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Inhibition of the activation pathway of the T-type calcium channel $\text{Ca}_v3.1$ by ProTxII

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Abstract

Toxins have been used extensively to probe the gating mechanisms of voltage-gated ion channels. Relatively few such tools are available to study the low-voltage activated T-type Ca channels, which underlie thalamic neuron firing and affect sleep, resistance to seizures, and weight gain. Here we show that ProTxII, a peptide toxin recently isolated from the venom of the tarantula spider *Thrixopelma pruriens*, dose-dependently inhibited $\text{Ca}_v3.1$ causing a decrease in current ($81.6\% \pm 3.1\%$ at -30 mV in $5 \mu\text{M}$ toxin) and a positive shift in the voltage range of activation ($+34.5$ mV ± 4.4 mV). Toxin-modified currents were slower to activate and faster to deactivate and they displayed a longer lag in the onset of current, i.e. the Cole-Moore shift, consistent with the inhibition of gating transitions along the activation pathway, particularly the final opening transition. Single channel current amplitude and total gating charge were unaffected by toxin, ruling out a change in ion flux or channel drop-out as mechanisms for the decrease in macroscopic conductance. A positive shift in the voltage range of gating charge movement ($+30.6$ mV ± 2.6 mV shift in the voltage of half maximal charge movement in the presence of $5 \mu\text{M}$ toxin) confirmed that ProTxII-induced gating perturbations in this channel occur at the level of the voltage sensors, and kinetic modeling based on these findings suggested that reductions in current magnitude could be largely accounted for by kinetic perturbations of activation.

Keywords

T-type Calcium Channel; ProTxII; voltage gated; gating modifier toxin

1. Introduction

The three cloned T-type Ca channel isoforms $\text{Ca}_v3.1$, 3.2 , and 3.3 make up the family of low threshold activation (LVA) voltage-gated Ca channels. These channels have a broad tissue distribution and play important roles in contributing to depolarization and the regulation of Ca influx. T-type Ca channels gate in response to changes in membrane potential and these channels, in particular, are thought to produce a steady influx of Ca near the resting membrane potential (for review see: Cueni et al., 2009). This allows T-type channels to contribute to the generation of low-threshold Ca spikes, pacemaking, rebound

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burst firing, and low amplitude Ca oscillations (for review see: Contreras, 2006; Perez-Reyes, 2003). Abnormal expression and gating of T-channels has been linked to epilepsy, cardiac arrhythmias, sleep disorders, and abnormal weight gain (Anderson et al., 2005; Kim et al., 2001; Lee et al., 2004; Mangoni et al., 2006; Song et al., 2004; Uebele et al., 2009). However, little detail is known about the structure and function that underlies their gating.

Toxins have been valuable tools for exploring the structural basis of gating mechanisms in voltage dependent ion channels. By interfering with conformational changes during gating, toxins are able to modify activation and/or inactivation transitions, effectively altering ion conductances mediated by channels within physiological voltage ranges. Several gating modifier toxins are known to target voltage-gated K and Na channels, and discoveries of their specific toxin-channel interaction sites have led to important insights into the structure, function, and coordination of the voltage sensors across channel types. For example, the finding that sea anemone toxins modified gating in Na channels by binding to the S3–S4 linker of domain IV (DIV) was crucial to understanding the unique role of the DIV voltage sensor in fast inactivation from the open state in these channels (Hanck and Sheets, 2007). The β -scorpion toxins, on the other hand, bind primarily to the domain II voltage sensor and enhance activation by shifting the voltage dependence of activation and steady state inactivation to more negative potentials (Cestele et al., 1998; de la Vega and Possani, 2007). The α -scorpion toxin kurtosin, is one of only a few gating modifier toxins that are known to interact with T-type Ca channels and its mechanism remains unknown (Chuang et al., 1998; Sidach and Mintz, 2002).

ProTxII, a recently isolated tarantula toxin, has been identified as a gating modifier toxin with a high affinity for several different isoforms of voltage-gated Na channels (Middleton et al., 2002). ProTxII is a 30 amino acid peptide found in the venom of *Thrixopelma pruriens*. Its secondary structure likely conforms to the inhibitory cysteine knot (ICK) motif common to many peptide toxins (Middleton et al., 2002; Priest et al., 2007). For Na channels the toxin has been reported to reduce peak current, and/or to inhibit activation by shifting voltage dependent activation to more positive potentials and decrease the voltage dependence of activation (Edgerton et al., 2008; Middleton et al., 2002; Smith et al., 2007). In at least one Na channel isoform, Nav1.2, ProTxII decreased total gating charge (Sokolov et al., 2007). Interestingly, inactivation is unaffected by ProTxII in these channels suggesting that the toxin targets regions of the channel that independently regulate activation. The related toxin, ProTxI, isolated from the same organism, has been shown to affect voltage-gated Na and K channels as well as the T-type Ca channel Cav3.1 (Middleton et al., 2002). However, the sequences of ProTxI and ProTxII are highly dissimilar, sharing only three residues other than the cysteines involved in disulfide bonds characteristic of the inhibitory cysteine knot (ICK) backbone motif. As a toxin that modifies other channels for which more extensive literature on gating mechanisms is available, ProTxII has the potential to be a useful tool in studying T-type Ca channel activation. Specifically, a comparison of the ways ProTxII interacts with voltage-gated Na and T-type Ca channels will provide insight into the similarities and differences in gating mechanisms among these channels.

We find that ProTxII dose-dependently positively shifts the voltage range of activation and decreases maximum macroscopic conductance ($G_{\max}$) in Cav3.1. Our data indicate that the decrease in conductance is, for the most part, secondary to toxin-induced slowing of activation gating transitions all along the activation pathway, rather than a reduction in single channel conductance or channel dropout, and that the shift in voltage-dependent activation occurs at the level of the voltage sensors. Some of these results have been presented in abstract form (Edgerton et al., 2009b).

