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Evidence for multiple effects of ProTxII on activation gating in $\text{Na}_v1.5$

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Abstract

The peptide toxin ProTxII, recently isolated from the venom of the tarantula spider *Thrixopelma pruriens*, modifies gating in voltage-gated Na^+ and Ca^{2+} channels. ProTxII is distinct from other known Na^+ channel gating modifier toxins in that it affects activation, but not inactivation. It shifts activation gating positively and decreases current magnitude such that the dose-dependence of toxin action measured at a single potential reflects both effects. To test the extent to which these effects were independent, we tracked several different measures of current amplitude, voltage dependent activation, and current kinetics in $\text{Na}_v1.5$ in a range of toxin concentrations. Changes in voltage dependence and a decrease in G_{max} appeared at relatively low concentrations (40–100nM) while a positive shift in the voltage range of activation was apparent at higher toxin concentrations ($\geq 500\text{nM}$). Because ProTxII carries a net +4 charge we tested whether electrostatic interactions contributed to toxin action. We examined the effects of ProTxII in the presence of high extracellular Ba^{2+} , known to bind and/or screen surface charge. Some, but not all aspects of ProTxII modification were sensitive to the presence of Ba^{2+} indicating the contribution of an electrostatic, surface charge-like mechanism and supporting the idea of a multi-faceted toxin-channel interaction.

Keywords

gating-modifier toxin; voltage dependent activation; voltage clamp

1. Introduction

Voltage-gated ion channels display exquisite sensitivity to changes in membrane potential, which is critical to their diverse roles in controlling neuronal excitability, triggering cell signaling pathways, and regulating the propagation of action potentials. Gating modifier toxins have been useful tools in probing voltage dependent gating mechanisms and in elucidating the variation in these mechanisms across channel types. A number of toxins target voltage-gated Na^+ channels and efforts to understand the details of these toxin-channel interactions have led to a greater understanding of pore structure, permeation pathways, and the domain-specific roles of voltage sensors in various gating transitions. We now know that toxins employ diverse mechanisms of action. Some, like tetrodotoxin and μ -conotoxin, block permeation by binding

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in the channel pore (reviewed in Cestele and Catterall, 2000). Others selectively disrupt specific gating transitions, such as the anemonae toxins anthopleurin A and B (Ap-A and Ap-B), which inhibit inactivation from the open state, or the β -scorpion toxins, which modify both activation and inactivation (reviewed in de la Vega and Possani, 2007; Hanck and Sheets, 2007).

ProTxII is a 30 amino acid peptide toxin recently isolated from the venom of the tarantula spider *Thrixopelma pruriens*, which has been reported to modify gating in voltage-gated Na^+ and Ca^{2+} channels (Middleton et al., 2002; Smith et al., 2007). It is unique from other gating modifier toxins that target Na^+ channels because it alters voltage dependent activation without any accompanying effects on inactivation gating transitions (Smith et al., 2007). Moreover, although the specific binding determinants for ProTxII remain unknown, they are not thought to coincide with sites previously identified for other Na^+ channel gating modifier toxins (Smith et al., 2007). However, similar to these other toxins, it is likely that ProTxII interacts with the channel close to one or more of the voltage sensors both because of its ability to alter gating transitions and because, like other toxins, ProTxII has the ability to interact with membrane phospholipids (Smith et al., 2005). This latter observation localizes the toxin to the channel-membrane interface where critical voltage sensor movements occur during gating. The actions of other tarantula toxins have been shown to rely on interactions with both membrane phospholipids and voltage sensors (Milescu et al., 2007), and one study has implicated the disruption of gating charge movement by ProTxII itself in a neuronal Na^+ channel isoform $\text{Na}_v1.2$ (Sokolov et al., 2007).

Based on the toxin's ability to inhibit peak current at a single depolarized potential, ProTxII was originally reported to have high affinity for several different Na^+ channel isoforms including $\text{Na}_v1.5$ (Middleton et al., 2002). Because $\text{Na}_v1.5$ is relatively easy to express in a heterologous system and an extensive literature has identified many useful tools for studying its gating, we chose this isoform to further investigate ProTxII interactions. Here we show that gating modification by ProTxII consists of multiple distinct effects that differ in their dose-dependency and in their mechanism of action. Some of these data have been presented in abstract form (Edgerton et al., 2007).

2. Materials and methods

2.1. Heterologous expression

The cDNA for the human heart voltage-gated Na^+ channel, $\text{Na}_v1.5$ (hH1a) was kindly provided by H. Hartmann (University of Maryland Biotechnology Institute) and A. Brown (Chantest Inc.) (Hartmann et al., 1994). For all experiments the $\text{Na}_v1.5$ channel containing a mutation in which the cysteine in the domain I P loop at position 373 was replaced with tyrosine (C373Y) was used. This position controls sensitivity to the guanidinium toxins, tetrodotoxin (TTX) and saxitoxin (STX) (Satin et al., 1992). The C373Y mutation exerts minimal effects on channel kinetics while at the same time eliminating an important extracellular reactive cysteine (McNulty et al., 2006). The cDNA for the $\text{Na}_v1.5$ C373Y channel was subcloned into the mammalian expression vector pcDNA5/FRT and transfected into HEK Flp-In-293 (Invitrogen) cells. Stable cell lines were created using Hygromycin B (Sigma) or HygroGold (Invitrogen) selection (100 $\mu\text{g}/\text{ml}$ for selection, 50 $\mu\text{g}/\text{ml}$ for maintenance). The use of stable cell lines meant that channel expression levels were relatively homogenous, thus minimizing variation in current magnitude across experiments. Whole cell voltage clamp was performed on trypsinized cells (0.25% trypsin-EDTA, Invitrogen), 3–6 days after plating.

2.2. Solutions and chemicals

Bath solution contained (in mM): 5–20 NaCl, 120–135 CsCl, 2CaCl₂ 10 Hepes, pH 7.4 with CsOH. Extracellular NaCl concentration was increased in order to study currents in the

presence of high concentrations of toxin (greater than 1 μ M) and the concentration of CsCl was adjusted such that ionic strength was maintained. Pipette solution contained (in mM): 100 CsCl, 40 CsF, 10 EGTA, 10 Hepes, pH 7.4 with CsOH. For the surface charge neutralization experiments, either 20mM BaCl₂ was added to the control bath solution or 40mM BaCl₂ was added to bath solution in which CsCl concentration was lowered to 80mM to keep ionic strength approximately the same.

