Interactions of Key Charged Residues Contributing to Selective Block of Neuronal Sodium Channels by \(\mu\)-Conotoxin KIIIA

J. R. McArthur, G. Singh, D. McMaster, R. Winkfein, D. P. Tieleman, and R. J. French

Department of Physiology and Pharmacology, and the Hotchkiss Brain Institute (J.R.M., D.M., R.W., R.J.F.), and Department of Biological Sciences and the Institute of Biocomplexity and Informatics (G.S., D.P.T.), University of Calgary, Calgary, Alberta, Canada

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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), myotonias (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. \(\mu\)-Conotoxin KIIIA blocks rNav1.2 (IC\textsubscript{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\textsubscript{i}, isoform specificity. KIIIA-R14A showed the highest affinity for Na\textsubscript{i}, 1.7, a channel involved in pain signaling. Wild-type KIIIA has a 2-fold higher affinity for Na\textsubscript{i}, 1.4 than for Na\textsubscript{i}, 1.7, which can be attributed to a missing outer vestibule charge in domain III of Na\textsubscript{i}, 1.7. Reciprocal mutations Na\textsubscript{i}, 1.4 D1241I and Na\textsubscript{i}, 1.7 I1410D remove the affinity differences between these two channels for wild-type KIIIA without affecting their affinities for KIIIA-R14A. KIIIA is the first \(\mu\)-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\textsubscript{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\textsubscript{i} 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 Nav channel isoforms.

Venoms from fish-hunting cone snails contain many different toxins, which represent possible therapeutic compounds targeting various ion channels. \(\mu\)-Conotoxins (\(\mu\)CTXs) make up one such group of toxins. As a group, \(\mu\)CTXs 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). \(\mu\)CTXs from different species target various Na\textsubscript{i} channels and show differing selectivity profiles. \(\mu\)CTX GIIIA from Conus geographus specifically targets skeletal muscle channels (Nav1.4) (Cruz et al., 1985), whereas the very similar \(\mu\)CTX, PIIIA, from Conus purpureascens most strongly inhibits skeletal muscle channels but also blocks some neuronal channels (Nav1.2 and Na\textsubscript{i}, 1.7) with lower affinity (Shon et al., 1998). \(\mu\)CTX KIIIA, from Conus kinoshitai, points to the potential importance of \(\mu\)CTXs as possible therapeutic compounds by showing analgesic activity (Zhang et al., 2007). KIIIA is the shortest known \(\mu\)CTX, at only 16 amino acids in length, but retains the typical \(\mu\)CTX disulfide bond pattern (Bulaj et al., 2005) (Fig. 1). KIIIA has a nominal net charge of 2+ and exhibits the highest pH sensitivity among the \(\mu\)CTXs as possible therapeutic compounds by showing analgesic activity (Zhang et al., 2007).

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ABBREVIATIONS: Na\textsubscript{i}, voltage-gated sodium channel; \(\mu\)CTX, \(\mu\)-conotoxin; GIIIA, \(\mu\)-conotoxin GIIIA; PIIIA, \(\mu\)-conotoxin PIIIA; HPLC, high-performance liquid chromatography; HEK, human embryonic kidney; PCR, polymerase chain reaction; MD, molecular dynamics.
neuronal channel, Na$_{1.2}$, than for skeletal muscle channels (Zhang et al., 2007). Indeed, KIIIA block of Na$_{1.2}$ is almost irreversible on a normal experimental time scale. KIIIA also shows a high affinity (IC$_{50}$ in the nanomolar range) for Na$_{1.7}$, a channel involved in pain perception (Yang et al., 2004).

Unlike µCTXs 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$_{1.2}$ (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 (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$_{1.7}$ but present in Na$_{1.4}$ and Na$_{1.2}$; 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 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 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$_{x}$ 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$_{1.7}$, 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$_{x}$ channel isoforms.