2. Materials and Methods

2.1 Heterologous Expression

Ca_v3.1 exists in a number of splice variants (Emerick et al., 2006). Here we used the 217 isoform because it is one of the two most prevalent isoforms of Ca_v3.1 in the human adult brain. It only differs from the other highly abundant 89 isoform by 23 amino acids in the domain II–III linker (Emerick et al., 2006). The cDNA for the Ca_v3.1 (splice variant 217) was kindly provided by M.C. Emerick and W.S. Agnew (The Johns Hopkins University School of Medicine, Baltimore, MD). It was subcloned into the mammalian expression vector pcDNA3.1/Zeoicin and stably expressed in HEK-293 cells. Stable cell lines were created using Zeoicin (Invitrogen, Carlsbad, CA) selection (200 µg/ml for selection, 100 µg/ml maintenance). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen), 1% penicillin-streptomycin (Invitrogen), and selection antibiotic in 60–100 mm culture dishes (Corning, Corning, NY). The use of stable cell lines meant that channel expression levels were relatively consistent, thus minimizing variation in current magnitude across experiments. Whole cell and cell attached patch voltage clamp was performed on trypsinized cells (0.25% trypsin-EDTA, Invitrogen or Sigma-Aldrich, St. Louis, MO), 3–6 days after plating.

2.2 Solutions and Chemicals

2.2.1 Whole cell recordings—For the experiments at room temperature (21–23 °C) the solutions used contained either (in mM): 140 NaCl, 2 CaCl₂, 10 HEPES titrated to pH 7.4 with NaOH (bath 1) and 130 NaCl, 1 CaCl₂, 5 Mg ATP, 11 EGTA, 10 HEPES, titrated to pH 7.4 with NaOH (pipette 1), or 140 NaCl, 5 BaCl₂, 10 HEPES, titrated to pH 7.4 with NaOH (bath 2) and 140 NaCl, 1 CaCl₂, 11 EGTA, 10 HEPES, titrated to pH 7.4 with NaOH (pipette 2). To prevent hydrolysis of the ATP, pipette solution (1) was kept frozen in 1 mL aliquots and a new aliquot was thawed for each day of recording. For experiments carried out at 9–11 °C, Ca_v3.1 currents were recorded with Ba as the permeant ion (bath 2). When Ba replaced Ca in the extracellular solution (bath 2) the 5 mM Mg ATP was removed (pipette solution 2) to prevent the formation of insoluble complexes with the Ba.

2.2.2 Single channel recordings—Bath solution contained (in mM) 150 KCl and 10 HEPES, titrated to pH 7.4 with KOH to depolarize cells to 0 mV for cell-attached patch recording. The pipette solution contained (in mM): 20 CaCl₂ or 60 BaCl₂, 150 or 50 TEACl, and 10 HEPES, titrated to pH 7.4 with TEAOH. For recordings made in the presence of ProTxII, toxin was diluted in pipette solution to desired concentration and kept on ice over the course of recording.

2.2.3 Gating current recordings—Bath solution contained (in mM): 140 NMDG·MES, 11 EGTA, 1 CaMES, 10 HEPES, titrated to pH 7.4 with MES. The pipette solution contained (in mM): 140 NMDG·MES, 1 EGTA, 10 HEPES, titrated to pH 7.4 with MES.

2.2.4 Toxin preparation—ProTxII 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) (Smith et al., 2005). Fusion protein was purified on Ni²⁺-nitrilotriacetic acid resin, reduced with 10 mM dithiothreitol, diluted to 0.2 mg/mL, and dialyzed against 2.5 mM glutathione, 50 mM Tris, 100 mM NaCl, pH 8.3. Following dialysis, the proteins were oxidized by dropwise addition of oxidized glutathione to a final concentration of 0.5 mM, dialyzed against 50 mM NH₄ HCO₃, cleaved overnight at room temperature with enterokinase and purified to homogeneity by RP-HPLC.

Lyophilized ProTxII was resuspended to a concentration of 100 μM in either purified water or solution containing (in mM): 140 NMDG, 2 CaCl_2 , 4 MgCl_2 , 10 Hepes, pH 7.4 with HCl. Toxin in solution was frozen in 10–30 μl 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. Previous work has shown that both synthetic (Middleton et al., 2002) and recombinant (Smith et al., 2005) ProTxII have nearly identical effects on voltage-gated Na^+ channel targets. For the experiments described below recombinant protein was used exclusively.

2.3 Electrophysiological Recordings and Analysis

All recordings were made using an Axopatch 200 or 200B feedback amplifier (Molecular Devices, Sunnyvale, CA). For the gating currents the PXI1002 digitizer and LabView data acquisition software (National Instruments) were used. For all other recordings, the Digidata 1322A digitizer and pClamp 8.1 data acquisition software (Molecular Devices) were used.

2.3.1 Whole cell recordings—Whole-cell ionic current recordings used pipettes pulled from thin-walled borosilicate (World Precision Instruments, Sarasota, FL) or Fisherbrand microhematocrit (Fisher Scientific, Pittsburgh, PA) glass capillaries using the Flaming/Brown micropipette puller P97 (Sutter Instruments, Novato, CA) and had resistances of 0.8–1.7 M Ω when filled with pipette solution. Data were filtered at 5–10 kHz using an 8-pole low-pass Bessel filter and sampled at 50 kHz, or, for tail current recordings, filtered at 100 kHz and sampled at 200 kHz. After capacity correction, data were filtered at 5 kHz or 20 kHz (tail currents) offline for analysis.

Recordings were made at room temperature or after solution in chamber was cooled to 9–11 $^{\circ}\text{C}$ as indicated in the figure legends. For experiments conducted in the cold, chamber was cooled using the SensorTEK TS-4 (Physitemp Instruments, Inc., Clifton, NJ) temperature controller device. Solution temperature was monitored with a YSI Tele-Thermometer using a YSI series 400 probe (YSI Integrated Systems & Services, Yellow Springs, OH) 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. Bath solution was then perfused 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 Ba (bath 2) were perfused into the chamber following the 3 minute perfusion with Ca bath solution (bath 1) to avoid the formation of insoluble complexes between Ba and the culture media. Toxin in bath solution (150–200 μl) 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, chamber bottom (glass coverslip) was removed and chamber was washed with dilute basic solution (50 mM Na_3PO_4) and then rinsed thoroughly with dilute acid solution (0.1% HCl) and/or distilled water.

