 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. 2A, B). KIIIA blocked Na,1.2 with the highest affinity (K_d=5nM) compared to Na,1.4 (K_d=37nM) and Na,1.7 (K_d=97nM) (see 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 (Na,1.2, 90%, Na,1.4, 95% and Na,1.7, 94%) at saturating concentrations of wildtype KIIIA. Kinetics are very slow in Na,1.2, making it impractical to collect truly steady state data. Thus this dose-response curve was fit using an IC_{50} of 5nM, equal to the kinetically determined (K_d). Other cases provided steady state data which allowed direct determination of IC_{50} values from the dose-response relation, and these values correspond well with K_d, values determined from the kinetics. Overall, wildtype KIIIA (KIIIA-wt) shows a range of binding affinities of ~30-fold, in the following order: Na,1.2 > Na,1.4 > Na,1.7 (IC_{50} / K_d values: 5, 54, and 147nM, 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$\mu$M$^{-1}$min$^{-1}$, respectively) relative to that of Na1.4 (1.6$\mu$M$^{-1}$min$^{-1}$). The dissociation rate constants, $k_{off}$, also differed among channel isoforms with Na1.2 being the slowest (0.002min$^{-1}$) followed by Na1.7 (0.017min$^{-1}$) with Na1.4 being the fastest (0.060min$^{-1}$). 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* oocytes (Zhang *et al.*, 2007). In spite of the skeletal muscle targeting for which the $\mu$CTX 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 ($IC_{50}$ values 0.36, 3.7 and 6.8$\mu$M, 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$\mu$M$^{-1}$min$^{-1}$ and 0.020min$^{-1}$, 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$\mu$M$^{-1}$min$^{-1}$ for Na1.2 and 1.4 respectively), and $k_{off}$ (0.026 and 1.2min$^{-1}$ 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 to 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 $\mu$-conotoxins (McArthur *et al.*, 2011a; Choudhary *et al.*, 2007), it seemed likely that KIIIA’s R14 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 (I1410) is present at the “outer ring” position of domain III, taking the place of the aspartate found in all other Na, channels (D1241 in rat Na1.4). Thus, we constructed two “exchange” mutants, rNa1.4 D1241I and hNa1.7 I1410D, to test for possible interactions with R14 in KIIIA.

First, we tested KIIIA-wt on both channels to see if this exchange could explain the differences in affinity between Na1.4 and Na1.7 for KIIIA-wt (Fig. 2C and Table 1). The Na1.4 D1241I mutation increased the $K_d$ of
wildtype KIIIA to 102nM (close to that of Na\textsubscript{1.7}, 97nM), while the Na\textsubscript{1.7} I1410D mutation decreased the $K_d$ to 56nM (similar to that of Na\textsubscript{1.4}, 37nM). The mutations had opposite effects on the $k_{on}$ and $k_{off}$ rates. The Na\textsubscript{1.4} D1241I mutation slowed the association rate, and sped up the dissociation rate, compared with KIIIA-wt block of Na\textsubscript{1.4}, accounting for the observed lower affinity of the mutant channel. In turn, Na\textsubscript{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 when compared to KIIIA-R14A’s block of native Na\textsubscript{1.4} and Na\textsubscript{1.7}, there was little change in the parameters $k_{on}$, $k_{off}$ and $K_d$. The on and off-rates for Na\textsubscript{1.4} D1241I were not significantly different from native Na\textsubscript{1.4}. The Na\textsubscript{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 5.7μM between Na\textsubscript{1.4} and Na\textsubscript{1.4} D1241I, and 0.51 and 0.78μM for Na\textsubscript{1.7} and Na\textsubscript{1.7} I1410D, respectively – see Table 1).

