Differential Interactions of Na\textsuperscript{+} Channel Toxins with T-type Ca\textsuperscript{2+} Channels

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Two types of voltage-dependent Ca\textsuperscript{2+} channels have been identified in heart: high (I_{Ca,L}) and low (I_{Ca,T}) voltage-activated Ca\textsuperscript{2+} channels. In guinea pig ventricular myocytes, low voltage-activated inward current consists of I_{Ca,L} and a tetrodotoxin (TTX)-sensitive I_{Ca} component (I_{Ca(TTX)}). In this study, we reexamined the nature of low-threshold I_{Ca}, but was inhibited by 50 μM Ni\textsuperscript{2+} (by ~90%) or 5 μM mibefradil (by ~50%), consistent with the reported properties of I_{Ca,T}. Addition of 30 μM TTX in the presence of Ni\textsuperscript{2+} increased the current approximately fourfold (41% of control), and shifted the dose–response curve of Ni\textsuperscript{2+} block to the right (IC\textsubscript{50} from 7.6 to 30 μM). Saxitoxin (STX) at 1 μM abolished the current left in 50 μM Ni\textsuperscript{2+}. In the absence of Ni\textsuperscript{2+}, STX potently blocked I_{Ca,T} (EC\textsubscript{50} = 185 nM) and modestly reduced I_{Ca,L} (EC\textsubscript{50} = 1.6 μM). While TTX produced no direct effect on I_{Ca,T} elicited by expression of hCa\textsubscript{V}3.1 and hCa\textsubscript{V}3.2 in HEK-293 cells, it significantly attenuated the block of the current by Ni\textsuperscript{2+} (IC\textsubscript{50} increased to 550 μM Ni\textsuperscript{2+} for Ca\textsubscript{V}3.1 and 15 μM Ni\textsuperscript{2+} for Ca\textsubscript{V}3.2); in contrast, 30 μM TTX directly inhibited hCa\textsubscript{V}3.3-induced I_{Ca,T} and the addition of 750 μM Ni\textsuperscript{2+} to the TTX-containing medium led to greater block of the current that was not significantly different than that produced by Ni\textsuperscript{2+} alone. 1 μM STX directly inhibited Ca\textsubscript{V}3.1, Ca\textsubscript{V}3.2, and Ca\textsubscript{V}3.3-mediated I_{Ca,T} but did not enhance the ability of Ni\textsuperscript{2+} to block these currents. These findings provide important new implications for our understanding of structure–function relationships of I_{Ca,T} in heart, and further extend the hypothesis of a parallel evolution of Na\textsuperscript{+} and Ca\textsuperscript{2+} channels from an ancestor with common structural motifs.

INTRODUCTION

Voltage-gated Na\textsuperscript{+} and Ca\textsuperscript{2+} channels are ubiquitously expressed in excitable cells across the animal kingdom and from an evolutionary standpoint have been proposed to have arisen from a common ancestor, primarily by gene duplication (Strong and Gutman, 1993; Hille, 2001). The pore-forming or α-subunit of Na\textsuperscript{+} and Ca\textsuperscript{2+} channels share in common the basic structure of a single linear sequence of amino acids characterized by four repeat sequences containing each six transmembrane domains (S1–S6), with the fourth transmembrane segment of each repeat bearing positively charged residues conferring voltage-sensitive properties to the channel. For both classes of channels, the amino acid sequence between S5 and S6 of each repeat dips back into the membrane from the extracellular space and forms the basic structure of the pore or P-loop of the channel. Each P-loop repeat shares one critical residue that forms a ring of four amino acid residues conferring ion selectivity and permeation across the pore. For Na\textsuperscript{+} channels, the signature sequence of repeats I–IV is DEKA (Catterall, 2000), whereas for Ca\textsuperscript{2+} channels, it is EEXX (Perez-Reyes, 2003), where X is either E or D (Fig. 1; SF, selectivity filter). Mutation of the Lys of repeat III or Ala of repeat IV to Glu conferred Ca\textsuperscript{2+} and Ba\textsuperscript{2+} selectivity to Na\textsuperscript{+} channels, again supporting commonality in their evolutionary heritage (Heinemann et al., 1992).

Among the three subfamilies of Ca\textsuperscript{2+} channels encoded by the Ca\textsubscript{V} genes (Ca\textsubscript{V}1, Ca\textsubscript{V}2, and Ca\textsubscript{V}3), the Ca\textsubscript{V}3 subfamily encoding low threshold voltage-activated Ca\textsuperscript{2+} channels commonly referred to as T-type (for “transient”; I_{Ca,T}) Ca\textsuperscript{2+} channels has been hypothesized to be the closest Ca\textsuperscript{2+} channel subfamily to the Na\textsuperscript{+} channel.

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Abbreviations used in this paper: HP, holding potential; LVA, low voltage-activated inward Ca\textsuperscript{2+} current; SF, selectivity filter; STX, saxitoxin; TTX, tetrodotoxin.

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There is also recent evidence for similarities in the pore region between Na⁺ channels and T-type Ca²⁺ channels. McNulty et al. (2006) recently showed that mutating Asn406 to Ala or Cys in Na V 1.5 genes (Hille, 2001). Similar to Na⁺ channels and contrary to high threshold voltage-gated Ca²⁺ channels (e.g., L-type), T-type Ca²⁺ channels activate in the negative range of membrane potentials and exhibit relatively fast activation and inactivation kinetics and have a small unitary conductance (~7 pS with 100 mM Ca²⁺ or Ba²⁺ as charge carrier). There is also recent evidence for similarities in the pore region between Na⁺ channels and T-type Ca²⁺ channels. McNulty et al. (2006) recently showed that mutating Asn406 to Ala or Cys in Na V 1.5 genes.

Figure 1. Amino acid sequence alignments of the pore region of several vertebrate voltage-gated Na⁺ and Ca²⁺ channels. This figure shows the alignments of P-loop residues located between transmembrane segments S5 and S6 of the four domains (Domains I–IV) of four mammalian (Na V 1.4, Na V 1.5, Na V 1.8, and Na V 1.9) and one garter snake (tsNa V 1.4; from Sonoma County, CA), Na⁺ channel genes, and three mammalian T-type (Ca V 3.1, Ca V 3.2, and Ca V 3.3), and one L-type Ca²⁺ channel (Ca V 1.2) gene. These sequences were imported from the GenBank/EMBL/DBJ (http://www.ncbi.nlm.nih.gov) with the accession no. (Access #) for each protein indicated to the right. Small numbers before and after the sequences indicate the absolute position (P) of the first and last amino acids from the N terminus of the translated protein. Overlaid are also shown relative positions before or after the selectivity filter (SF; 0) in each domain. These sequences were aligned using Vector NTI Advance (v. 8.0, InforMax). Please note that only predicted sequences were available for the canine Na⁺ and Ca²⁺ channel genes but those were identical to their corresponding human sequences. Residues highlighted in dark green indicate identical residues, those in light green indicate equivalent substitutions based on a Blosum Matrix 45 score ≥ 1, those shaded in red indicate residues found to play a role in TTX and STX binding, and those highlighted in pink are Ca²⁺ channel residues that differ from residues involved in TTX and STX binding.