Wild-type ProTxII was expressed as a fusion protein with maltose binding protein upstream of the toxin in *Escherichia coli* BL21 (DE3) as described (Smith et al., 2005). Fusion protein was purified on Ni²⁺-nitrilotriacetic acid resin, reduced with 10mM dithiothreitol, diluted to 0.2mg/mL, and dialyzed against 2.5mM glutathione, 50mM Tris, 100mM NaCl, pH 8.3. Following dialysis, the proteins were oxidized by dropwise addition of oxidized glutathione to a final concentration of 0.5mM, dialyzed against 50mM NH₄HCO₃, cleaved overnight at room temperature with enterokinase, and purified to homogeneity by reverse phase-high pressure liquid chromatography (RP-HPLC).

Lyophilized ProTxII was resuspended to a concentration of 100 μ M in solution containing (in mM): 140 NMDG, 2CaCl₂, 4 MgCl₂, 10 Hepes, pH 7.4 with HCl. Toxin in solution was frozen in 20–25 μ l aliquots. Each aliquot was thawed for use and re-frozen no more than 5 times. For each experiment, dilutions to the desired concentration were made using the desired bath solution and immediately added to the chamber.

2.3. Electrophysiological recordings and analysis

All recordings were made using the whole cell voltage clamp configuration. Patch pipettes were pulled from thin-walled borosilicate (World Precision Instruments, Inc.) or microhematocrit (Fisher Scientific) glass capillaries using the Flaming/Brown micropipette puller P97 (Sutter Instruments) and had resistances of 0.8–1.5M Ω when filled with pipette solution. All recordings were made using an Axopatch 200 or 200B feedback amplifier (Molecular Devices) with a Digidata 1322A digitizer and pClamp 8.2 data acquisition software (Molecular Devices). Data were filtered at 10kHz using an 8-pole low-pass Bessel filter and sampled at 50kHz, or, for tail current recordings, filtered at 100kHz and sampled at 200kHz.

Recordings were made at room temperature (20–22°C) or after solution in chamber was cooled to 9–11°C as indicated in the figure legends. For experiments conducted in the cold, the bath was cooled using the Sontek TS-4 (Physitemp Instruments, Inc.) temperature controller device. Solution temperature was monitored with a YSI Tele-Thermometer using a YSI series 400 probe (YSI Integrated Systems & Services) throughout the experiment with the probe at the level of the cell.

Trypsinized cells suspended in media were added to the recording chamber and allowed to settle for 20–30 minutes. For the wash-in/wash-out experiments (Fig. 1), cells were recorded as either control bath solution or bath solution containing ProTxII was being perfused through the chamber as indicated in the figure legends. For all other experiments, control bath solution was allowed to perfuse over the cells for at least 3 minutes, after which perfusion was stopped prior to recording and temperature controller was switched on for experiments conducted at cold temperatures. Bath solutions containing high Ba²⁺ were perfused into the chamber following the 3 minute perfusion with control bath solution to avoid the formation of insoluble complexes between Ba²⁺ and the culture media. Toxin in bath solution (150–200 μ l) was added to the chamber with a pipette immediately after control bath was aspirated. Cells were allowed to sit in toxin for at least 3 minutes prior to recording. Following experiments in which toxin was used, the chamber bottom (glass coverslip) was removed and the chamber was rinsed thoroughly with distilled water and allowed to soak overnight.

During recording cells were kept at a holding potential of -140mV to ensure full channel availability. In all experiments recordings were made such that time since gaining access was consistent in order to minimize the influence of time dependent shifts in channel kinetics known to occur under these recording conditions (Fig. 2; Hanck and Sheets, 1992b). The depolarization protocol stepped to potentials ranging from -110 to $+35\text{mV}$ in 5mV increments for 50ms once every second. To record tail currents cells were depolarized to $+40\text{mV}$ and then repolarized to potentials ranging from -130 to 0mV . Duration of the initial step to $+40\text{mV}$ ($1.2\text{--}3\text{ms}$) was adjusted for each cell based on the time to peak current recorded at or near $+40\text{mV}$ in a step protocol immediately preceding the tail current protocol. The data were analyzed using locally written protocols in MATLAB (The Math Works, Inc.). Data were capacity-corrected using $8\text{--}16$ subthreshold responses (voltage steps of 10 or 20mV) and leak-corrected based on linear leak resistance calculated at potentials negative to -80mV or by linear interpolation between the current at the holding potential and 0mV . Junction potential was compensated for before touching the cell with the pipette. Leak- and capacity-corrected currents were filtered at 5kHz (step depolarizations) or 20kHz (tail currents) offline before analysis. All currents are shown leak- and capacity-corrected. Mean whole cell capacitance was $21\text{pF} \pm 9\text{pF}$. To ensure recordings were made with adequate voltage control, pooled data included only cells in which the slope factor of the voltage dependence of activation was $\geq 6.0\text{mV}$.

Curve fitting and statistical analyses were performed using the Origin software (OriginLab Corp) unless otherwise noted in the figure legends. Data from individual cells were fit and reported parameter values are averages $\pm$ S.E.M. of parameters from individual fits. A p -value of <0.05 was considered significant. Conductance-voltage relationships were fit using the Boltzmann equation:

$$y = \frac{A_1 - A_2}{1 + e^{(x-x_0)/dx}} + A_2 \quad (1)$$

where A_1 is the amplitude, A_2 the baseline, x the voltage, x_0 the half-point of the relationship, and dx the slope factor in mV . This equation was modified to take into account driving force and used to fit current-voltage relationships:

$$I = \frac{(x - V_{rev})^* G_{max}}{1 + e^{(x-x_0)/dx}} \quad (2)$$

where V_{rev} is the reversal potential and G_{max} is the maximum macroscopic conductance calculated from the slope of the linear portion of the current-voltage relationship negative to the reversal potential. The slope factor was converted to apparent charge (z) using the relationship

$$z = \frac{k_B T / q_e}{dx} \quad (3)$$

where k_B is Boltzmann's constant, T is temperature in $^\circ\text{K}$, and q_e is elementary charge.