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

General implications for binding affinity that emerge of these experiments are: a) the differences of binding of KIIIA-wt among Na\textsubscript{1.4}, Na\textsubscript{1.7} and their reciprocal mutants depend on the presence or absence of an interaction between toxin R14 and the domain III outer ring aspartate; and b) the substitution R14A removes this interaction, so that 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\textsubscript{1.2}>Na\textsubscript{1.4}>Na\textsubscript{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 K7 charge decreased the maximal block of the toxin when compared to KIIIA-wt. In Na\textsubscript{1.2} the maximal block was 68%, which was larger than both Na\textsubscript{1.4} and Na\textsubscript{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 to Na,1.4 and Na,1.7, at the homologous position (PIIIA-R14A, see Fig. 3C, 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 K7 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, while 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. 3A, 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. 3C, D). This mutation had little effect on either the IC_{50}s 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 R10 Decreases Affinity and Maximal Block of KIIIA.**

Replacement of R10 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 to KIIIA-wt in all three channel isoforms. The largest reduction was seen for Na,1.7, to 52%, while Na,1.2, 66%, and Na,1.4, 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, while 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 to 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 H12 with an alanine, severely reduced the affinity of the toxin for all native channel isoforms (Fig. 4b). This substitution increased the $IC_{50}$s for Na$_v$1.2 and Na$_v$1.4 by >2000-fold, while in contrast, the increase in $IC_{50}$ for Na$_v$1.7 was only 133-fold (Fig. 4B). The resulting sequence of affinities for KIIIA-H12A was Na$_v$1.2>Na$_v$1.7>>Na$_v$1.4. These drastic changes in affinity were not due to changes in $k_{on}$ (2 to 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$_v$1.2, 1.4 and 1.7 respectively). Maximal block by KIIIA-H12A was slightly reduced in all three channels (to 81% for Na$_v$1.2, 78% for Na$_v$1.4, and 88% for Na$_v$1.7). For Na$_v$1.4, 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$_v$1.4 is shown in Fig. 5A. Increasing the pH caused a decrease in $k_{on}$ in Na$_v$1.2, 1.4 and 1.7 (Fig. 5C). For Na$_v$1.2 the $k_{on}$ decreased from 2.2 μM$^{-1}$min$^{-1}$ at pH 6.0, to 0.28 μM$^{-1}$min$^{-1}$ at pH 7.4 and to 0.053 μM$^{-1}$min$^{-1}$ at pH 9.0. Similar to Na$_v$1.2, the channels Na$_v$1.4 and Na$_v$1.7 also showed a decrease 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 $k_{off}$ were less pronounced (Fig. 5D). In Na$_v$1.2 there was no significant difference in $k_{off}$ between pH 6.0 and 7.4 (0.004 and 0.002min$^{-1}$, respectively), while Na$_v$1.4 showed a slight decrease in $k_{off}$ from pH6.0 to 7.4 (0.11 and 0.060min$^{-1}$, respectively). However from pH6.0 or pH7.4 to pH 9.0, both Na$_v$1.2 and Na$_v$1.4 showed an increase in $k_{off}$ (to 0.010 and 0.43min$^{-1}$, respectively). For Na$_v$1.7 there was no significant changes in $k_{off}$ across all three pH values (0.012, 0.017 and 0.015min$^{-1}$, 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$_v$1.2, the $K_d$ increased by ~90-fold from 2-183nM, from pH 6.0 to pH 9.0. Similar results were seen for Na$_v$1.4 and Na$_v$1.7 (Fig. 5E). Even in the absence of a free N-terminal amino group (see following section), 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

In order 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, grey). There was a reduced sensitivity of $k_{on}$ to pH for all the native channels (Fig. 5C). These changes, averaged over the 3 channel isoforms, reflect a decrease in $k_{on}$ of 1.7-fold per pH unit (c.f. 13-fold for KIIIA-wt) over the pH range from 6.0 to 9.0 (see above).

Similar to the effects of pH on wildtype KIIIA’s $k_{off}$, pH has little effect on KIIIA-DA’s $k_{off}$. In Nav1.2 there was no significant difference in $k_{off}$ across pH (0.025, 0.014 and 0.018min$^{-1}$, for pH 6.0, 7.4 and 9.0 respectively) (Fig. 5D). However in both Na1.4 and Na1.7 there is a significant difference between pH 7.4 and 9.0 (0.10 and 0.48min$^{-1}$ for Na1.4 and 0.016 and 0.063min$^{-1}$ for Na1.7).