![Fig. 1. Model of KIIIA structure.](https://example.com/fig1)

**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$_{1.4}$ Y401F, rNa$_{1.4}$ D1241I, and hNa$_{1.7}$ 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-fluorenylmethoxycarbonyl chemistry. Coupling of 9-fluorenylmethoxycarbonyl amino acids was performed using the 1-hydroxybenzotriazole/2-(1H)-benzotriazol-1-yl)-1,1,3,3-tetramethylurea- hexafluorophosphate/N,N-disopropylhydroxylamine method on a Quartet 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 promote the formation of 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 spectrometer 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 (pCDM8, a gift from W. A. Catterall) (Linford et al., 1998), rNa,1.4 (pCDNA3.1) (Trimmer et al., 1989), and hNav,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 I1140D) 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 flankning 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 PCR-generated misincorporations had occurred during cloning.

HEK293 cells were transiently cotransfected with the plasmid encoding the particular sodium channel α-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 9 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, 10 mM CaF, 1 mM MgCl2, 10 mM HEPES, and 1 mM EGTA, with pH adjusted to 7.2 with CaOH (−295 mOsm/kg).

Whole-cell patch clamp was performed with an EPC7 Amplifier (HEKA, Lambrecht/Pfalz, Germany). Current signals 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 Na+, 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 15 ± 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 ss = (1 – Is/I ctr) versus concentration of toxin, where Is is the residual current in the presence of the toxin at steady state and I ctr is the current level before toxin application. Data were fit with a rectangular hyperbola, allowing 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 ss 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 ss and IC 50 were varied to obtain the best fit.

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

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 t on and t off. The rates constants, k on and k off, and the equilibrium dissociation constant, K d (Fig. 2, A and B) were calculated using the following equations:

\[
k_{on} = \frac{Fb_{ss}}{t_{on} \cdot [tx]} \quad k_{off} = \frac{1}{t_{off}} \quad K_d = \frac{k_{off}}{k_{on}}
\]

For ease of reading in the text, group data are represented as mean values, with full statistics provided in the tables. Differences between group 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 9 force field. At first, 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 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 Na+ channels. Despite KiIIIA 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 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 Na1.2, 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 Na1.4. 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 Na1.2, Na1.4, Na1.7, Na1.4 D1241I, and Na1.7 I1410D, respectively. Note that the curve for Na1.2 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 IC50 and the maximal saturating block, $F_b$, were determined directly from fits to the steady-state dose-response data. E, comparison of IC50 values for KIIIA-wt and KIIIA-R14A in each channel.
degrees of differences among the three channel subtypes. To assess the important-
differences among the channels, we used the following
constructs: Na\textsubscript{1.4} Y401F, Na\textsubscript{1.4} D1241I, and Na\textsubscript{1.7}
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 \(\mu\)CTX
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 Na\textsubscript{1.2}, 1.4, and 1.7 by Wild-Type KIIIA.**
Channel isoforms Na\textsubscript{1.2}, 1.4, and 1.7 were expressed heter-
ologously 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 Na\textsubscript{1.2} with the highest affinity (\(K_d = 5\) nM)
compared with Na\textsubscript{1.4} (\(K_d = 37\) nM) and Na\textsubscript{1.7} (\(K_d = 97\)
\(\mu\)M) (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\textsubscript{1.2}, 90%; Na\textsubscript{1.4},
95%; and Na\textsubscript{1.7}, 94%) at saturating concentrations of wild-
type KIIIA. Kinetics are very slow in Na\textsubscript{1.2}, making it
impractical to collect truly steady-state data. Thus, this dose-
response curve was fit using an IC\textsubscript{50} of 5 nM, equal to the
kinetically determined \(K_d\) of Na\textsubscript{1.2} (0.060 min\(^{-1}\)).

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type KIIIA. Kinetics are very slow in Na\textsubscript{1.2}, making it
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response curve was fit using an IC\textsubscript{50} of 5 nM, equal to the
kinetically determined \(K_d\) of Na\textsubscript{1.2} (0.060 min\(^{-1}\)).