Tail currents were trimmed to exclude the rising phase (always $<250\mu\text{s}$) and then fit using a single (4) or double (5) exponential decay function:

$$I = I_s + A_1 e^{-t/\tau} \quad (4)$$

$$I = I_s + A_1 e^{-t/\tau_1} + A_2 e^{-t/\tau_2} \quad (5)$$

where I is the macroscopic current, I_s the steady current, A_1 and A_2 the amplitudes, t the time, and τ_1 and τ_2 the time constants of current decay.

3. Results

3.1 Multiple distinct effects of ProTxII on activation

Previously it has been shown that a relatively high concentration of ProTxII (1 μ M) decreased peak current in Na v 1.5, positively shifted the voltage range of activation, and decreased the apparent voltage dependence of conductance (i.e. slope factor), with no accompanying effects on inactivation gating transitions (Middleton et al., 2002; Smith et al., 2007). Those experiments also showed that over a wide range of toxin concentrations ProTxII produced a reversible dose-dependent decrease in peak current measured at a single potential. Notably, in those experiments the contribution of changes in channel gating could not be distinguished from toxin-induced changes in macroscopic conductance.

We recorded currents in response to depolarization steps (see Methods) as bath solution containing ProTxII (40nM–200nM) was washed into and out of the recording chamber. Consistent with previous results at higher concentrations (Smith et al., 2007), toxin reversibly decreased peak currents at –10mV recorded from HEK 293 cells stably expressing Na v 1.5 (Fig. 1A). Also consistent with their suggestion that ProTxII inhibited activation, we re-analyzed their data with 1 μ M toxin to look for evidence of changes in the timecourse of current development. In those experiments 1 μ M ProTxII was perfused into the recording chamber while cells were pulsed to –30mV. There was a ~30% delay in time to peak current at steady state in those cells (Fig. 1B–C), consistent with inhibition of channel activation.

In order to identify the characteristics of block at low concentrations, we compared currents recorded in response to step depolarizations before, during, and after perfusion of the chamber with bath containing the indicated concentration of ProTxII (see Methods; Fig. 2). We observed a reversible decrease in the apparent voltage dependence of conductance (i.e. the slope factor, dx , from which the apparent charge, z , was determined). Upon perfusion of bath containing toxin the apparent charge decreased relative to pre-toxin controls by $-0.4e^- \pm 0.3e^-$ in 40nM ($N=5$ cells), by $-1.2e^- \pm 0.5e^-$ in 100nM ($N=5$ cells), and by $-1.2e^- \pm 0.3e^-$ in 200nM ($N=3$ cells). This change in apparent charge was distinct from the shifts in gating that occur with time (0.3–0.6mV/min; (Hanck and Sheets, 1992b)). Consistent with previous studies, we observed no change in current decay at potentials positive to –45mV, indicating no effect on inactivation from the open state, and there was no change in steady state channel availability, (data not shown).

Similarly, at 40nM, $G_{\max}$ was virtually unaffected by toxin (Fig. 2A, inset; mean change relative to pre-toxin controls was $11\% \pm 7\%$, $N=5$ cells). At 100nM cells being perfused with ProTxII displayed a greater decrease in $G_{\max}$ relative to pre-toxin controls (Fig. 2B, inset; mean change was $27\% \pm 9\%$, $N=5$ cells), and at 200nM ProTxII the effect on $G_{\max}$ was similar (Fig. 2C, inset; mean change relative to pre-toxin controls was $35\% \pm 3\%$, $N=3$ cells). Thus, the effect of ProTxII on the voltage dependence of conductance and on $G_{\max}$ displayed overlapping dose-dependencies.

At higher concentrations of toxin there was an additional positive shift in the voltage range over which channels activated (Fig. 3). In order to investigate these multiple kinetic effects of ProTxII on Na v 1.5 current we slowed channel gating by cooling to 9–11° C. Cooling did not have any obvious effect on the way ProTxII modified Na v 1.5. The effect of a very high concentration of ProTxII (5 μ M) on macroscopic Na v 1.5 currents was comparable at room temperature (Fig. 3A), and at colder temperatures (Fig. 3B). At room temperature 5 μ M ProTxII shifted the half-point ($V_{1/2}$) of activation positive by ~21mV ($V_{1/2C_{\text{ctrl}}} = -48.4\text{mV} \pm 2.0\text{mV}$; $V_{1/2T_x} = -27.3\text{mV} \pm 1.9\text{mV}$) and decreased apparent charge ($z_{C_{\text{ctrl}}} = 3.75e^- \pm 0.1e^-$; $z_{T_x} = 2.9e^- \pm 0.1e^-$). At colder temperatures ProTxII (5 μ M) positively shifted the range of activation by more than 20mV relative to the range observed in the absence of toxin (Table 1)

and $G_{\max}$ was markedly reduced compared to control (Fig. 3C and Table 1). A ProTxII-induced decrease in apparent charge, i.e. a toxin-induced decrease in the voltage dependence of conductance (Fig. 3D and Table 1) was also apparent at this concentration. It should be noted that the change in the voltage dependence of conductance we observed at low toxin concentrations (Fig. 2) produced a small positive shift in the $V_{1/2}$ of activation, but that the shift observed at $5\mu\text{M}$ toxin was much greater in magnitude than what would be expected secondary to the reduction in apparent voltage dependence. Thus, at micro-molar concentrations of ProTxII multiple effects on $\text{Na}_v1.5$ channel function were apparent: the range of activation shifted positively, the voltage dependence of activation was decreased, and $G_{\max}$ was reduced. There was no significant difference in the effect on $G_{\max}$, apparent charge, or the $V_{1/2}$ of activation in the presence of $1\mu\text{M}$ or $5\mu\text{M}$ ProTxII indicating these toxin effects had saturated (Fig. 3C–E). Previous studies have relied on changes in peak current at a single potential to track dose-dependent effects of ProTxII. However, changes in the voltage dependence of conductance, the range of activation and $G_{\max}$ will all affect current magnitude so that no single measure accurately captures toxin action making traditional dose-response relationships less useful.