The reduced pH effect on KIIIA-DA’s $k_{on}$ compared to KIIIA-wt, leads to a smaller increase in $K_d$ as pH is increased. In Na1.2 the $K_d$ increases from 162nM at pH 6.0 to 186nM at pH7.4 to 635nM at pH 9.0 (Fig. 5E). Similar to these results, Na1.4 and Na1.7 also increase in $K_d$ from pH6.0 to 7.4 to 9.0 (199nM, 294nM and 3.2μM for Na1.4 and 145nM, 558nM and 5.5μM for Na1.7).

Double Mutant Cycle Analysis and Molecular Dynamics Simulations of μ-CTX KIIIA

Bound Structure

The KIIIA structure was overlaid on the previously generated GIIIA docked conformation (Choudhary et al., 2007) and MD simulations were run for 20ns. 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, K7 points towards the outer ring charges of domain I and II, while the R10 lies close to the Domain I outer ring glutamate. R14 interacts with the outer ring charges in both domains III and IV, while the H12 lies between the outer ring charges of domains II and III.

We performed double mutant cycle analysis for toxin/channel pairs of KIIIA-K7 with Na1.4 Y401 and D1241I, and for both KIIIA-R10 and KIIIA-R14 with Na1.4 D1241 and Na1.7 I1401 (Fig. 6D and Supplementary Table 1). KIIIA-K7 showed no substantial coupling to Na1.4 Y401 (0.27kT), and only a small coupling (1.0kT) to the outer
ring domain III charge, Na,1.4 D124. The KIIIA-R10A and R14A derivatives both showed larger couplings than KIIIA-K7A, of 1.5kT or greater, to the domain III outer ring charge (Na,1.4 D1241). Experiments with hNa,1.7 and its reciprocal outer ring mutation (I1410D) showed increased affinity for KIIIA-wt (Fig. 2E), while mutant cycle analysis showed similar or slightly greater coupling energies to those obtained with rNa,1.4. Thus, these two sets of data confirm that residues K10 and R14 of KIIIA are both interacting partners with the domain III outer ring position on the channel.
DISCUSSION

Differing Kinetics of KIIIA Block Among Nav Isoforms from Brain, Skeletal Muscle and Peripheral Nerve.

Wildtype KIIIA is selective among channel isoforms, as reflected by mean IC$_{50}$s for Nav1.2 (5nM, brain) over Nav1.4 (37nM, skeletal muscle) and Nav1.7 (97nM, peripheral nerve). Underlying the differences in affinity are changes in both binding and unbinding kinetics. The high affinity for Nav1.2 is predominantly due to extremely slow dissociation ($k_{off}$, 0.002min$^{-1}$), making it irreversible for most practical purposes. Nav1.7, another neuronal channel, shows $k_{on}$ values similar to Nav1.2 (0.18 vs 0.28μM$^{-1}$min$^{-1}$, respectively) but the $k_{off}$ for Nav1.7 is about 9 times faster (0.017min$^{-1}$). Nav1.4, a skeletal muscle channel, shows an intermediate IC$_{50}$, but has the most divergent kinetics of the three. Its $k_{on}$ is 6-9 fold faster (1.6μM$^{-1}$min$^{-1}$) than those for both of the neuronal channels, but it also has the fastest $k_{off}$ (0.060min$^{-1}$). The faster kinetics in Nav1.4 may reflect an evolutionary adaptation allowing the snail to rapidly immobilize its prey by blocking skeletal muscle action potentials (Table 1 and Fig. 5C).

Compared with μCTX GIIIA and PIIIA, μCTX-KIIIA’s higher affinities for both neuronal channels examined arise from a much slower dissociation rate. KIIIA’s dissociation from Nav1.2 is ~100-fold slower than that of PIIIA, while the association rate constants are not significantly different (McArthur et al., 2011a).

Derivative KIIIA-R14A Selectively blocks Nav1.7, an Important Contributor to Pain Signaling.