**Substitution R14A in KIIIA Changes Its Target Spec-
ificity.** Replacement of KIIIA’s arginine-14 with alanine re-
moved a basic side chain resulting in the derivative KIIIA-
R14A, which is selective for Na\textsubscript{1.7} over both Na\textsubscript{1.2} and
Na\textsubscript{1.4} (IC\textsubscript{50} values 0.36, 3.7, and 6.5 \(\mu\)M, respectively; see
Fig. 2D). KIIIA-R14A showed a slightly decreased \(k_{on}\) but
unchanged \(k_{off}\) values (Table 1) in its interaction with Na\textsubscript{1.7}
(0.038 \(\mu\)M\(^{-1}\), min\(^{-1}\) and 0.020 min\(^{-1}\), respectively). In con-
trast, for Na\textsubscript{1.2} and Na\textsubscript{1.7}, 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 Na\textsubscript{1.2} and 1.4, respectively), and \(k_{off}\) (0.026 and
1.2 min\(^{-1}\) for Na\textsubscript{1.2} and 1.4, respectively) getting faster for
both. Despite the changes in affinity there were no signif-
ican differences in maximal block compared with KIIIA-wt
(range, 87–91%; see Table 1). Here, the dramatic result is that
the peripheral nerve channel isoform, Na\textsubscript{1.7}, becomes
the preferred target for KIIIA R14A by a factor of 10-

**Channel Residues in the Domain III Outer Ring In-
teract with KIIIA-R14A.** Two channel mutants were con-
structed 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 (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 Na\textsubscript{1.2}/1.4 and Na\textsubscript{1.7} in these domains (Fig.
1B). In human Na\textsubscript{1.7}, an isoleucine (Ile1410) is present at the
“outer ring” position of domain III, taking the place of the
aspartate found in all other Na\textsubscript{c} channels (Asp1241 in rat
Na\textsubscript{1.4}). Thus, we constructed two “exchange” mutants,
rNa\textsubscript{1.4} D1241I and hNa\textsubscript{1.7} I1410D, to test for possible inter-
actions with Arg14 in KIIIA.

First, we tested KIIIA-wt on both channels to determine
whether this exchange could explain the differences in affinity
between Na\textsubscript{1.4} and Na\textsubscript{1.7} for KIIIA-wt (Fig. 2C; Table
1). The Na\textsubscript{1.4} D1241I mutation increased the \(K_d\) of wild-
type KIIIA to 102 nM (close to that of Na\textsubscript{1.7}, 97 nM),
wheras the Na\textsubscript{1.7} I1410D mutation decreased the \(K_d\) to 56
nM (similar to that of Na\textsubscript{1.4}, 37 nM). 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 disso-

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**TABLE 1**

Kinetic analysis of KIIIA wild-type and R14A binding (\(k_{on}\) and unbinding (\(k_{off}\))
Fractional block at saturating toxin concentrations (\(F_{max}\)) are determined from the dose-response data in Fig. 2.