Also consistent with multiple toxin actions, we were able to completely or partially reverse each of the three effects of ProTxII modification following depolarization for 250–1500ms. In the presence of ProTxII ($1\mu\text{M}$) a complete reversal of the reduction in slope factor and shift in $V_{1/2}$ of activation was observed for pre-pulses to $+10\text{mV}$ for 750ms or longer (Fig. 4). In contrast, the decrease in $G_{\max}$ was only partially, albeit rapidly reversible, i.e. $G_{\max}$ was not different following pre-pulses of 250–1500ms. Therefore, all data collected in the presence of toxin ($N=14$ cells) were pooled and normalized to control data in the absence of toxin. $G_{\max}$ recovered by 250ms to $67\%\pm 2\%$ of that expected based on currents recorded from control cells using the same protocols ($N=16$ cells).

Our analysis of currents elicited from step depolarizations suggested that ProTxII was altering voltage dependent activation transitions. However, inferring specific changes in gating from changes in conductance is difficult because many transitions contribute to the magnitude and timecourse of currents elicited by step depolarizations. Small fluctuations in voltage control secondary to changes in current magnitude can also independently affect the conductance-voltage relationship. By comparing tail current timecourse in the presence and absence of ProTxII, we could directly evaluate toxin-induced changes in activation/deactivation gating. In order to characterize the behavior of fully modified channels, we conducted all subsequent experiments in the presence of $5\mu\text{M}$ ProTxII, which is also roughly 100-fold above the reported IC_{50} for the toxin on this channel according to peak current inhibition measurements (Middleton et al., 2002).

Tail current relaxation is dominated at the most negative potentials (negative to -90mV) by channel deactivation, i.e. transitioning from the open to the closed state ($\text{O}\rightarrow\text{C}$). At potentials approaching the activation threshold for the channel, tail current timecourse reflects the net contribution of channels both deactivating and reactivating, and at relatively depolarized potentials current timecourse will also be greatly influenced by channels inactivating from the open state ($\text{O}\rightarrow\text{I}$). Tail currents were best fit with a single exponential function at relatively negative potentials, but were better fit by double exponential functions for potentials $\geq -60\text{mV}$. We and others have noted that ProTxII seemed to have no effect on inactivation from the open state. Thus, differences in tail current timecourse, particularly positive to the voltage at which channels activate would be expected to reflect the toxin's exclusive effect on activation and deactivation gating transitions and their interaction via mass action with the unaffected inactivation timecourse.

Tail current amplitudes were smaller in the presence of toxin as expected secondary to the decrease in $G_{\max}$ (Fig. 5A, left). In addition, tail current timecourse was faster in the presence of toxin. This was evident by eye when currents were normalized to their own peak amplitude and then superimposed (Fig. 5A, right). An analysis of variance (see Fig. 5B, legend) determined that the fast time constants of tail current relaxation were significantly faster in the presence of 5 μ M ProTxII when compared to control cells. This difference was smallest at very negative potentials and increased with voltage. Na^+ channels deactivate very quickly even at 10°C raising the concern that tail current timecourse may be difficult to distinguish from the decay of the capacity transient at very negative potentials. However, even at -130mV where current relaxation was fastest, the time constant of the capacity transients was 4–7 times faster than tail current timecourse (data not shown). The voltage dependence of the fast time constant at negative potentials was virtually flat in the presence of 5 μ M ProTxII compared to control cells (estimated voltage dependence of $1.0e^- \pm 0.1e^-$), consistent with the decrease in apparent charge of the channel suggested by the conductance-voltage relationship.

Because for concentrations at or below 200nM we had observed a change in the voltage dependence of conductance with minimal shift in the range of activation (Fig. 2), we further investigated the effect of low toxin concentrations (40nM, 100nM, and 500nM ProTxII) on tail current timecourse at potentials negative to -90mV. Tail current timecourse at negative potentials was abbreviated at each concentration (Fig. 5C) and was most dramatic at the positive end of the voltage range (i.e. -90mV; Fig. 5C, inset). At higher toxin concentrations (i.e. 5 μ M), tail current timecourse was even faster consistent with the additional effect of a positive shift the range of activation (Fig. 5C, inset). In addition, the second, long time constants of tail current relaxation, which were resolved at potentials ≥ -60 mV, were also faster in the presence of 5 μ M ProTxII, which was consistent with the shift in the range of activation (Fig. 5D).

3.2. Evidence for electrostatic interactions of ProTxII with $\text{Na}_v1.5$

Concentrations of toxin >500nM induced a positive shift in the range of activation, which was reminiscent of a surface charge effect—the well documented phenomenon by which elevated concentrations of extracellular divalent cations positively shift the voltage range of activation via neutralization of charges on the extracellular surface of the channel and/or on proximal headgroups that influence the voltage drop across the membrane experienced by the voltage sensors (summarized in Hille, 2001). Because ProTxII carries a net +4 charge and because this toxin is known to interact with membrane phospholipids (Smith et al., 2005), we investigated the possibility that the shift in the range of activation we observed resulted from electrostatic interactions between the toxin and the channel close to the channels' voltage sensors. To do this we added Ba^{2+} to the extracellular bath in order to preemptively neutralize surface charge and then tracked changes in channel gating upon addition of 5 μ M ProTxII. Ba^{2+} is known to screen and/or bind to surface charges in this channel and, in contrast to other divalent and trivalent cations, it blocks current in Na^+ channels relatively poorly (Sheets and Hanck, 1992). We were also concerned that Ba^{2+} might complex in solution with ProTxII thereby lowering its effective concentration, but circular dichroism spectra of the ProTxII peptide measured in the presence and absence of 20mM Ba^{2+} indicated no effect of the Ba^{2+} on toxin structure (data not shown), thus making changes in the toxin's effects due to physical interactions with Ba^{2+} itself unlikely.