Unlike KIIIA-wt (affinities in the sequence Na$_v$1.2>1.4>1.7), KIIIA-R14A, is selective for Na$_v$1.7 over both Na$_v$1.2 and Na$_v$1.4. The R14A substitution yielded a much less pronounced decrease in $K_d$ for Nav1.7 (~5-fold), than for Nav1.2 (200-fold) and Nav1.4 (150-fold), suggesting that R14 lacks the strong interaction with Na$_v$1.7 that occurs with both Na$_v$1.2 and Na$_v$1.4. Sequence alignment (Fig. 1A) shows this residue to be analogous to R19 in GIIIA and R20 in PIIIA. From MD docking simulations, we expected KIIIA-R14 to interact with the outer ring charges of domains III/IV for Na$_v$1.2 and Na$_v$1.4. However, the sequence alignment (Fig. 1B) shows that in hNa$_v$1.7, isoleucine replaces the outer ring aspartate that is present in domain III for both Na$_v$1.2 and Na$_v$1.4. Presumably, the lack of this aspartate accounts for the smaller change in affinity for Na$_v$1.7 associated with the KIIIA-R14A substitution.
The DIII outer ring reciprocal mutants (Fig. 1B) in Na\textsubscript{1.4} D1241I and Na\textsubscript{1.7} I1410D reveal a clear basis for the different affinities of KIIIA-wt for these two channels (Fig. 2E). The Na\textsubscript{1.4} D1241I mutation increased the $K_d$ to 102 nM, approximating that seen for Na\textsubscript{1.7} (97 nM), while the reciprocal mutation (Na\textsubscript{1.7} I1410D) mutation decreased the $K_d$ to 56 nM, closer to that for Na\textsubscript{1.4} (37 nM). Consistent with this, KIIIA-R14A showed very similar $K_d$s for the wildtype and mutant channel pairs (5.7 and 3.7 μM for Na\textsubscript{1.4} and Na\textsubscript{1.4} D1241I, versus 0.51 and 0.78 μM for Na\textsubscript{1.7} and Na\textsubscript{1.7} I1410D). This suggests that the DIII outer ring charge and R14 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 μCTX 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 like tetrodotoxin or saxitoxin are applied after KIIIA is bound. Simultaneous binding of a KIIIA derivative and one of the guanidinium toxins modifies blocking and unblocking kinetics, and opens a wider spectrum of pharmacological possibilities than the use of either type of toxin individually (Zhang et al., 2009; French et al., 2010).

The two KIIIA residues which strongly modulate maximal block (K7 and R10) are analogous to R14 and K17 in PIIIA, where neutral substitutions enable similar residual currents (McArthur et al., 2011a). Furthermore, KIIIA-wt, which is 6 amino acids shorter than PIIIA, lacks a charged residue analogous to PIIIA-R12, 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\textsubscript{v} channel isoform-specific (Fig. 3A). From previous work, we expected K7 to interact with DI and DII between the inner and outer rings. Sequence alignment (Fig. 1B) shows a tyrosine adjacent to the inner ring for both Na\textsubscript{1.4} and 1.7, where Na\textsubscript{1.2} has a phenylalanine. The mutation Na\textsubscript{1.4} Y401F had little effect on the $K_d$ of KIIIA-K7A but increased the maximal block to 69%, near that for Na\textsubscript{1.2}. This suggests that with KIIIA-K7A bound, ion passage through the channel is limited by Y401, 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\textsubscript{1.7} < Na\textsubscript{1.2} < Na\textsubscript{1.4}, likely as a result of the differences in Domain III, where Na\textsubscript{1.7} has an isoleucine, while Na\textsubscript{1.2} and Na\textsubscript{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).

H12 of KIIIA Contributes Strongly to Binding to All Three Channel Isoforms

A conserved histidine (H12 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 may allow the histidine to play a more dominant role. The H12A-associated reductions in affinity were dramatic, and result in preferential targeting of the two neuronal isoforms, Na,1.2 and Na,1.7, over skeletal muscle Na,1.4 (Fig. 4B). Of the toxin derivatives that we studied, only H12A and R14A showed a clear change in channel isoform targeting preference from that of KIIIA wt.

Protonation of KIIIA Increases Association Rate and Hence Affinity.