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confers “T-type-like” blocking action of mibebradil, a putative blocker of T-type Ca\(^{2+}\) channels, on Na\(^+\) channels and yielded slower inactivation. An alignment of the P-loop region of the four repeats of Ca\(_{3.1}\), Ca\(_{3.2}\), and Ca\(_{3.3}\) thought to generate T-type Ca\(^{2+}\) currents (Perez-Reyes, 2003; Vassort et al., 2006) shows significant homology with that of various mammalian Na\(^+\) genes (Fig. 1). Tetrodotoxin (TTX) and saxitoxin (STX) are two structurally related heterocyclic guanidinium marine toxins that potently inhibit voltage-gated Na\(^+\) channels by an interaction with several residues in the P-loop as indicated in Fig. 1 (labeled in red). In view of the structural similarities within or near the pore region of the two classes of channels and the possibility that they may have evolved from a common ancestor, we wondered whether these toxins also interact with T-type Ca\(^{2+}\) channels. We examined the effects of TTX and STX from several commercial sources on I\(_{\text{CaT}}\) recorded from canine atrial myocytes or from HEK 293 cells transfected with Ca\(_{3.1}\), Ca\(_{3.2}\), or Ca\(_{3.3}\). TTX exerted no significant effect on the magnitude of native I\(_{\text{CaT}}\) and I\(_{\text{CaT}}\) generated by either Ca\(_{3.1}\) or Ca\(_{3.2}\), the two isoforms predominantly expressed in heart (Vassort et al., 2006), while it inhibited Ca\(_{3.3}\)-induced I\(_{\text{CaT}}\). Interestingly, for both native I\(_{\text{CaT}}\) and Ca\(_{3.1}\) or Ca\(_{3.2}\)-induced I\(_{\text{CaT}}\) TTX partially relieved the blockade of this current by Ni\(^{2+}\). Finally, STX directly inhibited I\(_{\text{CaT}}\) in dog atrial cells and that elicited by expression of all three Ca\(_{3}\) subclasses. Our studies further extend the notion that voltage-gated Na\(^{+}\) and Ca\(^{2+}\) channels share signature properties and may have arisen from a common ancestor.

MATERIALS AND METHODS

This investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the NIH and the guidelines of the Canadian Council on Animal Care, and was approved by the Montreal Heart Institute Animal Care Committee.

Cell Dispersion Technique

Adult mongrel dogs (20–30 kg) were anesthetized with morphine (2 mg/kg s.c.) and α-chloralose (120 mg/kg i.v.) and mechanically ventilated. The heart was removed after intra-atrial injection of heparin (10,000 U), immersed in 2 mM Ca\(^{2+}\)-containing Tyrode solution, and the left atrium perfused via the coronary artery with Tyrode solution until free of blood. The perfusate was then switched to nominally Ca\(^{2+}\)-free Tyrode solution for 20 min, after which 110 U/ml collagenase (Type II, Worthington) and 0.1% BSA were added. Perfusion solutions were saturated with 95% O\(_2\) and 5% CO\(_2\) at 37°C. Cells were dispersed by gentle trituration in Tyrode’s containing 10 mM Ca\(^{2+}\). The cells were kept at room temperature in Tyrode solution containing 100 mM Ca\(^{2+}\) and 0.1% BSA for use within 8 h. The composition of the Tyrode solution was as follows (mM): NaCl 136, KCl 5.4, MgCl\(_2\) 1.0, CaCl\(_2\) 2, Na\(_2\)HPO\(_4\) 0.33, glucose 10, and HEPES 10, pH adjusted to 7.4 with NaOH.

Cell Culture and Transient Transfection

tsk-201 cells were grown and transiently transfected with expression plasmids for hCat3.1, hCat3.2, or hCat3.3 constructs and NHE4-CD8, containing the cDNA of the T cell antigen CD8 to identify effectively transfected cells. In brief, cells were grown to 85% confluence at 37°C (5% CO\(_2\)) in Dulbecco’s modified Eagle’s medium (DMEM) (10% FBS, 200 U/ml penicillin, and 0.2 mg/ml streptomycin, Invitrogen) in 35-mm cell culture plastic Petri dishes and transfected with hCat3.1, hCat3.2, or hCat3.3 channel \(\alpha\) subunits (8 μg) and CD8 marker (1 μg) by the calcium phosphate method for 8 h. After transfection, cells were dissociated with trypsin (0.025%)–ETDA and plated on glass coverslips. Experiments were performed 24–48 h after transfection.

Electrophysiology and Data Analysis

Macroscopic currents were recorded from Ca\(^2+\)-tolerant canine atrial myocytes using the whole-cell patch clamp technique (35.5 ± 0.5°C). With tips ~1 μm in diameter, patch pipette resistance ranged between 2 and 4 MΩ when the micropipette was filled with the internal solution containing (in mM) CsCl 120, TEA 20, MgCl\(_2\) 1, MgATP 5, HEPES 10, GTP.Na 0.1, and EGTA 10, pH adjusted to 7.2 with CsOH. Voltage clamp protocols were computer driven using Digidata 1200 series acquisition system with PC clamp software (v. 8.0 or 9.2) and an Axopatch 200A amplifier (Molecular Devices). Pipette and stray capacitance, as well as series resistance were compensated for in all experiments. Membrane currents were low-pass filtered at 1 or 2 kHz (4-pole Bessel filter) before being acquired at a sampling rate of 2 or 5 kHz. After applying whole-cell access, myocytes were held at the standard holding potential ~90 mV, and cell dialysis was allowed to proceed for at least 5 min before any voltage clamp protocol was initiated. To minimize the undesirable effects of I\(_{\text{CaL}}\) rundown on the measurement of low threshold I\(_{\text{CaT}}\) we used a voltage clamp protocol consisting of two test pulses (TP1 and TP2) to different voltages separated by a 500 ms interval at ~50 mV. The current elicited by TP1 comprised both low and high threshold Ca\(^{2+}\) currents, and that evoked by TP2 mainly consisted of I\(_{\text{CaT}}\). Digital subtraction of I\(_{\text{CaT}}\) from total Ca\(^{2+}\) current recorded during TP1 yielded the low threshold T-type I\(_{\text{CaT}}\) (I\(_{\text{CaT}}\)). The bath solution for Ca\(^{2+}\) current recordings contained (in mM) TEA 136, CsCl 5.4, MgCl\(_2\) 1, CaCl\(_2\) 1.8, HEPES 10, glucose 5.5, and 4-aminoptyridine (4-AP) 2, pH adjusted to 7.35 with CsOH. In all cells studied, cell membrane capacitance was estimated by integrating (with Clampfit 8.0 or 9.2, Molecular Devices) the mean of five consecutive capacitative current transients elicited by 20-ms test pulses from ~50 to ~60 mV.