Ba^{2+} at 20mM shifted the $V_{1/2}$ of activation positive by ~23mV (Fig. 6 and Table 1). This Ba^{2+} concentration had the advantage that it maximized its effect on surface charge while minimizing the effect of voltage-dependent block (Hanck and Sheets 1992a, Sheets and Hanck, 1992). There was a significant decrease in $G_{\max}$ in the presence of 20mM Ba^{2+} , as well as a decrease in the apparent voltage dependence of activation (Table 1), which probably reflected a contribution of voltage-dependent block at this concentration. However, in order to ascertain

whether the presence of Ba^{2+} changed the way ProTxII modified the channel, it was only necessary that G_{max} and z be significantly different from that which was observed in toxin alone, which was true in both cases.

For each effect of ProTxII modification (i.e. reduction in voltage dependence of conductance, positive shift in the range of activation, reduction in G_{max} , and faster tail current relaxation timecourse), we examined the extent to which the presence of Ba^{2+} disrupted toxin action. Normalized conductance-voltage curves are shown to illustrate the effects of Ba^{2+} on the apparent voltage dependence of conductance and the $V_{1/2}$ of activation (Fig. 7A). In the presence of Ba^{2+} , ProTxII produced a statistically significant ($p < 0.05$) decrease in the apparent voltage dependence of conductance compared to Ba^{2+} alone (Fig. 7B, right bars). The magnitude of this decrease was roughly half of the magnitude of the decrease observed in the absence of Ba^{2+} (Table 1). We also found that the apparent voltage dependence of conductance in the presence of both Ba^{2+} and toxin was significantly greater than what was observed in toxin alone ($p < 0.05$; Fig. 7B, black bars and Table 1). Thus, the decrease in the magnitude of the effect was not just a consequence of the lower initial voltage dependence produced by Ba^{2+} alone, but instead suggested that this particular effect of ProTxII was partially disrupted by Ba^{2+} .

We still observed a toxin-induced positive shift in the $V_{1/2}$ of activation in the presence of 20mM Ba^{2+} , although the magnitude of this effect was also smaller compared to the shift seen in the absence of Ba^{2+} (Fig. 7C, right bars and Table 1). This shift was statistically significant ($p < 0.05$), although Ba^{2+} alone positively shifted $V_{1/2}$, toxin-modified channels displayed a significantly more positive $V_{1/2}$ in the presence of Ba^{2+} than in its absence, ($p < 0.05$; Fig. 7C, black bars and Table 1), suggesting that the largest part of the toxin-induced shift was independent of the electrostatic effect of Ba^{2+} . In contrast to these partial effects, ProTxII (5 μM) no longer reduced G_{max} in the presence of Ba^{2+} (Fig. 7D, right bars and Table 1).

We next examined the timecourse of tail current relaxation for toxin-induced changes in the presence of 20mM Ba^{2+} . Block of $\text{Na}_V1.5$ by Ba^{2+} is very fast and thus would not affect the kinetics of tail current relaxation (Sheets and Hanck, 1992). Consistent with this, changes in the time constant of tail current relaxation in the presence of Ba^{2+} alone were completely accounted for when the shift in activation apparent in the conductance-voltage relationship (Fig. 6) was taken into account (data not shown). Therefore, we were able to determine whether Ba^{2+} disrupted the toxin-induced effects on tail current timecourse. At very negative potentials (negative to -100mV) there was no toxin-induced effect on tail current timecourse in the presence of Ba^{2+} (Fig. 8A). At more positive potentials tail current time constants in the presence and absence of toxin diverged as was seen in the absence of Ba^{2+} (Fig. 5B). The second, longer, tail current time constant was also faster in the presence of both ProTxII and Ba^{2+} (Fig. 8B). Taken together, the change in voltage dependence and the increase in the toxin-induced effect on tail current relaxation at more positive potentials supported the idea that the toxin was likely affecting gating transitions along the activation pathway distal to the final opening step.

To test whether the residual effects of ProTxII on tail current timecourse were a result of incomplete neutralization of surface charge, we increased the extracellular concentration of Ba^{2+} to 40mM. At this Ba^{2+} concentration the effect of toxin on voltage dependent gating could not be determined from current-voltage relationships, since in this case 40mM Ba^{2+} produced voltage dependent block that extended into the range of channel activation. The increase in Ba^{2+} concentration did shorten the time constant of tail current relaxation consistent with a further shift in activation. Addition of ProTxII (5 μM) in the presence of 40mM Ba^{2+} produced no further change in tail current timecourse at very negative potentials (negative to -90mV) while the divergence in tail current time constants at more positive potentials persisted (Fig.

8C). Interestingly, toxin speeded tail currents to the same extent regardless of Ba^{2+} concentration (Fig. 8D). These data suggested that the effect on the closing transition ($\text{O} \rightarrow \text{C}$) was restricted to the toxin's surface charge-like effect.

4. Discussion

In this study we have identified multiple effects of ProTxII on macroscopic currents in $\text{Na}_V1.5$. Previous reports have used measurements of peak current to track channel modification and measure toxin affinity. However, peak current, like most macroscopic current metrics, is a complex variable reflecting net changes in channel gating and pore conductance. Our data showed that ProTxII has multiple, distinct effects on $\text{Na}_V1.5$ channels. This was evidenced by changes the current- and conductance-voltage relationships including a positive shift in the voltage range of activation, modification of gating transition rates along the activation pathway, and a decrease in G_{max} . All three effects contribute to changes in peak current, making conventional assays of toxin affinity using dose-response relationships less useful. Data collected at various concentrations of ProTxII showed that the toxin-induced decrease in the voltage dependence of conductance and G_{max} occurred at concentrations for which little or no positive shift in the range of activation was observed. This observation was most consistent with independent toxin binding events at multiple binding sites with different affinities.

Changes in tail current timecourse reflect the net contribution of channel deactivation (at very negative potentials) and reactivation (at more positive potentials) transition rates. Thus, analysis of tail current timecourse allowed us to further localize the effects of ProTxII to activation gating transitions. We observed faster tail current timecourse in the presence of very low concentrations of toxin which supported the idea that the decrease in the voltage dependence of conductance was indeed a result of activation gating modification.