During recording, cells were kept at a holding potential of -110 mV. In all experiments recordings were made such that time since gaining access was consistent. Depolarization protocols stepped to potentials ranging from -90 to $+75$ mV in 5 mV increments for 100–250 ms once every 4 s. To record tail currents cells were depolarized to $+40$ mV and then repolarized to potentials ranging from -130 mV to $+75$ mV. In order to ensure maximal channel activation, and to take into account the slower activation timecourse of toxin-modified channels, the duration of the initial step to $+40$ mV was adjusted for each cell based on the time to peak current recorded at $+40$ mV in a protocol immediately preceding the tail current protocol. The durations ranged from 7.4–8.3 ms in control cells and from 7–

10 ms in the presence of toxin. The data were analyzed using locally written protocols in MATLAB (The Math Works, Inc., Natick, MA). Data were capacity-corrected using 8–16 subthreshold responses (voltage steps of 10 or 20 mV) and leak-corrected based on linear leak resistance calculated at potentials negative to −80 mV or by linear interpolation between the current at the holding potential and 0 mV. Leak- and capacity-corrected currents were filtered at 5 kHz (step depolarizations) or 20 kHz (tail currents) offline before analysis. Whole cell capacitance ranged from 14–43 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 ≥ 6.0 mV.

Curve fitting and statistical analyses (Student's *t* test) were performed using the Origin software (OriginLab Corp, Northampton, MA). 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. Tail currents were time shifted such that peak tail current (I) was at approximately $t = 0$ ms and then fit using either a single (2) or double (3) exponential decay function:

$$I = I_s + A_1 e^{-t/\tau} \quad (2)$$

$$I = I_s + A_1 e^{-t/\tau_1} + A_2 e^{-t/\tau_2} \quad (3)$$

Where I is the macroscopic current, I_s the steady current, A_1 and A_2 the amplitude(s), t the time, and τ or τ_1 and τ_2 the time constant(s) of current decay. Whether the data were better fit with a single or double exponential was determined based on the results of an F-test and a second order Akaike Information Criterion test.

2.3.2 Single-channel recordings—Patch pipettes were constructed from 1.2 mm quartz glass capillary tubes (Sutter Instruments) using a P-2000 laser puller (Sutter Instruments). Pipettes had resistances of 4–18 M Ω for single channel recordings. Single-channel data were filtered at 2 kHz using an 8-pole low pass Bessel filter and sampled at 20 kHz. Patches were held at −100 mV and single channel currents were recorded during 200 ms steps to −80 mV immediately after a 3 ms depolarization to 0 mV. Pulses were applied every 3 s to maximize channel availability.

Even though HEK293 cells are relatively electrically quiet, patches occasionally contained endogenous channels. Several different methods were used to ensure that activity was consistent with Ca_v3.1. Contamination by endogenous channels was reduced by the use of extracellular TEA, and patches containing activity at the holding potential were excluded. Endogenous currents were identified based on their voltage-dependent activation profile and their longer open duration and greater current magnitude relative to T-channels (these criteria are described in detail in Bittner and Hanck, 2008). In addition to excluding patches at the time of recording, patches that contained activity inconsistent with T-type Ca channel activity were also occasionally excluded during offline analysis.

Currents displayed are always capacity- and leak- corrected. Currents were capacity-corrected by subtracting the average of the current records containing no channel activity (nulls). Current records were leak-corrected by subtracting the average current in the last millisecond of the pulse. In addition, the baseline was adjusted manually on a sweep-by-sweep basis as necessary.

The data were analyzed using locally written programs in MATLAB and the Origin software. Recordings were decimated to 4 kHz sampling frequency prior to analysis. Current amplitudes were measured by constructing all-points histograms from selected segments of active sweeps. Events were identified by eye, with a bias against simultaneous openings. The segments of data surrounding each event were manually selected; data were concatenated and used to generate amplitude histograms. All-points histograms were constructed from the concatenated data using a bin width of 0.02 pA and the histograms were fit with the sum of two Gaussians. This method excluded excess closed-state data and enhanced the relative size of the open-state peak in the amplitude histogram, thereby increasing the accuracy of the fits. The current amplitude at 80 mV was taken as the difference between the open state peak and the closed state peak as determined by the fit to the data from individual cells.

2.3.3 Gating current recordings—Pipettes were pulled from 1.5 mm diameter quartz glass capillary tubes (Sutter Instruments) using a P-2000 laser puller (Sutter Instruments). Pipettes had resistances of 5.4–10.2 M Ω as measured in the NMDG recording solution. Data were filtered at 10 kHz using an 8-pole low-pass Bessel filter and sampled at 20 kHz. During recording cells were kept at a holding potential of -120 mV. Off gating currents were recorded using the whole-cell voltage clamp configuration in response to 4 identical sweeps in which cells were repolarized to the holding potential for 75 ms after a 250 ms pulse depolarization to potentials from -110 mV to 0 mV. The data were analyzed using locally written protocols in MATLAB (The Math Works, Inc., Natick, MA). Recordings were capacity corrected using 4 sweeps in which cells were hyperpolarized to -140 mV from the holding potential embedded in between each set of data sweeps. Recordings were leak corrected based on linear interpolation of current between the holding potential and 0 mV. If necessary, currents were further leak corrected based on current measured during a 5 ms window after all gating current had decayed (20–25 ms for the off gating currents). Off gating charge was determined from the running integral of current during the first 25 ms of the hyperpolarizing step.

Curve fitting and statistical analyses (Student's t test) were performed using the Origin software (OriginLab Corp, Northampton, MA). Currents from individual cells were integrated and reported charge measurements are averages $\pm$ S.E.M. of gating charge calculated for individual cells. A P-value of <0.05 was considered significant. Charge-voltage relationships were fit using the Boltzmann equation (1) described for fitting whole cell conductance voltage-relationships.

3. Results

3.1 Effects of ProTxII on voltage dependent activation and macroscopic conductance

For voltage-gated Na channels, ProTxII has been reported to decrease current amplitude, delay the timecourse of activation and, in some cases, positively shift the voltage range of activation with no accompanying effects on inactivation, recovery from inactivation or steady state availability (Edgerton et al., 2008; Middleton et al., 2002; Smith et al., 2007; Sokolov et al., 2007). We set out to compare these results to the effects of ProTxII on Ca_v3.1. We first characterized the effect of toxin on Ca_v3.1 Ca currents elicited from step depolarizations (see Methods) at room temperature. Several aspects of ProTxII's effects on

Ca_v3.1 were similar to its effects on Na channels. For example, ProTxII dose-dependently induced a decrease in current amplitude ($IC_{50} = 0.8 \mu M$) and a positive shift in the voltage range of activation ($IC_{50} = 1.7 \mu M$; Fig. 1), with minimal or no effect on steady state availability or recovery from inactivation (See Supplemental Data). Furthermore, as for Na_v1.5, increasing the extracellular divalent concentration partially abrogated the toxin-induced shift in activation without affecting the toxin's ability to reduce peak current (Edgerton et al., 2008). The effect of ProTxII on current amplitude was virtually the same in the presence of Ca (2 mM) and Ba (5 mM), but the shift in the voltage of half maximal activation (V_{half}) was smaller in Ba (at 2 μM ProTxII: $\Delta V_{halfCa(2)} \sim 23$ mV, $\Delta V_{halfBa(5)} \sim 7.5$ mV; Table 1) consistent with a surface charge screening-like mechanism involved with this aspect of toxin action.

