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

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Two types of voltage-dependent Ca\(^{2+}\) channels have been identified in heart: high (I\(_{\text{CaL}}\)) and low (I\(_{\text{CaT}}\)) voltage-activated Ca\(^{2+}\) channels. In guinea pig ventricular myocytes, low voltage-activated inward current consists of I\(_{\text{CaT}}\) and a tetrodotoxin (TTX)-sensitive I\(_{\text{Ca}}\) component (I\(_{\text{Ca(TTX)}}\)). In this study, we reexamined the nature of low-threshold I\(_{\text{Ca}}\) in dog atrium, as well as whether it is affected by Na\(^+\) channel toxins. Ca\(^{2+}\) currents were recorded using the whole-cell patch clamp technique. In the absence of external Na\(^+\), a transient inward current activated near \(-50\) mV, peaked at \(-30\) mV, and reversed around +40 mV (HP = \(-90\) mV). It was unaffected by \(30 \mu M\) TTX or micromolar concentrations of external Na\(^+\), but was inhibited by \(50 \mu M\) Ni\(^{2+}\) (by \(\sim 90\%\)) or \(5 \mu M\) mibefradil (by \(\sim 50\%\)), consistent with the reported properties of I\(_{\text{CaT}}\). Addition of \(30 \mu M\) TTX in the presence of Ni\(^{2+}\) increased the current approximately fourfold (41\% of control), and shifted the dose–response curve of Ni\(^{2+}\) block to the right (IC\(_{50}\) from 7.6 to 30 \(\mu M\)). Saxitoxin (STX) at \(1 \mu M\) abolished the current left in 50 \(\mu M\) Ni\(^{2+}\). In the absence of Ni\(^{2+}\), STX potently blocked I\(_{\text{CaT}}\) (EC\(_{50}\) = 185 nM) and modestly reduced I\(_{\text{CaL}}\) (EC\(_{50}\) = 1.6 \(\mu M\)). While TTX produced no direct effect on I\(_{\text{CaT}}\) elicited by expression of hCa\(_{V}\)3.1 and hCa\(_{V}\)3.2 in HEK-293 cells, it significantly attenuated the block of Ni\(^{2+}\) (EC\(_{50}\) = 185 nM). Addition of 30 \(\mu M\) TTX increased the current approximately fourfold (IC\(_{50}\) = 185 nM) and modestly reduced I\(_{\text{CaL}}\) (EC\(_{50}\) = 1.6 \(\mu M\)). While TTX produced no direct effect on I\(_{\text{CaT}}\) elicited by expression of hCa\(_{V}\)3.1 and hCa\(_{V}\)3.2 in HEK-293 cells, it significantly attenuated the block of this current by Ni\(^{2+}\) (IC\(_{50}\) increased to 550 \(\mu M\) Ni\(^{2+}\) for Ca\(_{V}\)3.1 and 15 \(\mu M\) Ni\(^{2+}\) for Ca\(_{V}\)3.2); in contrast, \(30 \mu M\) TTX directly inhibited hCa\(_{V}\)3.4-induced I\(_{\text{CaT}}\) and the addition of 750 \(\mu M\) Ni\(^{2+}\) to the TTX-containing medium led to greater block of the current that was not significantly different than that produced by Ni\(^{2+}\) alone. 1 \(\mu M\) STX directly inhibited Ca\(_{V}\)3.1-, Ca\(_{V}\)3.2-, and Ca\(_{V}\)3.3-mediated I\(_{\text{CaT}}\) but did not enhance the ability of Ni\(^{2+}\) to block these currents. These findings provide important new implications for our understanding of structure–function relationships of I\(_{\text{CaT}}\) in heart, and further extend the hypothesis of a parallel evolution of Na\(^+\) and Ca\(^{2+}\) channels from an ancestor with common structural motifs.

INTRODUCTION

Voltage-gated Na\(^+\) and Ca\(^{2+}\) 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 \(\alpha\)-subunit of Na\(^+\) and Ca\(^{2+}\) 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 segment 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\(^+\) channels, the signature sequence of repeats I–IV is DEKA (Catterall, 2000), whereas for Ca\(^{2+}\) 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\(^{2+}\) and Ba\(^{2+}\) selectivity to Na\(^+\) channels, again supporting commonality in their evolutionary heritage (Heinemann et al., 1992).