Changes in tail current timecourse in the presence of ProTxII also indicated that at least some toxin-modified channels are able to activate. This is significant since channel drop-out could explain both the decrease in G_{max} and the shift in the range of activation. The fact that we are able to observe perturbed gating kinetics suggests that if such a population of permanently closed toxin-modified channels is responsible for those changes in the macroscopic current, it is distinct from a population of channels that are able to activate, albeit with altered gating properties. Similar changes in voltage dependent activation and G_{max} have been observed in voltage-gated K^+ channels in the presence of Hanatoxin, another peptide toxin isolated from spider venom that shares extensive sequence homology with ProTxII (Priest et al., 2007). Because Na^+ channels are not four-fold symmetric, it is not surprising that ProTxII-induced changes in gating are more complex. Like Hanatoxin-bound K^+ channels, at least some ProTxII-modified $\text{Na}_V1.5$ channels are able to activate as evidenced by toxin-induced changes in tail current timecourse. Hanatoxin binds to and impedes the movement of the voltage sensor in K^+ channels (Swartz and MacKinnon, 1997a,b; Li-Smerin et al., 2000; Lee et al., 2005; Phillips et al. 2005), suggesting that interactions between ProTxII and the voltage sensors of voltage-gated Na^+ channels are likely as well given the effects of the toxin on the voltage dependence of conductance and on tail current relaxation kinetics. Consistent with this idea, Sokolov et al. (2007) noted a decrease in total gating charge in the presence of ProTxII in the $\text{Na}_V1.2$ isoform.

To further explore the possibility of interactions between ProTxII and $\text{Na}_V1.5$ close to its voltage sensors, we checked for evidence of an electrostatic (surface charge-like) mechanism causing the shift in the range of activation we observed at concentrations above 200nM. We took advantage of the fact that by adding Ba^{2+} to the extracellular bath we would screen/bind surface charge, which would produce an apparent shift in gating without any direct changes to

the gating transitions themselves. In these experiments we were able to distinguish between toxin effects that were sensitive to the presence of Ba^{2+} , i.e. surface charge-like effects, and others that were insensitive and, therefore, surface charge-independent.

Tail current timecourse analysis revealed evidence of both types of effects. Tail currents at negative potentials responded to the surface charge agent, Ba^{2+} only in the absence of toxin. The simplest explanation for this finding is that Ba^{2+} and ProTxII “compete” for a site(s) that electrostatically affects the voltage sensors. The affinity of ProTxII is clearly much greater for such sites as toxin (5 μM) speeds tail current relaxation to the same extent even in the presence of 40mM Ba^{2+} . We also observed a persistent, toxin-induced, divergence in tail current timecourse at potentials positive to -90mV . Ba^{2+} alone did not produce such divergence indicating that this particular toxin effect is independent of its surface charge-like action. Changes in tail current time constants that cannot be accounted for by a shift in gating, reflect a perturbation of at least one activation gating transition. The fact that such changes were only apparent at more positive potentials indicates that the toxin is affecting gating transitions along the activation pathway distal to the final opening step.

Changes in activation due to surface charge effects and gating perturbations will all influence the voltage dependence of conductance. This explains the more complicated effect adding Ba^{2+} had on this relationship. If the shift of the conductance voltage relationship were due entirely to the surface charge-like effects of ProTxII we would expect data in the presence of toxin to overlay regardless of Ba^{2+} concentration, as was the case for tail current timecourse. However, this is not what we observed. The shift in the $V_{1/2}$ of activation in the presence of both Ba^{2+} and ProTxII was largely additive reflecting the contribution of surface charge-like and surface charge-independent toxin actions. The extent to which the effects of Ba^{2+} and toxin were not additive most likely reflects the toxin’s surface charge-like effect.

ProTxII’s effect on G_{max} was also sensitive to the presence of Ba^{2+} indicating competition between the two agents. However, in contrast to the toxin’s effect on tail current timecourse, Ba^{2+} precluded any further decrease in G_{max} upon addition of ProTxII. The ability of Ba^{2+} to directly out-compete toxin in this case is unlikely since the effective concentration of toxin is nearly two orders of magnitude lower than the effective concentration of Ba^{2+} . Also consistent with a high affinity toxin effect on G_{max} , two-thirds of the toxin-induced decrease in G_{max} was recovered within 250ms of depolarization. Ba^{2+} binds to and voltage dependently blocks ion conduction through the pore in $\text{NaV}1.5$. However, it seems unlikely that Ba^{2+} and ProTxII would be directly competing for a binding site within the pore, as toxin effects on activation gating and the relief of toxin modification with depolarization suggested an interaction site at the voltage sensors, which are far from the central pore (Jiang et al., 2003; Lee et al., 2005; Long et al., 2005; Long et al., 2007). Furthermore, experiments conducted in $\text{NaV}1.2$ in the presence of TTX, a known pore blocker, showed a ProTxII-induced decrease in gating charge, which indicates ProTxII is able to interact with that channel in the presence of TTX (Sokolov et al., 2007). The effect of ProTxII on that particular isoform appeared to be limited to a decrease in G_{max} , lending further support to the idea that whatever the mechanisms of this effect, it is independent of TTX binding to the pore. As an alternative to direct competition between ProTxII and Ba^{2+} for a binding site within the permeation pathway, Ba^{2+} may indirectly disrupt a toxin binding site(s) via its interactions with the channel, thus precluding yet another distinct toxin action. These data also support the idea of a gating mechanism underlying the toxin-induced decrease in G_{max} and more strongly suggest that the loss of current reflects stabilization of one or more native closed states or the introduction of an additional absorbing non-conducting state that dose-dependently sequestered channels outside of the activation pathway.