All experiments on HEK-293 cells transfected with hCat3.1, hCat3.2, or hCat3.3 were performed at room temperature with a bathing solution containing 2 mM Ca\(^{2+}\) (in mM): 128 CsCl, 2 CaCl\(_2\), 1.5 MgCl\(_2\), 10 HEPES, and 25 d-glucose; pH 7.4 (adjusted with CsOH). In the experiments designed to examine the effects of ETDA on the response of I\(_{\text{CaT}}\) to Na\(^+\) channel toxins (see Fig. S2, available at http://www.jgp.org/cgi/content/full/jgp.200709883/DC1), all solutions containing Ni\(^{2+}\) were adjusted to take into account the buffering effect of EDTA on this cation and ensure that the solutions with and without EDTA had equivalent free Ni\(^{2+}\) concentrations. In the presence of 100 μM EDTA, the total added Ni\(^{2+}\) concentrations to achieve either 15, 550, or 750 μM free [Ni\(^{2+}\)]\(_{\text{e}}\) to block respectively Ca\(_{3.1}\), Ca\(_{3.2}\), or Ca\(_{3.3}\) were calculated using WinMAXC (v. 2.5, Chris Patton, http://www.stanford.edu/~cpatton/downloads) and were as follows (in mM): 15 μM free [Ni\(^{2+}\)]\(_{\text{e}}\); 115 μM total [Ni\(^{2+}\)]\(_{\text{e}}\); 550 μM free [Ni\(^{2+}\)]\(_{\text{e}}\); 650 μM total [Ni\(^{2+}\)]\(_{\text{e}}\); 750 μM free [Ni\(^{2+}\)]\(_{\text{e}}\); 850 μM total [Ni\(^{2+}\)]\(_{\text{e}}\). Whole-cell patch clamp recordings were performed on cells positive for CD8 antibody coated beads, using an Axopatch 200B (Molecular Devices) amplifier and Clampex 9.2 software (Molecular Devices), low-pass filtered at 1 kHz and digitized at 10 kHz. Borosilicate glass pipettes (2.5–4 MΩ) were filled with internal solution (in mM): 2 CaCl\(_2\), 1.5 MgCl\(_2\), 10 HEPES, and 25 d-glucose; pH 7.4 (adjusted with CsOH). Series resistance was compensated to 80% of the initial value. Steps of 250 ms duration to ~40 mV (5 s interval)
difference between two means (Statistica for Windows 99, version 5.5). Comparisons among multiple means were performed using one-way ANOVA with a Newman-Keuls (Statistica for Windows, Statsoft) or Bonferroni post-hoc tests (OriginLab Corp.). Comparisons of $I_{Ca_T}$ amplitudes obtained in control, Ni$^{2+}$-treated, and Ni$^{2+}$ plus TTX-treated groups over voltage range from $-110$ to $+10$ mV were performed using two-way ANOVA (Origin 7.5). A $P$ value of $<0.05$ was considered to indicate statistical significance.

Online Supplemental Material

The online supplemental material (available at http://www.jgp.org/cgi/content/full/jgp.200709883/DC1) includes data showing the effects of low concentrations of external Na$^{+}$ (0.05 to 4 mM) and TTX (30 μM) on the low threshold inward Ca$^{2+}$ current recorded from dog atrial myocytes, and the impact of buffering heavy metals with EDTA (100 μM) on the responses of expressed CaV3.1–CaV3.3 to STX and TTX. The data presented in Fig. S1 provides additional evidence that the low threshold Ca$^{2+}$ current in atrial myocytes consists of a T-type Ca$^{2+}$ current only and is not the result of a TTX-sensitive Ca$^{2+}$ current ($I_{Ca(TTX)}$) as identified in cardiac myocytes of some species in the absence of external Na$^{+}$.

Fig. S2 reports the lack of effect of EDTA on the responses to STX and TTX of T-type Ca$^{2+}$ current evoked by the expression of either

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**Figure 2.** Biophysical and pharmacological properties of low and high voltage-activated Ca$^{2+}$ currents in dog atrium. (A) Representative current traces recorded during steps to indicated voltages by the voltage clamp protocol shown at the top. (B) Mean $I-V$ curves of total $I_{Ca}$ ($I_{Ca,90}$) elicited by the first test pulse (TP1), and the low voltage-activated $I_{Ca_T}$ (LVA) obtained by subtracting the currents elicited by TP2 from those evoked by TP1 ($n = 6$). LVA peaks at $-30$ mV and reverses between $+30$ and $+60$ mV, consistent with $I_{Ca_T}$ previously described in cardiac cells. (C) Four sets of superimposed typical current traces recorded from different cells are shown. Currents were elicited by steps to $-30$ mV (or to $-40$ mV for the right top set of traces) from HP = $-90$ mV before and after application of different compounds as indicated. Short bars to left of current traces indicate zero current level. Low threshold inward current was inhibited by nickel (Ni$^{2+}$) and mibefradil (Mib), two putative T-type Ca$^{2+}$ channel blockers, but was insensitive to the Na$^{+}$ channel antagonist tetrodotoxin (TTX) and lidocaine (Lido). (D) Bar graph summarizing the effects of the various compounds for experiments similar to those illustrated in C. As in C, each compound was tested in different cells. Peak inward current is expressed as mean ± SEM % relative to the control value (filled bar). LVA was inhibited 90% by 50 μM Ni$^{2+}$ (Control: $-132 ± 19$ pA, Ni$^{2+}$: $-10 ± 2$ pA, $n = 16$) and 57% by 5 μM mibefradil (Control: $-73 ± 7$ pA, TTX: $-70 ± 8$ pA, $n = 5$) or 500 μM lidocaine (Control: $-116 ± 21$ pA, lidocaine: $-111 ± 17$ pA, $n = 3$). The source of TTX for all these experiments was Calbiochem.

were given to the cells from a holding potential of $-90$ mV to monitor the magnitude of the current.

**Reagents**

All reagents were purchased from Sigma-Aldrich or Merck KGaA. Tetrodotoxin (TTX) was purchased from Calbiochem (dog atrial cell experiments) or Alomone Laboratories (dog atrial and transfected HEK cell experiments), whereas saxitoxin (STX) was obtained from Calbiochem (dog atrial cell experiments), Sigma-Aldrich (dog atrial cell experiments), or from the Institute for Marine Biosciences, NRC-IMB (Halifax, Nova Scotia, Canada; transfected HEK cell experiments).

**Statistical Analysis**

Membrane currents were analyzed with Clampfit 8.0 and/or 9.2 (Molecular Devices). Offline leak subtraction was performed in Clampfit by digital subtraction using scaled currents that did not elicit time-dependent currents. The software Origin 7.5 (OriginLab Corp.) was used to calculate the best fit to the dose-dependent response of $I_{Ca_T}$ to Ni$^{2+}$, TTX, and STX using weighted least-squares fitting routines to a Logistic function.

All pooled data are expressed as means ± SEM. Both unpaired and paired Students $t$ tests were used to determine the statistical difference between two means (Statistica for Windows 99, version 5.5). Comparisons among multiple means were performed using one-way ANOVA with a Newman-Keuls (Statistica for Windows, Statsoft) or Bonferroni post-hoc tests (OriginLab Corp.). Comparisons of $I_{Ca_T}$ amplitudes obtained in control, Ni$^{2+}$-treated, and Ni$^{2+}$ plus TTX-treated groups over voltage range from $-40$ to $-10$ mV were performed using two-way ANOVA (Origin 7.5). A $P$ value of $<0.05$ was considered to indicate statistical significance.

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The online supplemental material (available at http://www.jgp.org/cgi/content/full/jgp.200709883/DC1) includes data showing the effects of low concentrations of external Na$^{+}$ (0.05 to 4 mM) and TTX (30 μM) on the low threshold inward Ca$^{2+}$ current recorded from dog atrial myocytes, and the impact of buffering heavy metals with EDTA (100 μM) on the responses of expressed CaV3.1–CaV3.3 to STX and TTX. The data presented in Fig. S1 provides additional evidence that the low threshold Ca$^{2+}$ current in atrial myocytes consists of a T-type Ca$^{2+}$ current only and is not the result of a TTX-sensitive Ca$^{2+}$ current ($I_{Ca(TTX)}$) as identified in cardiac myocytes of some species in the absence of external Na$^{+}$. Fig. S2 reports the lack of effect of EDTA on the responses to STX and TTX of T-type Ca$^{2+}$ current evoked by the expression of either
one of the three Ca_{V}3 subunits in HEK-293 cells, which indicates that the effects of the toxins on I_{CaT} are not due to the presence of heavy metal contaminants in the commercial toxin samples.