To determine the extent to which the decrease in current amplitude was secondary to a decrease in macroscopic conductance, we next measured the effect of ProTxII on the open channel current-voltage relationship. The non-linear nature of the Ca_v3.1 current voltage relationship makes it difficult to measure maximum divalent macroscopic conductance from these data. Therefore, we measured instantaneous Ba currents in a tail current protocol (see Methods). To enhance our ability to accurately measure the tail current amplitude, particularly at very negative voltages at which channel deactivation is very fast, we cooled the bath to 9–11 °C. At this temperature the effect of 2 μM ProTxII on Ba currents generated in response to step depolarizations (Table 1) was similar to the effect of the same toxin concentration on Ba currents recorded at room temperature ($\Delta V_{half(room)} \sim 7.5$ mV, $\Delta V_{half(cold)} \sim 11$ mV; % $\Delta Peak_{-30mV(room)} \sim 60\%$, % $\Delta Peak_{-30mV(cold)} \sim 75\%$; Table 1). Tail current amplitude was smaller in the presence of toxin (2 μM) at all potentials studied (Fig. 2). To determine maximum macroscopic divalent conductance (G_{max}) we measured the slope of the tail current-voltage relationship over the voltage range from –130 mV to –80 mV. At these voltages divalent cation conductance dominates the tail current-voltage relationship making it linear (Fig. 2). We measured a $45.2 \pm 5.8\%$ decrease in G_{max} in the presence of 2 μM ProTxII (Fig. 2). Thus, the majority of the $\sim 75\%$ decrease in macroscopic current amplitude was secondary to a decrease in G_{max} .

We also observed a dose-dependent speeding of tail current decay kinetics in the presence of ProTxII (Fig. 3A). We compared the time constants of tail current decay for potentials at which the rate of channel deactivation predominates the decay kinetics (i.e. negative to –60 mV). Tail current decay was faster in the presence of toxin at all but the most negative potential studied in this way (–130 mV). This finding suggests that toxin speeds the closing step, similar to what we have observed for Na channels (Edgerton et al., 2008). Interestingly, tail currents recorded in the presence of 2 μM toxin were consistently better fit with a double exponential function (see Methods). A second, faster time constant was apparent in these currents that accounted for 25–50 % of the total current amplitude (Fig. 3B). Consistent with the idea that the double exponential decay represents toxin induced changes in current kinetics, we were able to resolve this faster time constant in two out of three cells recorded in the presence of 400 nM ProTxII. However, in these cases, even when the faster time constant could be resolved, it accounted for a smaller fraction of the total current decay than was observed in the presence of 2 μM toxin (Fig. 3B, gray symbols). In the absence of toxin a second time constant was rare. We were able to resolve a second time constant in control cell currents recorded at –100 mV and –80 mV in two out of four cells and at –60 mV in one out of four cells studied in this way. Furthermore, in the absence of toxin the second time constant never accounted for more than 20% of total current decay (Fig. 3B, open symbols). Thus, tail currents recorded in the presence of toxin were faster not only because the dominant time constant was speeded, but also because a second, faster time constant accounted for a dose-dependently greater fraction of total current decay.

3.2 ProTxII does not change single channel current amplitude

One straightforward way toxin could reduce current magnitude would be by changing single channel conductance. We next explored the possibility that ProTxII was reducing the ion flux through open channels. To maximize our ability to resolve single channel openings, we increased the extracellular concentration of Ca to 20 mM and recorded opening events using the cell-attached patch conformation in response to a tail current protocol with a test potential of -80 mV (see Methods). We first recorded macroscopic tail currents under these conditions. ProTxII decreased peak current at -80 mV by $\sim 42\%$ (Fig. 4A). No dramatic changes in the characteristics of the single channel openings in the presence of $2\text{ }\mu\text{M}$ ProTxII were apparent by eye (Fig. 4B), nor was there a change in single channel current amplitude quantified using all point amplitude histograms of active segments of the recordings fit with the sum of two Gaussians (closed and open). Single channel current amplitude at this potential was unaffected by $2\text{ }\mu\text{M}$ toxin measured in 12 cells (Fig. 4C).

3.3 Effect of ProTxII on gating charge

A decrease in G_{max} could also indicate a reduction in the number of channels available to open in the presence of toxin. To test this possibility we next measured the effect of ProTxII on gating charge movement. Unlike previous studies of gating currents in this channel, these recordings were made in the absence of lanthanum (La), a pore blocker that itself introduces a significant shift in voltage dependent activation due to surface charge screening effects (Lacinova et al., 2002; Lam et al., 2005). In the recording solutions used for these experiments cations were replaced with NMDG; a large ion that is thought to be too large to pass through the pore. Surprisingly, in the absence of toxin we were still able to resolve very small ionic currents that displayed a timecourse like $\text{Ca}_v3.1$ during depolarizations. The voltage range of activation was also consistent with $\text{Ca}_v3.1$ (see Supplemental Data). These currents were not apparent in the presence of ProTxII. To avoid any ionic current contamination in our gating current measurements, we extended the duration of the depolarization step to 250 ms and studied the effect of ProTxII on off gating currents only. Off gating currents were recorded during a 75 ms step to -120 mV following a 250 ms depolarizing step to potentials from -120 mV to 0 mV (Fig. 5A). We have not observed, nor have there been any reports of charge immobilization in this channel (Lacinova et al., 2002; Lam et al., 2005). We, therefore, expect off-gating charge to be equal and opposite to on-gating charge. Total off gating charge was calculated from a running integral of the current at each potential (see Methods; Fig. 5B). Maximum off-gating charge ($Q_{\text{max(off)}}$) was unaffected by the presence of $5\text{ }\mu\text{M}$ ProTxII (Fig. 5B, inset). We did observe a positive shift in the voltage range of charge movement. This shift could be better appreciated when charge at each potential was normalized to $Q_{\text{max(off)}}$ for each individual cell (Fig. 5C; $\Delta V_{\text{half(Q)}} = 31.8\text{ mV} \pm 4.4\text{ mV}$). The magnitude of this shift was similar to that observed for activation of macroscopic ionic currents (I_{Ca}) at the same toxin concentration ($\Delta V_{\text{half(I}_{\text{Ca}})} = 34.3\text{ mV} \pm 1.5\text{ mV}$; Table 1) suggesting a mechanism of gating modification that is occurring at the level of the voltage sensors.