Our data showed multiple effects on $\text{NaV}1.5$ activation gating, including an effect on G_{max} , which differed in their affinity and mechanism. In contrast, modification of the $\text{NaV}1.2$ isoform

by ProTxII resulted only in a decrease in $G_{\max}$ (Sokolov et al., 2007). This difference in ProTxII's effects between channel isoforms also are consistent with multiple binding sites. Interestingly, studies of β -scorpion toxins have revealed evidence of multiple toxin-channel interaction sites that together determine the characteristics of gating modification and toxin affinity that vary among isoforms (Leipold et al., 2006). Perhaps there is a similar basis for the complexity of ProTxII action on $\text{Na}_V1.5$ and the differences in toxin effects among Na^+ channel isoforms (Middleton et al., 2002; Sokolov et al., 2007). Because the domains of these channels are non-identical, even analogous binding sites could vary in their toxin affinities and would be expected to produce very different consequences for channel gating. Mutagenesis experiments in $\text{Na}_V1.2$ suggested that ProTxII action in that channel is controlled by binding close to the domain II voltage sensor (Sokolov et al., 2007) making it likely that at least one interaction site for ProTxII on $\text{Na}_V1.5$ is in this domain. Interactions with the domain IV voltage sensor seem highly unlikely since movement of that voltage sensor is closely linked to inactivation from the open state (Cha et al., 1999; Sheets and Hanck, 2005), and there is no evidence for toxin interference with that transition. If indeed ProTxII binds to multiple domains, then the remaining candidates (assuming one interaction site on domain II) would be domain I and domain III, both of which have been closely linked to activation (Cha et al., 1999). Because of its exclusive effects on channel activation, determining the binding site(s) and mechanism(s) of action of ProTxII promises to help elucidate differences in gating between channel isoforms and channel types that underlie characteristics matching channel activity to physiological function.

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Fig. 1. ProTxII inhibits peak current and slows current development in Nav1.5

(A) Peak currents recorded from a representative cell during 20ms depolarizations to -10mV from a holding potential of -140mV at a 1Hz frequency as bath solution containing 200nM ProTxII was washed on (left) and off (right). Inset: Representative current traces. (B) Representative current traces recorded from the same cell in response to a depolarization step to -30mV from a resting potential of -140mV before (gray) and after (black) perfusion of $1\mu\text{M}$ ProTxII. Each trace is normalized to its own peak to allow for comparison of current development timecourse. (C) Currents were recorded in response to a depolarization step to -30mV from a resting potential of -140mV applied at a 1Hz frequency as $1\mu\text{M}$ ProTxII was perfused through the chamber. Time to peak current is normalized to time to peak current before application of toxin. (B) and (C) are a reanalysis of previously presented data (Smith et al., 2007). N=15 cells.

Fig. 2. ProTxII modifies voltage dependent activation reversibly at concentrations $\leq 200\text{nM}$
 Peak current was measured during step depolarizations (see Methods) before (open squares), during (filled circles), and after (open triangles) perfusion of bath containing 40nM (A), 100nM (B), and 200nM (C) ProTxII at room temperature. Each panel shows data from one cell of 3–5 cells studied in this way. In each case wash-out was complete, i.e. G_{max} returned to pre-toxin control values. Note that control gating after wash-out occurred over a more negative voltage range as expected for the background shift in kinetics that occurs in this isoform (Hanck and Sheets, 1992b). Solid lines are fits to the data using a modified Boltzmann equation (2). Insets: Conductance-voltage relationships of currents recorded before (black squares) and during (gray triangles) toxin perfusion (40nM ProTxII: Change in G_{max} for this cell during toxin

perfusion ($\Delta G_{\max}$)=0.1nS; 100nM ProTxII: $\Delta G_{\max}$ =5.3nS; 200nM ProTxII $\Delta G_{\max}$ =7.7nS). Solid lines are fits using the Boltzmann equation (1). For all insets: x-axis is from -125mV to +25mV in 60mV increments and y-axis is from -2nS to +40nS in 10nS increments.

Fig. 3. Gating modification by ProTxII

(A) Mean $\pm$ S.E.M. of normalized conductance in the absence (open squares, $N=5$ cells) and presence of 5 μ M toxin (black circles, $N=6$ cells) at room temperature determined as described in the Methods. Solid line is fit of mean data using the Boltzmann equation (1). (B) Normalized conductance as in (A) except at 9–11 $^{\circ}$ C for 11 control cells and 5 cells in 5 μ M toxin. Inset: Traces from representative cells in the absence (upper) and presence of 5 μ M ProTxII (lower) in response to step depolarizations ranging from -75 mV to $+20$ mV. (C) Mean $\pm$ S.E.M. fraction of G_{max} remaining for cells studied in the presence of 1 μ M (gray bars, $N=7$ cells) and 5 μ M toxin (black bars) at 9–11 $^{\circ}$ C shown normalized in (B). Fractions were determined for individual cells relative to mean G_{max} of control cells recorded in identical ionic conditions. Fraction of G_{max} remaining in 1 μ M and 5 μ M were not statistically different from each other using a student's t -test, but reflected a statistically significant decrease from control G_{max} in both cases. (D) Mean $\pm$ S.E.M. of apparent charge (z) for the same cells. (E) Mean $\pm$ S.E.M. of $V_{1/2}$ of activation for the same cells. Apparent charge and $V_{1/2}$ at 1 μ M and 5 μ M were not statistically different from each other, but were statistically different from control.

Fig. 4. Complete or partial reversal of all three effects of ProTxII modification with depolarization Currents were recorded in the presence of $1\mu\text{M}$ ProTxII in response to a modified step depolarization protocol (shown at top) in which each sweep included a pre-pulse of varying duration (t_c) to $+10\text{mV}$ and a 20ms recovery step to the holding potential (-140mV) before the standard 50ms depolarization step to various potentials (see Methods). Currents in response to a standard step depolarization protocol were recorded in the same cell immediately before or after the pre-pulse protocol. Normalized conductance-voltage relationships of a representative cell in response to pre-pulse protocols (gray circles) with pre-pulse durations of 250ms (top panel), 500ms (middle panel), and 750ms (bottom panel), and in response to standard step depolarization protocol recorded immediately before or after each pre-pulse

protocol (black circles). In each case data from this cell are compared to mean normalized conductance-voltage relationships from control cells in response to the standard step depolarization protocol (open squares). Recordings in the absence of toxin were made at approximately the same time following break-in as the corresponding recordings in the presence of toxin in order to control for time-dependent shifts in gating. Solid lines are fits to the data using the Boltzmann equation (1).