<table>
<thead>
<tr>
<th>Toxin (KIIIA) and Channel</th>
<th>(k_{on}) ((\mu)M(^{-1}), min(^{-1}))</th>
<th>S.E.M.</th>
<th>(k_{off}) (min(^{-1}))</th>
<th>S.E.M.</th>
<th>(K_d) ((\mu)M)</th>
<th>(F_{max})</th>
<th>S.E.M.</th>
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<tbody>
<tr>
<td>wt</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Na\textsubscript{1.2}</td>
<td>0.280</td>
<td>0.026</td>
<td>0.002</td>
<td>5.0 \times 10^{-6}</td>
<td>0.005</td>
<td>0.897</td>
<td>0.016</td>
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<tr>
<td>Na\textsubscript{1.4}</td>
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<td>0.202</td>
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<td>0.006</td>
<td>0.037</td>
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<tr>
<td>Na\textsubscript{1.7}</td>
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<td>0.017</td>
<td>0.004</td>
<td>0.097</td>
<td>0.937</td>
<td>0.059</td>
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<tr>
<td>Na\textsubscript{1.4} D1241I</td>
<td>0.940</td>
<td>0.126</td>
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<td>0.992</td>
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<td>Na\textsubscript{1.7} I1410D</td>
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<td>0.004</td>
<td>0.056</td>
<td>0.589</td>
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<td>0.060</td>
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<td>R14A</td>
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<td>Na\textsubscript{1.2}</td>
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<td>0.026</td>
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<td>0.511</td>
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<tr>
<td>Na\textsubscript{1.4} D1241I</td>
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<td>0.807</td>
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</tr>
<tr>
<td>Na\textsubscript{1.7} I1410D</td>
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<td>0.091</td>
<td>0.014</td>
<td>0.782</td>
<td>0.643</td>
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</tbody>
</table>
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 (Na1.2 > Na1.4 > Na1.7) (Fig. 3A). KIIIA-K7A has a similar effect on IC50 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 Na1.2 the maximal block was 68%, which was larger than both Na1.4 and Na1.7 (60 and 58%, respectively) (Fig. 3B). Examining a similar μCTX, PIIIA, a similar decrease in residual current is seen in Na1.2 compared with Na1.4 and Na1.7, 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 Na1.2, 1.4, and 1.7 in these two domains and found that Na1.4 and Na1.7 have a conserved tyrosine one residue external to DEKA locus in domain I, whereas Na1.2 has a phenylalanine at this position. To test for a role for this residue in determining maximal block by KIIIA-K7A in Na1.2 versus Na1.4 and 1.7, we generated a Na1.4 Y401F mutant. The Na1.4 Y401F mutant showed a maximal block of 69%, significantly larger than Na1.4 and 1.7 and the same as Na1.2 (Fig. 3, A and B). When PIIIA-R14A was tested in the Na1.4 Y401F construct, The

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**Fig. 3.** KIIIA-K7A affects 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 Na1.2, Na1.4, Na1.7, and Na1.4 Y401F. B, maximal block of ionic current by KIIIA-K7A through Na1.2, Na1.4, Na1.7, and Na1.4 Y401F. C, dose-response curve of PIIIA-R14A (data from 47 cells) in Na1.2, Na1.4, and Na1.4 Y401F. D, maximal block, by PIIIA-R14A, of ionic current through Na1.2, Na1.4, and Na1.4 Y401F.
residual current was reduced to the same level as the native Na\textsubscript{1,2} residual current level (Fig. 3, C and D). This mutation had little effect on either the IC\textsubscript{50} values or the kinetics compared with native Na\textsubscript{1,4}. In contrast, the Na\textsubscript{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\textsubscript{50} of the toxin for all three channel isoforms (~14- to 97-fold) yet retains the KIIIA-wt isoform specificity profile of Na\textsubscript{1,2} > Na\textsubscript{1,4} > Na\textsubscript{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\textsubscript{1,7}, to 52%, whereas reduction of Na\textsubscript{1,2} to 66% and Na\textsubscript{1,4} to 70% showed similar maximal block levels.