3.4 ProTxII slows macroscopic current activation

Macroscopic current amplitude reflects the net contribution of ions moving through open channels as a function of time. Thus, peak current amplitude is determined by the rate of channels activating and reaching the open state ($\text{C} \rightarrow \text{O}$) as well as the rates of channels leaving the open state and entering non-conducting inactivated or closed states ($\text{C} \leftarrow \text{O} \rightarrow \text{I}$). A decrease in peak current amplitude could indicate either a slowing of channel activation or a speeding of either inactivation or deactivation, or both. We observed a dose-dependent slowing of macroscopic current development in the presence of ProTxII (Fig. 6A–B). At 0 mV the time to reach peak current was more than two fold slower in the presence of $5\text{ }\mu\text{M}$ ProTxII. This effect was apparent even early in the timecourse of current development as

activation was slowed to a similar extent by the time currents reached 20% of peak. The decay phase of the currents was also affected by the presence of toxin (Fig. 6C); however in this case the extent of the slowing decreased as a function of potential. At more negative potentials the rate of activation is sufficiently slow as to impact the rate of current decay as channels open late. At more positive potentials activation rates are faster, and, therefore, they do not impact whole cell current decay rate. Consistent with this interpretation, while the toxin-induced slowing of current development was still apparent at +60 mV, ($\% \Delta_{\text{tmpk}} = 150\% \pm 17\%$; Fig. 6D, left), there was no effect on the current decay rate at this potential (Fig. 6D, right). Thus, the effect of ProTxII on the decay rate of the current at negative potentials is likely a consequence of toxin-induced slowing of activation and/or speeding of deactivation rather than any direct effect on channel inactivation rates. These findings are consistent with an exclusive effect on activation gating transitions that has been reported for ProTxII on Na channels as well (Edgerton et al., 2008; Middleton et al., 2002; Sokolov et al., 2007).

To further investigate the effect of ProTxII on activation gating in this channel we looked for changes in the voltage-dependent lag in current development at a given potential. In voltage-gated Na and K channels this lag, also called the Cole-Moore shift, has been correlated with transitions among multiple pre-open closed states along the activation pathway (Bezanilla et al., 1994; Cole and Moore, 1960; Kuzmenkin et al., 2004; Stefani et al., 1994). We adapted the protocol first described by Cole and Moore to track the voltage dependent changes in the timecourse of current development at -20 mV following a 5 ms prepulse to various subthreshold voltages (-140 mV to -90 mV) from a holding potential of -110 mV (Fig. 7A). In the absence of toxin we observed that the timecourse of current development (i.e. the time it took to reach 20 % of peak current) at this potential was faster following more depolarized pre-pulses consistent with an activation pathway that involves multiple, voltage-dependent pre-open closed state transition steps (Fig. 7B). The Cole-Moore shift was voltage dependent in the presence of the toxin as well, however the shift was nearly two-fold longer in toxin compared to control across the entire voltage range and the voltage dependence of the shift was somewhat increased (Fig. 7C).

At first glance it was surprising to find that toxin produced a large change in macroscopic channel conductance without an effect on single channel amplitude. To explore the extent to which slowing steps along the activation pathway could produce the effects we saw in our experimental data, we simulated macroscopic ionic currents based on a simplified gating model of this channel, which included five closed, non-conducting states, a single open state and a single inactivated, non-conducting state (Fig. 8). Transitions among the pre-open closed states as well as the final opening transition ($C \rightarrow O$) were made voltage-dependent and the $O \rightarrow C$ and $O \rightarrow I$ transitions were voltage independent consistent with available data (Table 2). The generation of this model and its specific characteristics have been discussed elsewhere (Freeze et al., 2006)(Freeze et al. 2006). We simulated currents from the “control” model and from models in which each activation transition in turn was slowed 100-fold. There were several informative features of the resulting simulated currents with respect to our experimental data. First, perturbation of the first three gating transitions ($C1 \rightarrow C2$, $C2 \rightarrow C3$, $C3 \rightarrow C4$) all had similar effects on currents (Fig. 8A–B). Specifically, these perturbations early in the activation pathway resulted in $\sim 40\%$ decrease in current magnitude (measured at 30 mV), currents that were slower to activate, and no change in maximum conductance (i.e. the slope of the linear portion of the current-voltage relationship at +40 mV and more positive). The normalized conductance-voltage relationship was shallower, as expected with channels arriving late to the open state. Perturbations of transitions later in the activation pathway, i.e. $C4 \rightarrow C5$ (Fig. 8C) and $C5 \rightarrow O$ (Fig. 8D), recapitulated the effects we observed. Slowing of the $C4 \rightarrow C5$ transition, which is assigned the largest voltage dependency in the model, shifted V_{half} by more than 30 mV, although changing this

transition alone did not decrease maximum conductance. Conversely, when the final opening transition (C5→O) was slowed maximum conductance was reduced by >50%. Taken together, modeling suggests ProTxII inhibits transitions at multiple steps along the activation pathway, with slowing of the later, more voltage-dependent transitions, including the final opening step making the largest contribution.

4. Discussion

Our data show that the peptide toxin, ProTxII, modifies the T-type Ca channel Ca_v3.1; decreasing peak current amplitude principally via slowing channel activation and destabilization of the open state. Our study was informed by previous studies of this toxin's effect on voltage-gated Na channels (Edgerton et al., 2008; Middleton et al., 2002; Sokolov et al., 2007). We found that, similar to what was shown previously for Na_v1.5, the toxin-induced decrease in Ca_v3.1 macroscopic currents was the net result of a shift in the voltage range of activation and a decrease in macroscopic conductance. The toxin's effect on this channel shares several other features with the effects seen in Na_v1.5 including an approximate IC₅₀ for the decrease in conductance in the micromolar range and a lack of toxin effect on inactivation gating. Interestingly, unlike what has been shown for Na_v1.2, we saw no effect of ProTxII on total gating charge (Sokolov et al., 2007).