Fig. 5. Modification of tail current timecourse by ProTxII

(A, left) Representative families of tail currents (see Methods) recorded in the absence (top) and presence (bottom) of 5 μ M ProTxII at 9–11°C. Currents were filtered to 10 kHz and downsampled by 10 offline for display purposes. (A, right) Currents at -90 mV (top) and -50 mV (bottom) recorded in the absence (black) and presence (gray) of 5 μ M ProTxII were normalized to their own peak amplitude and superimposed. (B–D) Tail currents were fit with equation (4) or (5) (see Methods). (B) Mean $\pm$ S.E.M. of short time constants for tail currents recorded in the absence (open squares, N=3 cells) and presence (filled circles, N=4 cells) of 5 μ M ProTxII. Analysis of variance was performed on data in the absence and presence of toxin using SAS GLM accounting for repeated measures and the difference between the data sets

was found to be significant ($p < 0.05$). (C) Mean $\pm$ S.E.M. of short time constants for tail currents recorded in presence of 40nM (gray circles, N=5 cells), 100nM (gray triangles, N=5 cells), and 500nM ProTxII (black inverted triangles, N=5 cells). Data in the absence of toxin (open squares) are replotted from (B). Inset: Time constant at -90mV for all toxin concentrations. (D) Mean $\pm$ S.E.M. of long time constants for tail currents recorded at potentials $\geq -60\text{mV}$ in the same cells. Same symbols as in (B). Statistical analysis was performed as described in (B) and the difference between the two data sets was found to be significant ($p < 0.05$).

Fig. 6. Shift in gating in the presence of extracellular Ba^{2+}

(A) Mean $\pm$ S.E.M. of peak current recorded from step depolarizations (see Methods) in the absence (open squares) and presence (open triangles, $N=6$ cells) of 20mM extracellular Ba^{2+} at 9–11°C. Data in the absence of Ba^{2+} are replotted from Fig. 2D. (B) Mean $\pm$ S.E.M. of normalized conductance in the same cells. Same symbols as in (A). Solid lines are fits to the mean data using the Boltzmann equation (1). See Table 1 for means of fit parameters.

Fig. 7. Surface charge neutralization by extracellular Ba^{2+} and the effects of ProTxII on conductance

(A) Mean $\pm$ S.E.M. of normalized conductance recorded at $9\text{--}11^\circ\text{C}$ in the presence of 20mM Ba^{2+} and in the absence (open triangles) and presence (black triangles, $N=12$ cells) of $5\mu\text{M}$ ProTxII. Solid lines are fits to the mean data using the Boltzmann equation (1). Data in the absence of toxin are replotted from Fig. 6B for ease of comparison. (B) Mean $\pm$ S.E.M. of apparent charge (z) for cells shown normalized in (A). Data in the absence of toxin (white bars) were compared to data in the presence of $5\mu\text{M}$ ProTxII (black bars) in both the absence (left bars) and presence of 20mM extracellular Ba^{2+} (right bars). (C) Mean $\pm$ S.E.M. of $V_{1/2}$ of

activation for the same cells. (D) Mean $\pm$ S.E.M. of $G_{\max}$ for the same cells. Results of statistical tests for pairwise comparisons of these data are reported in the text.

Fig. 8. Surface charge neutralization effects on ProTxII tail currents

Tail currents were recorded at 9–11°C in the presence of 20mM Ba^{2+} and fit with equation (4) or (5). (A) Mean $\pm$ S.E.M. of short time constants for tail currents recorded in the absence (open triangles, N=5 cells) and presence (filled triangles, N=10 cells) of 5 μ M ProTxII. Analysis of variance was performed on data in the absence and presence of toxin using SAS GLM accounting for repeated measures. The difference between the data sets was found to be significant ($p<0.05$). (B) Mean $\pm$ S.E.M. of long time constants measured in the same cells. Same symbols as in (A). Statistical analysis of the data was performed as described in (A) and the difference between the two data sets was found to be significant ($p<0.05$). (C) Extracellular bath concentration of Ba^{2+} was increased to 40mM, and tail currents were recorded in the

absence (open diamonds, N=5 cells) and presence of 5 μ M ProTxII (filled diamonds, N=4 cells). Statistical analysis of the data was performed as described in (A) and the difference between the two data sets was found to be significant. (D) Short time constants of tail currents recorded in the presence of 5 μ M ProTxII are replotted for 0mM (circles), 20mM (triangles) and 40mM Ba²⁺ (diamonds). Data were not statistically different.

Table 1
Summary of mean data from fits to current- and normalized conductance-voltage relationships of individual cells recorded at 9–11°C as described in the figure legends.

[Ba ²⁺] _o (mM)	[ProTxII] _o (μM)	V _{1/2} (mV)	ΔV _{1/2} (mV)	z (e ⁻)	Δz (e ⁻)	G _{max} (nS)	Fraction of G _{max}	N
0	0	-46.8±1.4	n/a	3.3±0.1	n/a	26.9±6.2	n/a	11
0	1	-24.1±3.0	22.7±3.3 ^a	1.8±0.1	-1.5±0.1 ^a	5.2±1.2 ^d	0.5±0.1 ^e	7
0	5	-21.0±2.2	25.8±2.6 ^a	1.9±0.1	-1.4±0.1 ^a	7.7±1.7	0.3±0.1 ^a	5
20	0	-24.1±0.1	22.7±1.4 ^b	2.8±0.1	-0.5±0.1 ^b	15.9±2.8	0.6±0.1 ^b	6
20	5	-8.4±1.6	15.7±1.6 ^c	2.2±0.1	-0.6±0.1 ^c	12.6±2.2	n/a ^f	12

^a Difference between mean value of control cells and cells recorded in the presence of ProTxII only.

^b Difference between mean value of control cells and cells recorded in the presence of 20mM Ba²⁺ only.

^c Difference between mean value of cells recorded in the presence of 20mM Ba²⁺ alone and cells recorded in the presence of both 20mM Ba²⁺ and ProTxII.

^d Cells recorded in bath containing 5mM extracellular NaCl (see Methods).

^e Fraction calculated relative to mean G_{max} of control cells recorded under identical ionic conditions.

^f There was no significant difference in mean G_{max} in this case.