Changing pH in the range 6-9 produced obvious, systematic increases in $k_{on}$ values as pH decreased, showing an average change of 13-fold per unit pH over the three channel subtypes, with little change in $k_{off}$ (<3-fold). Thus, the large effect of the H12A substitution on dissociation rates rules out H12 as the primary mediator of pH effects. Furthermore, the parallel effects of pH on the three different channel isoforms make it unlikely that the major effect is mediated through titration of acidic residues in the channels’ outer vestibules.

An alternate possibility is that a disulphide linkages in the toxin could be lost at higher pH. However, removal of only one of the three disulphide linkages yielded a lower $k_{on}$ the C2-C15 linkage (Khoo et al., 2009; Han et al., 2009). This decrease in $k_{on}$ fits with the KIIIA-wt pH results, the concomitant 10-fold increase in $k_{off}$, together with the rapid reversibility of our observed pH effects, makes disulfide bond disruption an unlikely rationale for our observations.

A third possibility is that KIIIA’s free N-terminus could be titrated by the changing pH, and indeed, this clearly affects the observed toxin kinetics. An examination of differences between the PIIIA and KIIIA structures supports
this interesting possibility, as PI IIIA block shows no dependence on pH (data not shown) and PI IIIA lacks a free N-terminal charge as the residue is cyclized to form a pyroglutamate. Thus, we created a K IIIIA derivative lacking the free N-terminal charge, K IIIIA-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 K IIIIA block is predominantly due to titrating its free N-terminal charge, with the remaining dependence perhaps arising from the D11-H12 pair. This raises a possible complication for the strategy of toxin cyclization as a way of stabilizing the active conformation (Clark et al., 2010). If protonation of the free amino terminal were important for a pharmacological action, this option would be lost in the N-terminal to C-terminal cyclized version.

**Possible Pharmacological Applications of K IIIIA Derivatives.**

Na,1.7 play a role in pain signaling. Gain of function mutations lead to primary erythermalgia (Yang et al., 2004), while loss of function leads to congenital inability to experience pain (Cox et al., 2006; Fertleman et al., 2006), prompting a renewed interest in finding 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 should employ only hNa,1.7. K IIIIA has previously been shown to have analgesic behaviour (Zhang et al., 2007). Further honing K IIIIA targeting toward hNa,1.7 poses a challenge, in that two substitutions which were found to enhance this targeting (H12A and R14A) substantially reduce K IIIIA affinity.

Changes in pH in disease states is well documented. During ischemia, a drop of almost one 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 drops, may be well-suited to combat such disease states.
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Authorship Contributions

Participated in research Design: JRM, RJF, DPT, GS

Conducted experiments: JRM

Contributed new reagents or analytic tools: JRM, RJF, DPT, DM, RW

Performed data analysis: JRM, RJF, GS

Wrote or contributed to the writing of the manuscript: JRM, RJF, DPT, GS, DM, RW
References


Footnotes

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**Figure Legends**

**Fig. 1. Model of KIIIA structure.** A, Structure of KIIIA with important residues highlighted (K7, cyan; R10/R14, blue; H12, purple). B, Sequence alignment of the domains I and III p-loop regions, with inner ring (blue) and outer ring (red) labelled. Sequences of channel mutants rNa, 1.4 Y401F, rNa, 1.4 D1241I, and hNa, 1.7 I1241D are shown.

**Fig. 2. KIIIA-R14A selectivity profile is altered compared to KIIIA-wt.** A, Example experiment of KIIIA-wt (1 μM) washin in Na,1.4. B, Example washout experiment of KIIIA-wt (1 μM) from same cell as A. C, 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 $K_d$ (black, bold open circle on this plot), because the time constant was block was too long near this concentration, to reliably attain steady-state block. In other cases, both $IC_{50}$ and the maximal saturating block, $Fbsc$ 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.

**Fig. 3. KIIIA-K7A effects residual current through single channels and is modulated by residues in the pore.** A, Dose response curves of KIIIA-K7A (data from 87 cells) in Na,1.2, Na,1.4, Na,1.7 and Na,1.4 Y401F. B, Maximal block of ionic current by KIIIA-K7A through Na,1.2, Na,1.4, Na,1.7 and Na,1.4 Y401F. C, Dose response curve of PIIIA-R14A (data from 47 cells) in Na,1.2, Na,1.4 and Na,1.4 Y401F. D, Maximal block, by PIIIA-R14A, of ionic current through Na,1.2, Na,1.4 and Na,1.4 Y401F.