ProTxII is one of only a few known toxins that inhibit activation in T-type Ca channels. The α -scorpion toxin kurtosin does target T-type Ca channels and inhibits their activation, but preliminary reports suggest this toxin does not modify deactivation kinetics, unlike what we show here for ProTxII (Chuang et al., 1998; Sidach and Mintz, 2002). Among toxins known to target voltage-gated Na channels, another target of ProTxII, some exclusively modify inactivation gating, e.g. the anthropleurin toxins (for review see: Hanck and Sheets, 2007) or enhance activation gating, e.g. the β -scorpion toxins, (for review see: Catterall et al., 2007). Still others modify activation gating similarly to ProTxII, but target only K channels, e.g. Hanatoxin and SGTx, (Swartz and MacKinnon, 1997a,b; Wang et al., 2004). Thus, ProTxII is likely interacting with Ca_v3.1 at unique site(s) on the channel that are involved in gating transitions all along the activation pathway.

In Na channels, we have previously provided evidence that the decrease in macroscopic conductance and shift in voltage dependent activation of Na_v1.5 caused by ProTxII are the result of independent toxin actions (Edgerton et al., 2008). We investigated several possible mechanisms for the decrease in macroscopic conductance of Ca_v3.1 observed in the presence of ProTxII. Partial pore occlusion or the introduction of positive charge in the channel vestibule would cause a decrease in single channel conductance in the absence of any gating shifts consistent with independent toxin actions. However, ProTxII did not decrease single channel current amplitude in Ca_v3.1. Complete pore block of Ca_v3.1 by ProTxII without an effect on gating, e.g. as TTX block occurs in Na channels, cannot be ruled out. The lack of known pore blockers slow enough to allow for competition measurements make it difficult to definitively test for this mechanism of block in Ca_v3.1.

Total gating charge was also unaffected by ProTxII in this channel. Thus, we were able to rule out the possibility that a population of toxin-bound non-gating channels were contributing to the decrease in macroscopic conductance. The presence of the toxin did cause a positive shift in the gating current-voltage relationship, which we would expect if ProTxII is causing gating modifications via interactions with the channel's voltage sensors. The K channel toxin Hanatoxin, mentioned above, is known to bind to and inhibit the movement of the voltage sensors and its effects are apparent in the voltage dependence of both ionic current and gating current (Lee et al., 2003; Li-Smerin and Swartz, 2000; Phillips et al., 2005). Our data indicate that the voltage sensors of toxin-modified channels do move

through the membrane electric field, though they require more potential energy to do so, since the total gating charge per channel is likely unaffected by the presence of the toxin. Thus, we speculate that ProTxII is inhibiting activation in $\text{Ca}_v3.1$ via interaction with one or more of the channel's voltage sensors resulting in a population of channels that are gating more slowly and with a significantly lower probability of simultaneous openings.

A number of observations have led us to conclude that the decrease in open probability in toxin-modified channels is a result of the inhibition of activation and not inactivation. First we note that the slowed timecourse of current activation approaches the timecourse of fast inactivation in toxin-modified channels. The resulting overlap would reduce the number of channels contributing to the macroscopic current amplitude at any given time without any accompanying change in the rate of channel inactivation. Indeed, we saw no difference in current decay rate at very positive potentials at which we expect minimal overlap between activation and inactivation. The timecourse of current development, on the other hand, was slower across the entire voltage range studied. Furthermore, the slower timecourse of activation was apparent even early in current development making it unlikely that changes in the rate of inactivation could be contributing to the slowing. The modest effect we observed in steady state inactivation was limited to potentials within the activation range of the channel (i.e. positive to -75 mV) making it likely that the slowed activation of toxin-modified channels is responsible for this change as well.

We tested for evidence of ProTxII inhibition at different points along the activation pathway. In both voltage-gated K and Na channels, it is thought that several transitions among pre-open closed states precede the final opening step during activation gating (Campos et al., 2007; Cole and Moore, 1960). A voltage dependent lag to current onset, i.e. the Cole-Moore shift, suggests a similar path to activation in $\text{Ca}_v3.1$. As expected, ProTxII lengthened this lag time dramatically across the entire voltage range, supporting the idea that the toxin induces a general slowing of channel activation not limited to early transitions among pre-open closed states. We also note, however, that the voltage dependence of the shift is increased in the presence of toxin compared to control. The voltage dependence of the Cole-Moore shift reflects the bias of channels to occupy states closer to the open state at more positive potentials. The increase in the voltage dependence of the shift in the presence of ProTxII, therefore, suggests that at least part of the toxin-induced inhibition of activation is occurring early in the pathway and can thus be overcome by conditioning at more positive potentials. Despite this increase in voltage dependence, the Cole-Moore shift was nearly two-fold longer at the most positive conditioning potential. This argues against the exclusive inhibition of these early steps. Macroscopic currents simulated based on a simplified gating model of this channel confirm that perturbation of gating transitions all along the activation pathway can produce the changes in both current amplitude and voltage-dependent activation that we observed in our experimental data. The lesser shift in gating we observed in 5 mM Ba compared to 2 mM Ca considered alongside the modeling results, suggests that surface charge screening effects may be affected transitions close to the final opening step.

Our tail current kinetic analysis reveals ProTxII-induced effects on the rate of the final opening step of the activation pathway. Specifically, the timecourse of tail current decay at potentials negative to the activation range of the channel reflects the rate of channels leaving the open state almost exclusively since the probability of channels reopening at these potentials is very low. ProTxII dose-dependently speeds the dominant time constant of tail current decay at these potentials, indicating a destabilization of the open state. A second, faster time constant was also apparent in the presence of toxin. At a lower concentration of toxin the faster time constant could also sometimes be detected, but consistent with the idea that the presence of ProTxII increases the contribution of this second component, the faster time constant accounted for a smaller fraction of total current decay than we observed at the

higher toxin concentration. The faster time constant was occasionally detectable in the absence of toxin as well, making it unlikely that this second component of tail current decay represents toxin binding. The data suggest that ProTxII dose-dependently modifies a pre-existing deactivation rate transition, perhaps from a second open state, making it more reliably resolvable under our recording conditions. Indeed, previous studies have reported the existence of at least one subconductance state in this channel supporting the idea of multiple open states (Bittner and Hanck, 2008; Perez-Reyes et al., 1998). The ability of ProTxII to alter the gating kinetics of this channel is reminiscent of the modulated receptor model of interactions between voltage-gated Na⁺ channels and local anesthetics, e.g. lidocaine (Hille, 1977; Hondeghem and Katzung, 1977). This model describes state-dependent drug receptor sites on the channel that differ in their binding affinities depending on state and that alter voltage sensor movement (Hanck et al., 2000). Like local anesthetics, ProTxII clearly alters the movement of voltage sensors upon binding by shifting the voltage range of gating (Figure 5), and speeding channel deactivation (Figure 3).