**RESULTS**

Identification of I_{CaT} in Canine Atrial Cells

We first examined whether low and high threshold inward Ca^{2+} currents could be unequivocally identified in canine atrial cells superfused with a Na^{+}-free medium containing physiological Ca^{2+} concentration (1.8 mM). Fig. 2 A shows three sample membrane currents recorded in the same cell using the protocol shown at the top of this panel. A fast transient inward current was apparent at −50 and −30 mV during an initial 200-ms test pulse (TP1) from HP = −90 mV, and was completely inactivated during the second test pulse (TP2) to −30 mV (now elicited from a preconditioning potential of −50 mV). The inward current elicited by TP1 to −10 mV was clearly larger, and activated and inactivated more quickly than that evoked during TP2. Fig. 2 B shows the mean current–voltage (I–V) relationships (n = 6) for peak inward current recorded during TP1 ranging from −60 to +70 mV (filled circles), and low voltage–activated Ca^{2+} current (LVA) obtained from digital subtraction of the currents evoked by TP2 from those elicited by TP1 (empty circles). Examination of the I–V curve of the LVA shows that this current activated between −60 and −50 mV, peaked near −30 mV, and reversed between +30 and +60 mV. In contrast, the high threshold inward current activated between −40 and −30 mV, reached a maximum between 0 and +10 mV, and reversed near +50 mV.

The pharmacological data presented in Fig. 2 (C and D) support the contention that LVA is mainly composed of I_{CaT} and is not due to a Ca^{2+} entry pathway that is sensitive to block by TTX (so-called I_{Ca(TTX)}; Lemaire et al., 1995; Aggarwal et al., 1997; Cole et al., 1997; Santana et al., 1998; Sha et al., 2003). LVA was selectively inhibited by the T-type Ca^{2+} channel blockers Ni^{2+} (50 μM; P < 0.05) and mibefradil (5 μM; P < 0.05), but was unaltered by 30 μM TTX (P > 0.05) or 500 μM of the local anesthetic lidocaine (P > 0.05), both of which block Na^{+} current (I_{Na}). Another set of experiments also shows that LVA elicited at negative potentials is unaffected by 50 μM Na^{+} in the external medium (P > 0.05), a concentration previously shown to partially inhibit I_{Ca(TTX)} (Cole et al., 1997; Alvarez et al., 2004), while higher concentrations of Na^{+} led to the appearance of a faster TTX-sensitive inward current consistent with cardiac I_{Na} (Fig. S1, available at http://www.jgp.org/cgi/content/full/jgp.200709883/DC1). These results are consistent

Figure 3. TTX, but not lidocaine relieves the blockade of I_{CaT} by Ni^{2+} in dog atrium. (A) Current traces elicited by voltage steps from −90 to −30 mV were recorded in the absence and presence of 50 μM Ni^{2+}, and after the further addition of 30 μM TTX. Notice that TTX partially alleviated the block mediated by nickel. (B) Sample current traces elicited by voltage steps from −90 to −30 mV recorded in the absence and presence of 50 μM Ni^{2+}, and after the further addition of 100 μM lidocaine (Lido). (C) Mean I–V relationships for peak inward current evoked by steps ranging from −50 to +50 mV from HP = −90 mV in control conditions (filled circles), after the addition of 50 μM Ni^{2+} (empty circles) and in the combined presence of Ni^{2+} and TTX (filled triangles; n = 7). Inset shows an expanded portion of the I–V to better illustrate the effects of TTX. (D) Voltage dependence of TTX-sensitive current derived from C by digital subtraction of current recorded in the presence of Ni^{2+} from that recorded in the presence of both Ni^{2+} and TTX. The source of TTX for all these experiments was Calbiochem.
interact with $I_{CaT}$ but in a very peculiar manner. Fig. 3 A shows a sample experiment demonstrating this effect. In control conditions, a 200-ms step to $-110$ mV from $-90$ mV evoked a typical $I_{CaT}$, which was inhibited $>80\%$ by 50 μM Ni$^{2+}$. In the continued presence of Ni$^{2+}$, application of 30 μM TTX partially relieved the block exerted by Ni$^{2+}$ ($P < 0.001$). This sustained effect was consistently with the existence of two types of inward Ca$^{2+}$ current in this cardiac preparation with distinct kinetics and voltage dependence resembling those previously reported in other systems: low threshold T-type ($I_{CaT}$) and high threshold L-type ($I_{CaL}$) Ca$^{2+}$ currents (Bean, 1985; Mitra and Morad, 1986; McDonald et al., 1994; Fareh et al., 2001).

Relief of Ni$^{2+}$-induced Blockade of Native $I_{CaT}$ by TTX

While examining the pharmacological profile of the LVA in our preparation, we found that TTX does in fact interact with $I_{CaT}$ but in a very peculiar manner. Fig. 3 A shows a sample experiment demonstrating this effect. In control conditions, a 200-ms step to $-30$ mV from $-90$ mV evoked a typical $I_{CaT}$, which was inhibited $>80\%$ by 50 μM Ni$^{2+}$. In the continued presence of Ni$^{2+}$, application of 30 μM TTX partially relieved the block exerted by Ni$^{2+}$ ($P < 0.001$). This sustained effect was consistently

Figure 4. Concentration dependence of TTX relief of $I_{CaT}$ block by Ni$^{2+}$ in dog atrial myocytes. (A) Typical current traces elicited by voltage steps from $-90$ to $-40$ mV under control conditions (Control, filled circle), in the presence of 50 μM Ni$^{2+}$ alone (empty circle), with different concentrations of TTX in the continued presence of Ni$^{2+}$ (empty square, triangle, and diamond, and cross) and after washout of all drugs (filled square). (B) Dose–response curve of TTX relief of block of $I_{CaT}$ by Ni$^{2+}$ obtained from two cells. The curve represents a sigmoidal fit to the data points, yielding an IC$_{50}$ of 33 μM for the TTX relief of Ni$^{2+}$ block on the channel. Each data point is a mean ± SEM of fractional $I_{CaT}$ ([Ni$^{2+}$ + TTX]/[Control]). (C) Representative current traces elicited by voltage steps from $-90$ to $-40$ mV in two different cells exposed to various concentrations of Ni$^{2+}$, in the absence (left) or presence (right) of 30 μM TTX. (D) Dose–response curves for Ni$^{2+}$ block of $I_{CaT}$ with or without TTX. $n = 5$ cells/group. The source of TTX for all these experiments was Calbiochem.