We looked at the effects of KIIIA-R10A in the domain III channel mutants, Na\textsubscript{1,4} D1241I and Na\textsubscript{1,7} I1410D. The Na\textsubscript{1,4} D1241I mutant did not significantly affect the IC\textsubscript{50} of KIIIA-R10A, whereas the Na\textsubscript{1,7} I1410D only slightly increased the IC\textsubscript{50} (<4-fold). For Na\textsubscript{1,4} D1241I and Na\textsubscript{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\textsubscript{1,4} D1241I and 77% for Na\textsubscript{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\textsubscript{50} values for Na\textsubscript{1,2} and Na\textsubscript{1,4} by >2000-fold, whereas in contrast, the increase in IC\textsubscript{50} for Na\textsubscript{1,7} was only 133-fold (Fig. 4B). The resulting sequence of affinities for KIIIA-H12A was Na\textsubscript{1,2} > Na\textsubscript{1,7} > Na\textsubscript{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 increase), 30.4 (500-fold increase), and 2.0 min$^{-1}$ (120-fold increase), for Na\textsubscript{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>$k_{on}$ S.E.M.</th>
<th>$k_{off}$ S.E.M.</th>
<th>$K_d$</th>
<th>$F_{bs}$ S.E.M.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na\textsubscript{1,2}</td>
<td>0.180 0.013</td>
<td>0.030 0.004</td>
<td>0.169 0.678</td>
<td>0.017</td>
</tr>
<tr>
<td>Na\textsubscript{1,4}</td>
<td>0.512 0.035</td>
<td>0.680 0.045</td>
<td>1.327 0.603</td>
<td>0.021</td>
</tr>
<tr>
<td>Na\textsubscript{1,7}</td>
<td>0.117 0.019</td>
<td>0.369 0.022</td>
<td>3.170 0.581</td>
<td>0.033</td>
</tr>
<tr>
<td>Na\textsubscript{1,4} Y401F</td>
<td>0.441 0.027</td>
<td>0.445 0.042</td>
<td>1.011 0.687</td>
<td>0.017</td>
</tr>
<tr>
<td>Na\textsubscript{1,4} D1241I</td>
<td>0.455 0.056</td>
<td>0.603 0.046</td>
<td>1.326 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\textsubscript{1,2}, Na\textsubscript{1,4}, Na\textsubscript{1,7}, Na\textsubscript{1,4} Y401F, and Na\textsubscript{1,4} D1241I. Maximal block ranges from ~0.5 to ~0.9. B, dose-response curves of KIIIA-H12A (data from 89 cells) for Na\textsubscript{1,2}, Na\textsubscript{1,4}, and Na\textsubscript{1,7}. IC\textsubscript{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\textsubscript{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\textsubscript{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 of 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}, Na_{1,4}, and 1.7 (Fig. 5C). For Na_{1,2}, the \( k_{on} \) decreased from 2.2 \( \mu M^{-1} \cdot min^{-1} \) at pH 6.0 to 0.28 \( \mu M^{-1} \cdot min^{-1} \) at pH 7.4, and to 0.053 \( \mu M^{-1} \cdot min^{-1} \) 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 \( k_{off} \) were less pronounced (Fig. 5D). In Na_{1,2}, there was no significant difference in \( k_{off} \) between pH 6.0 and 7.4 (0.004 and 0.002 \( min^{-1} \), respectively), whereas Na_{1,4} showed a slight decrease in \( k_{off} \) 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 \( k_{off} \) (to 0.010 and 0.43 \( min^{-1} \), respectively). For Na_{1,7} there was no significant changes in \( k_{off} \) 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 \( \sim 90 \)-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 17-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 \( k_{on} \) 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 1.5A. The largest cluster contained \( \sim 60 \% \) of all the structures calculated.

**Discussion**

**Differing Kinetics of KIIIA Block among Na_{i} Isoforms from Brain, Skeletal Muscle, and Peripheral Nerve.** Wild-type KIIIA is selective among channel isoforms,
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 (kₐₒᵣₑ, 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, kₐₜ 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 kₐₜ and 68 estimates of kₐₒᵣₑ. D, values of kₐₒᵣₑ determined from the time courses of toxin washout. E, Kᵦ values calculated as the ratios of mean values of kₐₒᵣₑ to kₐₜ; see Materials and Methods.
most practical purposes. $\text{Na}_{1.7}$, another neuronal channel, shows $k_{\text{on}}$ values similar to $\text{Na}_{1.2}$ (0.18 versus 0.28 $\mu$M$^{-1}$ min$^{-1}$, respectively) but the $k_{\text{off}}$ for $\text{Na}_{1.7}$ is approximately nine times faster (0.017 min$^{-1}$). $\text{Na}_{1.4}$, 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 $\mu$M$^{-1}$ min$^{-1}$) than those for both of the neuronal channels, but it also has the fastest $k_{\text{off}}$ (0.060 min$^{-1}$). The faster kinetics in $\text{Na}_{1.4}$ 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 $\mu$CTX GIIIA and PIIIA, $\mu$CTX-KIIIA’s higher affinities for both neuronal channels examined arise from a much slower dissociation rate. KIIIA’s dissociation from $\text{Na}_{1.2}$ is ~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 $\text{Na}_{1.7}$, an Important Contributor to Pain Signaling.** Unlike KIIIA-wt (affinities in the sequence $\text{Na}_{1.2} > 1.4 > 1.7$), KIIIA-R14A is selective for $\text{Na}_{1.7}$ over both $\text{Na}_{1.2}$ and $\text{Na}_{1.4}$. The R14A substitution yielded a much less pronounced decrease in $K_d$ for $\text{Na}_{1.7}$ (~5-fold) than for $\text{Na}_{1.2}$ (200-fold) and $\text{Na}_{1.4}$ (150-fold), suggesting that 14 lacks the strong interaction with $\text{Na}_{1.7}$ that occurs with both $\text{Na}_{1.2}$ and $\text{Na}_{1.4}$. 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 $\text{Na}_{1.2}$ and $\text{Na}_{1.4}$. However, the sequence alignment (Fig. 1B) shows that in h$\text{Na}_{1.7}$, isoleucine replaces the outer ring aspartate that is present in domain III for both $\text{Na}_{1.2}$ and $\text{Na}_{1.4}$. The lack of this aspartate seems to account for the smaller change in affinity for $\text{Na}_{1.7}$ associated with the KIIIA-R14A substitution.