We conclude that ProTxII inhibits activation in this channel at multiple points along the activation pathway, perhaps via independent binding sites on one or more of the non-identical voltage sensor domains. Evidence to support that ProTxII interacts with multiple voltage sensors in Nav1.2 channels has been put forward in a study of chimeric channels in which the voltage-sensor regions of individual Na⁺ channel domains were inserted into the background of a K⁺ channel (Bosmans et al., 2008) and from electrophysiological study of Nav1.5 based on the ability to separate toxin effects (Edgerton et al., 2008). As well studies of this and other Na⁺ channel gating modifier toxins have used site-directed fluorescence measurements to pinpoint toxin interactions with specific voltage sensors (Campos et al., 2007; Edgerton et al., 2009a). Similar approaches could be used in this case to explore domain-specific interactions between ProTxII and the voltage sensors of Cav3.1. Reduction of macroscopic current magnitude in this channel occurs principally because toxin slows activation and speeds deactivation without blocking the pore, immobilizing the voltage sensors, or interfering with fast inactivation gating. We anticipate the binding site(s) for ProTxII on Cav3.1 to have profound implications of the structural correlates of gating in this unique and physiologically important channel.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

Cav3.1	the T-type voltage-gated Ca channel isoform
Nav1.5	the heart isoform of the voltage-gated Na channel
Nav1.2	the neuronal isoform of the voltage-gated Na channel

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Fig. 1. ProTxII inhibits Ca_v3.1 current and shifts voltage dependent activation

(A) Currents were recorded from HEK cells expressing Ca_v3.1 in response to step depolarizations in the absence (open squares), or presence of 500 nM (inverted triangles), 1 μM (left triangles), 2 μM (right triangles), 5 μM (diamonds), or 10 μM (pentagons) ProTxII (N = 3–9 cells, see Table 1). Mean peak current at each potential is plotted as a function of voltage. *Inset*: Representative currents recorded in response to depolarizations to -90 mV, -65 mV, -60 mV, and -50 mV. All traces are shown on the same scale. (B) Mean peak current recorded at -30 mV from data in (A) plotted as a function of toxin concentration. Solid line is fit to the data using the equation: $y = (A_1 - A_2) / (1 + (x / x_0)^p) + A_2$ where A_1 is the maximum inward current, A_2 the minimum inward current, x_0 the concentration at half maximal effect, and p the hill coefficient. The value of p was allowed to vary. The data were best fit when $p = 1.2$ and $x_0 = 0.85$ μM. (C) Individual conductance-voltage relationships for cells in (A) were fit using the Boltzmann function (equation 1, see Methods). Mean voltage of half maximal activation (V_{half}) is plotted as a function of toxin concentration. Solid line is fit to the data using the equation described in (B). The data were best fit when $p = 2.3$ and $x_0 = 1.7$ μM. Dashed lines indicate IC₅₀ concentration in each case.

Fig. 2. ProTxII decreases macroscopic conductance

Mean instantaneous peak (tail) current-voltage relationship for currents recorded in the absence (open squares; $N = 8$ cells) and presence of 2 μ M ProTxII (black circles; $N = 9$ cells) in response to the protocol described in the text. *Inset (left)*: Mean maximum macroscopic divalent conductance ($G_{\max}$). *Inset (right)*: Representative families of tail current traces recorded in the absence (top) and presence of 2 μ M ProTxII (bottom). Traces were filtered offline at 20 kHz and are shown for a subset of test potentials for display purposes. Both sets of traces are shown on the same scale. All data were recorded at 9–11 $^{\circ}$ C.

Fig. 3. ProTxII modifies tail current decay kinetics

Tail currents were fit with a single or double exponential function (see Methods). (A) Mean time constant of tail current decay is plotted as a function of potential for cells recorded in the absence (open squares; N=4) and presence of 400 nM (gray circles, N=3), or 2 μ M ProTxII (black triangles; N=4). For traces in which two time constants could be resolved the longer time constant or the one accounting for more than 80 % of the total current amplitude are reported in these data. *Inset:* Representative currents recorded at -100 mV in the absence (light gray) and presence of 400 nM (dark gray) or 2 μ M ProTxII (black). Currents are normalized to their own peak to highlight the change in timecourse. (B) The fraction of total current amplitude accounted for by the faster time constant, for traces in which two time constants could be resolved, is plotted as a function of potential. Two time constants were resolved for all cells recorded in the presence of 2 μ M toxin and so mean data are presented here. Same symbols as in (A). Data from individual cells are indicated by symbols of the same shape. All data were recorded at 9–11 °C.

Fig. 4. ProTxII does not change single channel current amplitude

(A) Macroscopic tail currents were recorded under the same conditions in the presence (gray traces and bar) and absence (black traces, open bar) of 2 μ M ProTxII. At this concentration of toxin tail current amplitude was significantly decreased ($\text{Peak}_{-80\text{Ctrl}} = -5.4 \text{ nA} \pm 0.9 \text{ nA}$, $N=7$; $\text{Peak}_{-80\text{PT}} = -3.1 \text{ nA} \pm 0.3 \text{ nA}$, $N=5$). (B) Single channel events recorded in response to a tail protocol (see Methods) in the absence (left) and presence of 2 μ M ProTxII (right). When necessary, data points contaminated by residual capacity transient current are removed for display purposes. (C) Single channel amplitude at 80 mV. $N = 6$ patches for each condition.

Fig. 5. ProTxII does not affect total off gating charge

(A) Representative families of off-gating currents recorded in response to step depolarizations (see Methods) in the absence (top) and presence of 5 μ M ProTxII (bottom). Data were normalized to the peak current recorded at -30 mV in each condition. Data were filtered at 5 kHz and downsampled by 5 for display purposes. (B) Off-charge at each potential was calculated from off-gating currents as described in the Methods. Mean charge is plotted as a function of voltage for cells recorded in the absence (open squares) and presence of 5 μ M ProTxII (black circles). *Inset:* Data for individual cells were fit with a Boltzmann function (equation 1; $V_{half(Ctrl)} = -71.1 \text{ mV} \pm 2.6 \text{ mV}$, $V_{half(PT)} = -39.6 \text{ mV} \pm 2.9 \text{ mV}$). Mean maximum off charge ($Q_{max(off)}$) in the absence (white bar) and presence (black bar) of toxin was not statistically different ($Q_{max(off)Ctrl} = 112.6 \text{ pF} \pm 7.5 \text{ pF}$, $N = 5$ cells; $Q_{max(off)PT} = -169.3 \text{ pF} \pm 35.5 \text{ pF}$, $N = 6$ cells). (C) Off gating charge at each potential was normalized to $Q_{max(off)}$ for each cell and is plotted as a function of potential. Smooth lines are best fits to the mean data using the Boltzmann function (equation 1). Mean normalized charge is plotted as a function of voltage. Dashed lines indicate voltage of half maximal charge movement. Same symbols as in (B).