Figure 5. Effects of STX on $I_{CaT}$ and $I_{CaL}$ in dog atrial myocytes. (A) Current traces recorded during steps from $-90$ to $-40$ mV (inset) in a typical cell in the absence (Control, filled circle) or presence of 50 μM Ni$^{2+}$ (empty circle), and after the further addition of 1 μM saxitoxin (STX, empty square). 1 μM STX abolished the residual current left in the presence of 50 μM Ni$^{2+}$. Similar results were obtained in three cells. (B) Current traces elicited by voltage steps from $-90$ to $-40$ mV (inset) in a typical cell exposed to increasing concentrations of STX (empty symbols and cross). As evident, 10 μM STX was sufficient to abolish $I_{CaT}$. (C) Dose–response curves for STX inhibition of $I_{CaT}$ and $I_{CaL}$, with estimated EC$_{50}$s of 185 nM for $I_{CaT}$ (filled circles) and 1.6 μM for $I_{CaL}$ (empty circles). The effects of STX on $I_{CaT}$ and $I_{CaL}$ were evaluated in the same cell using a triple-pulse protocol that was composed of the double-pulse protocol (see Materials and methods) for $I_{CaT}$ recording and a third step from $-50$ to $+10$ mV separated by a 500-ms interval to elicit $I_{CaL}$ ($n = 6$). The source of STX for all these experiments was Calbiochem.
observed in all 16 myocytes studied and took place regardless of the order of application of Ni\textsuperscript{2+} or TTX. Fig. 3 B illustrates that the effect of TTX on Ni\textsuperscript{2+}-induced block of I\textsubscript{CaT} was not shared by the structurally unrelated Na\textsuperscript{+} channel antagonist lidocaine. Moreover, the inhibition of I\textsubscript{CaT} by an 8–10-min exposure to 5 μM mibefradil could not be reversed by 30 μM TTX; the amplitude of I\textsubscript{CaT} elicited at −30 mV from HP = −90 mV was −67 ± 14 pA in the presence of 5 μM mibefradil, and −61 ± 13 pA after exposure to mibefradil and TTX (n = 3; P > 0.05). Fig. 3 C shows mean I–V relationships for peak inward current recorded from HP = −90 mV in control conditions, after the addition of Ni\textsuperscript{2+}, and in the combined presence of Ni\textsuperscript{2+} and 30 μM TTX. Nickel abolished I\textsubscript{CaT} and partially suppressed I\textsubscript{CaL}; for example, the inward current recorded at +20 mV, which mainly consists of I\textsubscript{CaL} (see Fig. 2 B), was inhibited 46% by Ni\textsuperscript{2+}, a result consistent with previous studies in cardiac myocytes (McDonald et al., 1994; Hobai et al., 2000). Most importantly, this plot shows that the partial relief of Ni\textsuperscript{2+} block by TTX is mainly apparent between −40 and −10 mV, which supports the idea that TTX interacts with I\textsubscript{CaT} but not I\textsubscript{CaL}. Two-way ANOVA analysis revealed a significant difference (P < 0.05) of I\textsubscript{CaT} densities obtained in control, Ni\textsuperscript{2+}-treated, and Ni\textsuperscript{2+} plus TTX-treated conditions over the voltage range from −40 to −10 mV. It also suggests that the relief of the Ni\textsuperscript{2+} block of I\textsubscript{CaT} by TTX is voltage dependent, being attenuated by membrane depolarization. This observation would be consistent with an electrostatic repulsion of the TTX molecule as it carries a net positive charge at physiological pH.

TTX dose dependently relieved the block of I\textsubscript{CaT} induced by 50 μM Ni\textsuperscript{2+} (Fig. 4 A). Data pooled from several experiments showed that TTX relieved the block produced by Ni\textsuperscript{2+} with an IC\textsubscript{50} of 33 μM (Fig. 4 B). We next explored the concentration dependence of the block exerted by Ni\textsuperscript{2+} in the presence and absence of TTX. Fig. 4 C shows representative current recordings obtained in two different cells. Both cells were exposed in sequence to increasing concentrations of Ni\textsuperscript{2+} ranging from 1 to 200 μM, with (righthand side) or without 30 μM TTX (lefthand side) throughout. These experiments clearly show that Ni\textsuperscript{2+} was more effective at inhibiting I\textsubscript{CaT} in the absence than in the presence of TTX. Fig. 4 D shows mean data from such similar experiments. The Na\textsuperscript{+} channel toxin induced a rightward shift of the dose–response curve without affecting the slope of the relationship; the IC\textsubscript{50} was 7.6 and 30 μM in the absence and presence of TTX, respectively. These results support the notion that TTX interferes with Ni\textsuperscript{2+} block of the I\textsubscript{CaT} channel through a competitive interaction.

The commercial source of the TTX used in the experiments shown in Figs. 3 and 4 was Calbiochem. We also examined the effects of TTX from a different source...
In contrast to the complete STX block observed in our study, they found that maximum block was partial (50%). STX (1 μM) from Sigma-Aldrich blocked I_{CaT} by 51.5 ± 4.2% (n = 7; P < 0.01), somewhat less potently than that from Calbiochem (Fig. 5C; 83% block) but similar to that produced by STX from the Institute for Marine Biosciences on CaV3.2 (51% block; see Fig. 7).

Effects of TTX and STX on Transiently Expressed I_{CaT}
It is now well established that cardiac I_{CaT} results mainly from the expression of CaV3.1 and/or CaV3.2 (Perez-Reyes, 2003; Vassort et al., 2006), although one study reported the expression of mRNA transcripts for CaV3.1, CaV3.2, and CaV3.3 in dog atrium, ventricle, and Purkinje fibers (Han et al., 2002). Figure 6A shows sample recordings of I_{CaT} from hCaV3.1-transfected cells elicited by repetitive 250-ms steps to 40 mV from a holding potential of 90 mV. Application of Ni^{2+} (550 μM) inhibited CaV3.1 current by 75%. Such a high Ni^{2+} concentration was necessary to achieve substantial block of the current in accordance with the reported sensitivity of CaV3.1 to this blocker (IC_{50} = 250 μM; Perez-Reyes, 2003). Similar to cardiac I_{CaT} (Fig. 2C and Fig. 3), 30 μM TTX had no direct effect on the current (middle set of traces). However, the same concentration of Ni^{2+} was clearly less effective at blocking CaV3.1 in the presence of TTX (middle recordings). In contrast to the complete STX block observed in our study, they found that maximum block was partial (~50%). STX (1 μM) from Sigma-Aldrich blocked I_{CaT} by 51.5 ± 4.2% (n = 7; P < 0.01), somewhat less potently than that from Calbiochem (Fig. 5C; ~83% block) but similar to that produced by STX from the Institute for Marine Biosciences on CaV3.2 (~51% block; see Fig. 7).

Saxitoxin Inhibits both Native I_{CaT} and I_{CaL}
We next tested the hypothesis that another marine toxin, saxitoxin (STX), which like TTX blocks Na^{+} channels (Hille, 2001), might also influence I_{CaT} in canine atrial myocytes. As illustrated in Fig. 5 and in contrast to TTX, STX (Calbiochem) potently inhibited I_{CaT} in the presence of 50 μM Ni^{2+} (Fig. 5A; P < 0.05), and dose dependently reduced this current in the absence of this divalent cation (Fig. 5B). Fig. 5C shows the dose–response curves for the inhibition of I_{CaT} and I_{CaL} by STX. In these experiments, the effects of STX on the two inward currents were evaluated in the same cell using a triple-pulse protocol. The magnitude of T-type Ca^{2+} current was first estimated by the double-pulse protocol described in the Materials and methods. Saxitoxin inhibited I_{CaT} with an IC_{50} = 185 nM. The toxin also suppressed I_{CaL} in a concentration-dependent manner with an IC_{50} = 1.6 μM, which is a slightly less potent inhibition than that reported by Su et al. (2004) for STX block of I_{CaL} in mouse ventricular myocytes (K_{i} ~0.3 μM). In contrast to the complete STX block observed in our study, they found that maximum block was partial (~50%). STX (1 μM) from Sigma-Aldrich blocked I_{CaT} by 51.5 ± 4.2% (n = 7; P < 0.01), somewhat less potently than that from Calbiochem (Fig. 5C; ~83% block) but similar to that produced by STX from the Institute for Marine Biosciences on CaV3.2 (~51% block; see Fig. 7).
addition of Ni$^{2+}$ led to further inhibition of the current. Fig. 6 B provides a summary of mean data from five to eight experiments with TTX and STX, respectively. A similar analysis was performed on CaV3.2 and the results are displayed in Fig. 7 following an identical format to the results presented in Fig. 6. A reduced concentration of Ni$^{2+}$ (15 μM) was used to probe CaV3.2 channels because of the higher affinity of Ni$^{2+}$ for CaV3.2 (IC$\text{_{50}}$ = 12 μM; Perez-Reyes, 2003). A similar trend was observed for this Ca$^{2+}$ channel isoform, including lack of effect of TTX, attenuation of Ni$^{2+}$ blockade by TTX, direct inhibition by STX, and maximal block by the combined addition of Ni$^{2+}$ and STX, which was not significantly different from the level of block achieved by Ni$^{2+}$ alone (Fig. 7 B). In contrast to the lack of effect of TTX on CaV3.1 and CaV3.2, the toxin at a concentration of 30 μM inhibited I$_{\text{CaT}}$ mediated by CaV3.3 by >60% (Fig. 8 A, middle set of traces). The addition of 750 μM Ni$^{2+}$ in the presence of TTX led to further block of I$_{\text{CaT}}$, which was similar to that produced by Ni$^{2+}$ alone (Fig. 8 A, leftward set of traces). The higher concentration of Ni$^{2+}$ was chosen to produce similar block of I$_{\text{CaT}}$ and is consistent, as for I$_{\text{CaT}}$ arising from the expression of CaV3.1, with the low affinity of CaV3.3-mediated I$_{\text{CaT}}$ for Ni$^{2+}$ (IC$\text{_{50}}$ = 216 μM; Perez-Reyes, 2003). Finally, 1 μM STX produced similar inhibitory effects on CaV3.3-elicited I$_{\text{CaT}}$ to those observed on currents arising from expressed CaV3.1 (Fig. 6) or CaV3.2 (Fig. 7), with a similar response to Ni$^{2+}$ in the presence of the toxin (Fig. 8 A, rightward set of traces). Fig. 8 B summarizes the data pooled from six to eight cells. All observed effects of the toxins, with or without Ni$^{2+}$, on CaV3-induced I$_{\text{CaT}}$ were unaffected by the addition in the superfusate of 100 μM EDTA to chelate heavy metals that might contaminate the toxin samples (Fig. S2). These results are in agreement with the paradigm that the well-characterized Na+ channel toxins TTX and STX interact with the α-subunit of native and cloned T-type Ca$^{2+}$ channels.