The DIII outer ring reciprocal mutants (Fig. 1B) in $\text{Na}_{1.4}$ D1241I and $\text{Na}_{1.7}$ I1410D reveal a clear basis for the different affinities of KIIIA-wt for these two channels (Fig. 2E). The $\text{Na}_{1.4}$ D1241I mutation increased the $K_d$ to 102 nM, approximating that seen for $\text{Na}_{1.7}$ (97 nM), whereas the reciprocal mutation ($\text{Na}_{1.7}$ I1410D) mutation decreased the $K_d$ to 56 nM, closer to that for $\text{Na}_{1.4}$ (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 $\mu$M for $\text{Na}_{1.4}$ and $\text{Na}_{1.7}$ I1410D, respectively) but the $K_d$ for $\text{Na}_{1.7}$ is approximately 6- to 9-fold faster (1.6 $\mu$M$^{-1}$ min$^{-1}$) than for $\text{Na}_{1.2}$ (0.18 versus 0.28 $\mu$M$^{-1}$ min$^{-1}$). The faster kinetics in $\text{Na}_{1.4}$ may reflect an evolutionary adaptation allowing the snail to rapidly immobilize its prey by blocking skeletal muscle action potentials (Table 1; Fig. 5C).

**Residues on Both Toxin and Channel Modulate Current through Toxin-Bound Channels.** KIIIA-wt was the first native $\mu$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 unblocked. Simultaneous binding of a KIIIA derivative and one of such as tetrodotoxin or saxitoxin are applied after KIIIA is bound. Simultaneous binding of a KIIIA derivative and one of these 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 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 (liquorice stick format) residues highlighted. In the experiments, each of the toxin residues labeled, was either replaced by a neutral substitution, or titrated (-NH$_2$ 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).
Conducting experiments: McArthur, Singh, Tieleman, and French.

Acknowledgments

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Authorship Contributions

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

An alternate possibility is that a disulfide linkages in the toxin could be lost at higher pH. However, removal of only one of the three disulfide linkages yielded a lower $k_{off}$ the C2–C15 linkage (Han et al., 2009; Khoo et al., 2009). This decrease in $k_{off}$ fits with the KIIIA-wt pH results, the concomitant 10-fold increase in $k_{on}$, 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 titratable 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, 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. Consequently, the observed pH dependence of KIIIA block is due predominantly to titrating its free N-terminal charge, with the remaining dependence perhaps arising from the Asp11-His12 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 KIIIA Derivatives. Na,1.7 plays a role in pain signaling. Gain of function mutations lead to primary erythermalgia (Yang et al., 2004), whereas 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 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 those 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.
Contributed new reagents or analytic tools: McArthur, McMaster, Winkfein, Tielemann, and French.

Performed data analysis: McArthur, Singh, and French.

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

Performed stimulations: McArthur and Singh.

References