**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. $IC_{50}$s 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 part B.
Fig. 5. Decreases in extracellular pH, increase the affinity of KIIIA-wt by speeding up the association rate, largely due to protonation of the free N-terminal amine. Examples of current traces showing toxin wash-in at pH6 and pH9. Command voltage sequences were applied at 5s intervals to elicit successive current traces. A, KIIIA-wt (5μM), and B, KIIIA-DA (10μM). At pH6, note the much faster decrement in peak current for KIIIA-wt than for KIIIA-DA. C, $k_{on}$ values for Na,1.2, Na,1.4 and Na,1.7 at pH6.0, 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 $k_{on}$ and 68 estimates of $k_{off}$. D, Values of $k_{off}$ determined from the time courses of toxin washout. E, $K_d$ values calculated as the ratios of mean values of $k_{off}$ to $k_{on}$ – see Methods.

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 coloured 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 (liquorice stick format) residues highlighted. In the experiments, each of the toxin residues labelled, was either replaced by a neutral substitution, or titrated (-NH2 terminal), to change the charge at that position. D, Coupling energies, expressed in units of kT 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).
Table 1. Kinetic analysis of KIIIA wildtype and R14A binding ($k_{on}$) and unbinding ($k_{off}$).

Fractional block at saturating toxin concentrations ($F_{bsc}$) are determined from the $IC_{50}$ determination.
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**Table 2.** Kinetic analysis of KIIIA K7A binding ($k_{on}$) and unbinding ($k_{off}$). Fractional block at saturating toxin concentrations ($F_{bsc}$) are determined from the $IC_{50}$ determination.
Table 3. Kinetic analysis of KIIIA R10A and H12A binding ($k_{on}$) and unbinding ($k_{off}$). Fractional block at saturating toxin concentrations ($F_{bsc}$) are determined from the $IC_{50}$ determination.
**Figure 1**

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<td>pE</td>
<td>R</td>
<td>L</td>
<td>C</td>
<td>C</td>
<td>G</td>
<td>F</td>
<td>O</td>
<td>K</td>
<td>S</td>
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<table>
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<th>A</th>
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<tbody>
<tr>
<td></td>
<td>K7</td>
<td>R10</td>
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</tbody>
</table>

**Note:** The table and diagram represent the sequence and structure of the P-loop regions in sodium channels.
Figure 2
Figure 3
Figure 4
Figure 5
Supplemental Data for:
Title: INTERACTIONS OF KEY CHARGED RESIDUES CONTRIBUTING TO SELECTIVE BLOCK OF NEURONAL SODIUM CHANNELS BY μ-CONOTOXIN KIIIA
Authors: McArthur, JR., Singh, G., McMaster, D., Winkfein, R., Tieleman, DP., and RJ. French
Journal: Molecular Pharmacology

Supplementary Figure 1. Dose response curve for block of \( \text{Na}_v1.4 \ Y401F \) by KIIIA-wt and \( \text{Na}_v1.4 \ D1241I \) by KIIIA-K7A
## Supplementary Table 1. Coupling coefficients (Ω) and coupling energies (ΔΔG, in units of kT) for six toxin-channel residue pairs

<table>
<thead>
<tr>
<th></th>
<th>Na\textsubscript{v}1.4 Y401F</th>
<th>Na\textsubscript{v}1.4 D1241I</th>
<th>Na\textsubscript{v}1.7 I1410D</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ΔΔG (kT)</td>
<td>Ω (d.f)</td>
<td>ΔΔG (kT)</td>
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<tr>
<td>K7A</td>
<td>0.27</td>
<td>1.31±1.97 (73)</td>
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<td>R10A</td>
<td>n/a</td>
<td>n/a</td>
<td>1.5</td>
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<tr>
<td>R14A</td>
<td>n/a</td>
<td>n/a</td>
<td>1.8</td>
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