**DISCUSSION**

In the present study, we provide evidence that the Na+ channel antagonists tetrodotoxin and saxitoxin both interact with native and cloned T-type Ca$^{2+}$ channels. Our data indicate that while TTX produced no effect on cardiac I$_{\text{CaT}}$, and CaV3.1 and CaV3.2, the toxin significantly attenuated the block produced by Ni$^{2+}$. In contrast, STX exerted relatively high affinity block of cardiac I$_{\text{CaT}}$ and CaV3.1–3.3, and did not affect Ni$^{2+}$-induced inhibition of I$_{\text{CaT}}$; TTX produced similar effects on CaV3.3. These results point to common toxin-binding sites on I$_{\text{CaT}}$ and I$_{\text{Na}}$, channels and support the hypothesis that voltage-dependent T-type Ca$^{2+}$ and Na+ channels may have evolved from a common ancestor.
Low Threshold Inward Current in Dog Atrial Myocytes is a T-type Ca\textsuperscript{2+} Current

In the absence of external sodium ions, low voltage-activated inward calcium current (LVA) was consistently recorded in all canine atrial myocytes studied. With physiological Ca\textsuperscript{2+} in the bathing medium, this current shared many properties with T-type Ca\textsuperscript{2+} current measured in cardiac muscle cells (Bean, 1985; Mitra and Morad, 1986; McDonald et al., 1994; Zhang et al., 2000; Fareh et al., 2001) and \alpha_{T1} and \alpha_{T2} subunits expressed in mammalian cell lines (Cribbs et al., 1998; Monteil et al., 2000; Satin and Cribbs, 2000; Cribbs et al., 2001); the current (1) activated at potentials more negative than −40 mV and was completely inactivated at a holding potential of −50 mV, (2) displayed faster kinetics of activation and inactivation than L-type Ca\textsuperscript{2+} current, and (3) was blocked by mibebradil or Ni\textsuperscript{2+}. LVA was likely not the product of a TTX-sensitive Ca\textsuperscript{2+} entry mechanism since the latter pathway has been shown to be inhibited by TTX (Lemaire et al., 1995; Cole et al., 1997; Alvarez et al., 2004) or low concentrations (10−200 μM) of external Na\textsuperscript{+} (Cole et al., 1997; Alvarez et al., 2004), but is unaffected by Ni\textsuperscript{2+} concentrations up to 250 μM (Lemaire et al., 1995; Aggarwal et al., 1997; Cole et al., 1997; Heubach et al., 2000; Alvarez et al., 2004). We therefore conclude that LVA in canine atrial cells is generated by a T-type Ca\textsuperscript{2+} channel that is most likely primarily encoded by Ca\textsubscript{v}3.2 since the IC\textsubscript{50} for the block of I\textsubscript{CaT} by Ni\textsuperscript{2+} (7.6 μM) in our study is similar to the range of values measured for expressed Ca\textsubscript{v}3.2 but more than 20-fold lower than Ca\textsubscript{v}3.1 (Lee et al., 1999; Jeong et al., 2003; Perez-Reyes, 2003; Kang et al., 2006), the other major subunit known to be expressed in heart (Perez-Reyes, 2003; Vassort et al., 2006).

Na\textsuperscript{+} Channel Toxins Interact with T-Type Ca\textsuperscript{2+} Channels

The most salient observation of the present study was the demonstration that TTX and STX interact with I\textsubscript{CaT}. The nature of the TTX interaction is very peculiar in that the toxin does not apparently influence the voltage dependence and kinetics of cardiac I\textsubscript{CaT} but reduces the efficacy of Ni\textsuperscript{2+}-induced block of this current. With the exception of Ca\textsubscript{v}3.3, which was blocked by TTX, the toxin produced similar effects on I\textsubscript{CaT} arising from Ca\textsubscript{v}3.1 or Ca\textsubscript{v}3.2 expressed in HEK-293 cells. It appears unlikely that the effects of both toxins would be due to the presence of undesired contaminants as suggested by Jones and Marks (1989), who reported that STX produced a variable inhibition of a low threshold Ca\textsuperscript{2+} current in bullfrog sympathetic neurons whose potency varied with different batches of the toxin. We tested TTX and STX from respectively two and three different commercial sources and obtained results that were quantitatively similar. The responses of I\textsubscript{CaT} to both toxins were also unaffected by buffering heavy metals with EDTA, arguing against the possibility that such metals significantly contaminate the commercial toxin preparations.

Although it has been suggested that part of the inhibitory activity of Ni\textsuperscript{2+} takes place in the pore region between S5 and S6 (Lee et al., 1999), a more recent study from the same group postulated that His191 of Ca\textsubscript{v}3.2, as opposed to Gln172 in Ca\textsubscript{v}3.1 located in the extracellular loop between S3 and S4 of domain I, is responsible for the ~60-fold higher sensitivity of this channel to Ni\textsuperscript{2+} than Ca\textsubscript{v}3.1 (Kang et al., 2006). In view of the location of this site in close proximity to the voltage sensor in S4 and its remote location from the P-loop, combined with the fact that the block by Ni\textsuperscript{2+} was use independent, Kang et al. (2006) proposed that the divalent cation exerts its inhibitory activity by an effect on gating resulting in pore closure. Whether TTX is able to bind to this site is unknown. However, our data clearly showed that TTX competitively antagonized without mimicking the effect of Ni\textsuperscript{2+} on native I\textsubscript{CaT} an effect that was also observed with Ca\textsubscript{v}3.1 and Ca\textsubscript{v}3.2. Such an interaction could potentially explain why the toxin did not exert any effect on native or these cloned I\textsubscript{CaT} in the absence of the blocker. In this scheme, STX would not only bind to the same site with higher affinity, presumably facilitated by its additional positive charge, but would also imitate Ni\textsuperscript{2+} by mediating block of the pore. However, histidine at that same position is also replaced by a glutamine (Gln172) in Ca\textsubscript{v}3.3 and yet STX blocked Ca\textsubscript{v}3.2 and Ca\textsubscript{v}3.3 with nearly equal efficacies, and TTX also blocked this current.