Fig. 6. ProTxII slows macroscopic ionic current activation

(A) Mean time to peak current in response to step depolarizations plotted as a function of potential. Currents were recorded in the absence (open squares, $N = 7$ cells) and presence of 1 μ M (gray circles, $N = 4$ cells) and 5 μ M ProTxII (black triangles, $N = 9$ cells). (B) Mean time to reach 20 % of peak current for same cells as in (A). Symbols are the same as in (A). (C) The decay phase of the macroscopic currents from the same cells as in (A) were fit with a single exponential decay function (equation 2). Average time constants for currents under each condition are plotted as a function of voltage. Symbols are the same as in (A and B). (D) Average time to peak current (left) and time constant of current decay (right) from same cells as in (A) recorded at +60 mV in the absence (white bar) and presence of 5 μ M ProTxII (black bar).

Fig. 7. ProTxII inhibits early activation gating transitions

(A) Current traces from representative cells recorded at V_t in the absence (left) and presence of 2 μ M toxin (right) in response to protocol shown (*inset*). Traces were normalized to their own peak to highlight changes in timecourse. (B) Time to reach 20% peak current plotted as a function of potential during the conditioning step in the absence (open squares, $N = 5$ cells) and presence of 2 μ M ProTxII (black circles, $N = 4$ cells). (C) For each cell, the time to reach 20 % peak current was normalized to the time to reach 20 % peak current at the most negative conditioning potential ($V_c = -140$ mV) to highlight changes in the voltage dependence of the Cole-Moore shift. Symbols are the same as in (B).

Fig. 8. Inhibiting activation rate constants slows timecourse, shifts voltage dependent gating, and reduces peak current and conductance in simulated $\text{Ca}_v3.1$ macroscopic currents

Currents were simulated based on a simplified gating model (top; for details see text and Table 2). Reversal potential (V_{rev}) was set to +25 mV and the initial closed state (C_1) was given a starting probability of occupancy of 1.0. Currents were simulated in response to the same depolarization step protocol used experimentally (see Methods). (A–D) Peak current-voltage relationships for simulated data using the original model (gray line) and one in which a single forward rate constant (indicated in each panel) was decreased 100-fold (black line). *Upper inset*: Traces simulated in response to a depolarizing step to –10 mV using the original model (gray line) or perturbed model (black line). Each trace was normalized to its own peak to highlight the changes in timecourse. *Lower inset*: Maximum conductance was determined from the slope of the linear portion of the current-voltage relationship at potentials positive to +40 mV for simulations based on the original model (gray line) and the activation perturbed model (black line). Fractional conductance (G) at each potential (V) was calculated and normalized to the maximum conductance determined from the original model ($G_{\text{max(Ctrl)}}$):

$$G_{\text{norm}} = (I / (V - V_{\text{rev}})) / G_{\text{max(Ctrl)}}$$

Note: Simulated currents from model with 100-fold slower $C_1 \rightarrow C_2$ or $C_2 \rightarrow C_3$ transitions were indistinguishable. Therefore only results from the model in which $C_1 \rightarrow C_2$ was slowed are shown.

Table 1

Summary of mean data for currents recorded in response to step depolarizations.

Permeant ion	T	[ProTxII]	V _{half}	ΔV _{half} ^a	Peak _{-30mV}	Peak _{-30mV}	N
(mM)	(°C)	(μM)	(mV)	(mV)	(pA)	(%)	
Ca (2)	20-21	0	-53.2 ± 1.1	n/a	-1368.5 ± 190.2	n/a	7
Ca (2)	20-21	0.1	-52.3 ± 0.3	n/a ^b	-1553.8 ± 114.7	n/a ^b	3
Ca (2)	20-21	0.5	-51.6 ± 1.4	1.6 ± 1.1	-1066.9 ± 146.1	-22.0 ± 15.2	7
Ca (2)	20-21	1	-43.1 ± 1.8	10.1 ± 2.1	-654.0 ± 144.5	-52.2 ± 6.7	3
Ca (2)	20-21	2	-30.5 ± 2.4	22.7 ± 2.6	-470.1 ± 90.6	-65.6 ± 8.2	7
Ca (2)	20-21	5	-18.7 ± 0.9	34.5 ± 1.4	-251.3 ± 24.3	-81.6 ± 3.1	9
Ca (2)	20-21	10	-11.7 ± 5.0	41.5 ± 5.1	-127.4 ± 55.0	-90.7 ± 1.3	5
Ba (5)	20-21	0	-42.1 ± 1.4	n/a	-1062.1 ± 179.0	n/a	9
Ba (5)	20-21	2	-34.6 ± 2.6	-7.5 ± 2.9	-436.7 ± 54.3	-58.9 ± 8.6	7
Ba (5)	9-11	0	-38.8 ± 0.7	n/a	-336.6 ± 23.8	n/a	21
Ba (5)	9-11	2	-27.9 ± 1.7	10.9 ± 1.8	-84.3 ± 20.3	-74.9 ± 6.3	7

^aDelta values are from mean data in the presence and absence of toxin in data sets with the same recording conditions (i.e permeant ion, permeant ion concentration, and recording temperature).

^bThere was no significant change in V_{half} or peak current measured at -30 mV at this concentration.

Table 2

Transition rates and voltage dependencies used to produce simulated $\text{Ca}_v3.1$ currents

This model is a simplified version of the model described previously (Freeze et al. 2006).

Transition	k	q
	at 0mV (s^{-1})	(e)
C1C2	74800	0.6
C2C1	171	-0.6
C2C3	66490	0.6
C3C2	86	-0.6
C3C4	58180	0.6
C4C3	69	-0.6
C4C5	150000	1.75
C5C4	20	-1.75
C5O	15000	--
OC	53	-0.7
CI	90	--
IC	0.01	--