An alternative hypothesis is that TTX binds to, as it does on Na\textsuperscript{+} channels, a region within the outer vestibule near the pore of I\textsubscript{CaT} channels. TTX binding would not obstruct Ca\textsuperscript{2+} binding and flux through the pore but would partially occlude the binding of Ni\textsuperscript{2+} through a competitive interaction, or alternatively by a remote alteration of the structure of the Ni\textsuperscript{2+} binding site between S3 and S4 of domain I (His191) when the toxin occupies the pore. On the other hand, STX would bind these channels with higher affinity than TTX, perhaps due to the presence of an additional positively charged guanidinium group, resulting in reduced Ca\textsuperscript{2+} entry through the pore. The alignment of the pore region of the four domains of several Na\textsuperscript{+} channel subtypes and Ca\textsubscript{v}3.1, Ca\textsubscript{v}3.2, and Ca\textsubscript{v}3.3 is displayed in Fig. 1. The figure highlights in red the critical residues reported to be involved in TTX and STX binding (Terlau et al., 1991). Based on the results of single point mutations, the amino acids of the four repeat domains forming the SF of Na\textsuperscript{+} channels (0') and those downstream from the N terminus by four positions (3') are postulated to form two rings of charges that are critical for toxin interaction with the pore (Terlau et al., 1991; Hille, 2001). Lipkind and Fozzard (1994) performed molecular modeling of the interaction of TTX with the rat brain II and skeletal muscle (Na\textsubscript{v}1.4) Na\textsuperscript{+} channels, which are sensitive
to TTX in the nanomolar range, and suggested that the positive charge of the guanidinium group of TTX interacts electrostatically with three carboxyl groups of Domains I (D384, E387) and II (E942), while the hydroxyl groups of C10 and C11 of TTX would form hydrogen bonds with Glu 945 of Domain II. It has been demonstrated that the aromatic residue Tyr or Phe of TTX-sensitive channels located immediately adjacent to Asp 384 of Domain I is responsible for conferring high sensitivity of these Na’ channels to the toxin. This residue is substituted by a cysteine in the cardiac-specific TTX-resistant isoform NaV1.5 (Backx et al., 1992; Satin et al., 1992; Fig. 1) or by a Ser in the TTX-insensitive Na’ channels found in the nervous system (NaV1.8 and NaV1.9; Fig. 1). The cysteine at that position is also responsible for the higher sensitivity of the cardiac-type Na’ channel to group IIb metals such as Cd2+ and Zn2+ (Satin et al., 1992; Backx et al., 1992). The model of Lipkind and Fozzard (1994) was also able to predict the important role played by the aromatic residue that stabilized toxin binding most likely by an interaction with its ring structure. It was predicted that due to the presence of a second guanidinium group, the additional positive charge would also interact with Asp 1717 of Domains IV. In a subsequent study examining differences in interactions of the two toxins, Penzotti et al. (1998) showed that while mutations of the selectivity residues (DEKA) produced equivalent effects on both toxins, the aromatic residue (C, Y, or F) adjacent to the Asp (D400 of NaV1.4; Fig. 1) of Domain I involved in selectivity is more important for TTX binding, while the outer residues of Domains II (E758 of NaV1.4) and IV (D1540 of NaV1.4) play a more critical function in STX binding. Using various analogues of STX, Choudhary et al. (2002) confirmed the critical role of the outer vestibular Asp1539 of Domain IV (Fig. 1) for the interaction of C11 of STX with NaV1.4. A more recent study revisited the possibility of an interaction of TTX with the same residue of Domain IV (Choudhary et al., 2003). The study showed that the hydroxyl group at C11 of TTX probably interacts through hydrogen bonding with the outer vestibular Asp residue of Domain IV (Choudhary et al., 2003). According to this model, the guanidinium group of TTX would interact with the selectivity filter, and the toxin would be docked tilted across the outer vestibule stabilized by hydrogen bonds between C10 and Glu403 of Domain I, and C11 with Asp1539 of Domain IV. When comparing the pore residues of CaV3.1–CaV3.3 with mammalian TTX-sensitive (NaV1.4) and TTX-insensitive (NaV1.5, NaV1.8 and NaV1.9) Na’ channels (Fig. 1), although many identical amino acids as well as equivalent substitutions can be identified, in particular the residues involved in channel selectivity (SF in Fig. 1) and toxin binding, all outer ring residues critical for TTX and STX binding to Na’ are replaced by either neutral, hydrophobic, or positively charged amino acids in T-type Ca2+ channels. This could form the basis for the reduced apparent affinity of STX for native and cloned T-type Ca2+ channels. However, this scheme would be difficult to reconcile with the lack of effect of TTX on native and two of the CaV3 channels since binding of TTX to the selectivity filter residues Glu and Asp would be expected to alter ion permeation, which was not observed, as both native and CaV3.1 and CaV3.2-mediated T-type Ca2+ channels were unaffected by TTX in the absence of Ni2+. The fact that TTX blocks CaV3.3 but not CaV3.1 and CaV3.2 is difficult to explain on the basis of the primary amino acid sequence forming the pores as they are nearly identical with the exception perhaps of a neutral Gln (identified in black in Fig. 1) replacing the positively charged Arg at −5′ position from the selectivity filter of Domain IV. Interestingly, this Gln residue is also present in the TTX-insensitive mammalian Na’ channels (NaV1.5, NaV1.8, and NaV1.9). Clearly a thorough mutational analysis will be necessary to determine the possible contribution of His191 between S3 and S4 of Domain I and that of P-loop residues of all domains in the binding of Na’ channel toxins to T-type Ca2+ channels.

Evolutionary Properties of Na’ and T-type Ca2+ Channels

Voltage-dependent Na’ and Ca2+ channels have been hypothesized to have evolved from a common ancestor (Hille, 2001). This hypothesis is supported by comparing the sequences of cloned Na’ and T-type Ca2+ channels and their respective functional properties. Our data further extend this hypothesis by providing evidence that Na’ channel toxins also interact with native cardiac and cloned T-type Ca2+ channels. A link between the structure of the pore and the gating of CaV3.1 has recently been established (Talavera et al., 2003). Divalent cations compete with TTX and STX for common binding sites along the inner pore of Na’ channels (Doyle et al., 1993), which is similar to the rightward shift by TTX of the dose–response relationship of the Ni2+-induced block of native IcaT and the attenuated block by Ni2+ of CaV3.1 and CaV3.2 expressed in HEK-293 cells. Geffeney et al. (2005) analyzed the Na’ channel pore residues involved in the lack of sensitivity to TTX of skeletal muscle Na’ channels of different populations of garter snake that have coevolved with toxic newt preys in California, Oregon, and Idaho. For these particular Na’ channels, all of the “classical” residues in Domains I, II, and III involved in the TTX sensitivity of mammalian Na’ channels were identical to those of highly TTX-sensitive Na’ channels (e.g., NaV1.4 in Fig. 1) and were thus excluded to explain their TTX insensitivity. Analysis of four different populations of snake identified two major residues in the P-loop of Domain IV, Asn for Asp at position +3′, and Val for Isole at position −4′, from the selectivity filter. Geffeney et al. (2005) found this double mutation (and an additional less important one) in the Willow Creek garter snake Na’

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channel to be of prime importance in conferring extremely poor sensitivity to TTX compared with Na’ channels of other snakes. Curiously, these two identical substitutions are also found in the three T-type Ca\textsuperscript{2+} channel clones (Fig. 1). Site-directed mutagenesis experiments of these two sites and adjacent sites (e.g., Gln at –5’ position of the selectivity filter in Domain IV of Ca\textsubscript{v}3.3) combined with structural modeling of the pore should enable us to determine if they reflect pure coincidences or whether they bear any evolutionary foundation pointing toward an ancestral TTX-insensitive voltage-gated cation channel.

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Online Supplemental Material

Do Heavy Metal Contaminants Mediate the Response of T-type Ca\(^{2+}\) Channel Clones to Na\(^{+}\) Channel Toxins?

Because of the relatively low affinity of the responses of T-type Ca\(^{2+}\) channels to TTX and STX, one possible confounding factor might be the undesired presence of heavy metals in the aliquots of toxins from various commercial sources. Heavy metals block many types of channels including T-type Ca\(^{2+}\) channels (Jeong et al., 2003). We initially contacted the vendors regarding this issue and the Program Research Leader from the Institute for Marine Biosciences (NRC-IMB, Halifax, Nova Scotia, Canada) responded by saying that he was "doubtful that there could be significant concentrations of heavy metals" and that the "STX CRM is produced specifically for instrumental analytical chemistry" and is thus highly pure. We nevertheless tested the effects of EDTA, a chelator that binds many heavy metals with high affinity (e.g., Fe\(^{2+}\), Fe\(^{3+}\), Pb\(^{2+}\), Zn\(^{2+}\), Mn\(^{2+}\), Ni\(^{2+}\), Ca\(^{2+}\)) on the toxin responses of I\(_{Ca(TTX)}\). We chose a concentration of 100 µM EDTA that would have a very small impact on free Ca\(^{2+}\) concentrations (~0.1 mM reduction) and a negligible effect on free Mg\(^{2+}\) levels in our external solution. This would lower free heavy metal concentrations to levels ~10 nM (e.g., Mn\(^{2+}\)) or lower assuming that the concentration of the contaminant is 1 µM (based on the assumption that 5% of the toxin sample on a weight basis is composed of heavy metal contaminants). The buffering effect of EDTA on Ni\(^{2+}\) was compensated for by increasing the total Ni\(^{2+}\) concentration by an appropriate amount (see Materials and methods for a detailed description) in order to keep free Ni\(^{2+}\) concentration constant as

![Figure S1. Effects of low [Na\(^{+}\)]\(_{o}\) on low voltage–activated I\(_{Ca}\) in dog atrium. (A) Current traces were recorded from a typical cell and were elicited by voltage steps from -90 to -30 mV. Currents were recorded in the absence (Ctl) or presence of 50 µM, 200 µM, 1 mM of [Na\(^{+}\)]\(_{o}\), or after addition of 30 µM TTX in the presence of 1 mM [Na\(^{+}\)]\(_{o}\). This experiment was performed at room temperature. External Na\(^{+}\) at concentrations of 50 or 200 µM had no effect on the low voltage–activated I\(_{Ca}\). The faster current component recorded in the presence of higher [Na\(^{+}\)]\(_{o}\) can be abolished by TTX or washout of [Na\(^{+}\)]\(_{o}\); a result consistent with Na\(^{+}\) permeation through classical Na\(^{+}\) channels. (B) Bar graph summarizing the effects of external sodium on peak inward current recorded at -30 mV from HP = -90 mV. Peak inward current is expressed as mean ± SEM % relative to the control value (filled column, 0 mM [Na\(^{+}\)]\(_{o}\)). Not all Na\(^{+}\) concentrations could be tested in the same cell; each bar represents the mean of 6–11 experiments. W, washout. *, significantly different from control with P < 0.05.

Studies have provided evidence in cardiac myocytes for a Ca\(^{2+}\) entry pathway that is sensitive to block by TTX (so-called I\(_{Ca(TTX)}\); Lemaire et al., 1995; Aggarwal et al., 1997; Cole et al., 1997; Santana et al., 1998; Sha et al., 2003). Fig. S1 summarizes the results of typical experiments designed to explore the possibility that a component of the low voltage–activated inward Ca\(^{2+}\) current (LVA) might be attributed to Ca\(^{2+}\) permeation through a TTX-sensitive cation channel. In guinea pig ventricular myocytes, Cole et al. (1997) suggested that Ca\(^{2+}\) could permeate TTX-sensitive Na\(^{+}\) channels in the absence of Na\(^{+}\) in the superfusate. In addition to a modulation of this TTX-sensitive Ca\(^{2+}\) current by veratridine (10 or 50 µM), this group also showed that micromolar concentrations of extracellular Na\(^{+}\) were able to partially suppress this current with 10 mM Ca\(^{2+}\) as the charge carrier. Fig. S1 A shows that LVA was influenced little, if any, by exposing the myocyte to [Na\(^{+}\)]\(_{o}\) ≤ 200 µM. However, cell exposure to 1 mM [Na\(^{+}\)]\(_{o}\) led to significant enhancement of the inward current, an effect that was abolished by 30 µM TTX. Mean data for these experiments are shown in Fig. S1 B. While 50 and 200 µM [Na\(^{+}\)]\(_{o}\) failed to affect the inward current, the magnitude of the peak inward current nearly doubled and more than quadrupled in the presence of 1 and 4 mM [Na\(^{+}\)]\(_{o}\), respectively. These results suggest that the LVA is carried by T-type Ca\(^{2+}\) channels and does not appear to be contaminated by Ca\(^{2+}\) influx through either classical Na\(^{+}\) channels (Cole et al., 1997) or a separate class of Ca\(^{2+}\) channels that are sensitive to TTX (Aggarwal et al., 1997; Sha et al., 2003).
calculated by WinMAXC software (version 2.5, Chris Patton, http://www.stanford.edu/~cpatton/downloads). Fig. S2 shows mean data comparing the effects of STX and TTX in the absence (black columns) or presence (light gray columns) of EDTA on T-type Ca\textsuperscript{2+} currents elicited by the expression of all three Ca\textsubscript{V}3 subunits. Since TTX produced no significant effect on Ca\textsubscript{V}3.1 and Ca\textsubscript{V}3.2 (Figs. 6 and 7), the effects of EDTA in the presence of this toxin were not tested on these clones. Please note that each column represent the mean ± SEM % inhibition of I\textsubscript{CaT} evaluated with or without EDTA; each column had its own control so that the effects of the toxin would not be due to EDTA per se. For all three Ca\textsubscript{V}3 subunits, EDTA exerted no significant effect on any of the responses of I\textsubscript{CaT} to STX or TTX described in this report. These results argue against a possible contribution of heavy metal contaminants and suggest that the differential Na\textsuperscript{+} channel toxins on T-type Ca\textsuperscript{2+} channels are authentic.

REFERENCES

Figure S2. Effects of EDTA on the responses of cloned T-type Ca\textsuperscript{2+} channels to Na\textsuperscript{+} channel toxins. The standard protocol used to test the effects of toxins on I\textsubscript{CaT} recorded from transfected HEK-293 cells was identical for all three Ca\textsubscript{V}3 subtypes and consisted of 100-ms steps to −40 mV from HP = −90 mV. For all column pairs (black and light gray), the data were collected from different cells. As explained in the text, for each column showing the effects of TTX or STX, with or without Ni\textsuperscript{2+}, the control (no toxin) and test (toxin alone or toxin + Ni\textsuperscript{2+}) solutions both lacked EDTA (black) or both contained 100 µM EDTA (light gray). Each column represents a mean ± SEM % block of I\textsubscript{CaT} (n = 4–8) by toxin alone or toxin in the presence of Ni\textsuperscript{2+}. The numbers in parentheses reflect the number of experiments. (A–C) Pooled data obtained from HEK-293 cells transfected respectively with Ca\textsubscript{V}3.1, Ca\textsubscript{V}3.2, or Ca\textsubscript{V}3.3 as indicated. Unpaired Student’s t tests for each column pair in all three panels revealed no significant difference between control and 100 µM EDTA groups (P > 0.05).