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

Abbreviations used in this paper: HP, holding potential; LVA, low voltage-activated inward Ca\(^{2+}\) 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 (Hille, 2001).
conferred “T-type–like” blocking action of mibebradil, a putative blocker of T-type Ca2+ channels, on Na+ channels and yielded slower inactivation. An alignment of the P-loop region of the four repeats of Ca3.1, Ca3.2, and Ca3.3 thought to generate T-type Ca2+ 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 Ca2+ channels. We examined the effects of TTX and STX from several commercial sources on IcaT recorded from canine atrial myocytes or from HEK 293 cells transfected with Ca3.1, Ca3.2, or Ca3.3. TTX exerted no significant effect on the magnitude of native IcaT and IcaT generated by either Ca3.1 or Ca3.2, the two isoforms predominantly expressed in heart (Vassort et al., 2006), while it inhibited Ca3.3-induced IcaT. Interestingly, for both native IcaT and Ca3.1- or Ca3.2-induced IcaT, TTX partially relieved the blockade of this current by Ni2+. Finally, STX directly inhibited IcaT in dog atrial cells and that elicited by expression of all three Ca3 subclasses. Our studies further extend the notion that voltage-gated Na+ and Ca2+ 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 Ca2+-containing Tyrode solution, and the left atrium perfused via the circumflex artery with Tyrode solution until free of blood. The perfusate was then switched to nominally Ca2+-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 100% O2 at 37°C. Cells were dispersed by gentle trituration in Tyrode’s containing 10 μM Ca2+. The cells were kept at room temperature in Tyrode solution containing 100 μM Ca2+ and 0.1% BSA for use within 8 h. The composition of the Tyrode solution was as follows (mM): NaCl 136, KCl 5.4, MgCl2 1.0, CaCl2 2, NaH2PO4 0.33, glucose 10, and HEPES 10, pH adjusted to 7.4 with NaOH.

**Cell Culture and Transient Transfection**
sA-201 cells were grown and transiently transfected with expression plasmids for hCaV3.1, hCaV3.2, or hCaV3.3 constructs and pIF3-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% CO2) 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 hCa3.1, hCa3.2, or hCa3.3 channel α1 subunits (8 μg) and CD8 marker (1 μg) by the calcium phosphate method for 8 h. After transfection, cells were dissociated with trypsin (0.25%)-EDTA and plated on glass coverslips. Experiments were performed 24–48 h after transfection.

**Electrophysiology and Data Analysis**

Macroscopic currents were recorded from Ca2+-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, MgCl2 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 PClamp 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 gaining 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 IcaL rundown on the measurement of low threshold IcaL, 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 Ca2+ currents, and that evoked by TP2 mainly consisted of IcaL. Digital subtraction of IcaL from total Ca2+ current recorded during TP1 yielded the low threshold T-type IcaL (IcaL). The bath solution for Ca2+ current recordings contained (in mM) TEA 136, CsCl 5.4, MgCl2 1, CaCl2 1.8, HEPES 10, glucose 5.5, and 4-aminopyridine (4-AP) 2, pH adjusted to 7.35 with CsOH. In all cells studied, 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 hCa3.1, hCa3.2, or hCa3.3 were performed at room temperature with a bathing solution containing 2 mM Ca2+ (in mM): 128 CsCl, 2 CaCl2, 1.5 MgCl2, 10 HEPES, and 25 d-glucose; pH 7.4 (adjusted with CsOH). In the experiments designed to examine the effects of EDTA on the response of IcaL to Na+ channel toxins (see Fig. S2, available at http://www.jgp.org/cgi/content/full/jgp.200709883/DC1), all solutions containing Ni2+ 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 Ni2+ concentrations. In the presence of 100 μM EDTA, the total added Ni2+ concentrations to achieve either 15, 550, or 750 μM free [Ni2+]i; 150 μM total [Ni2+]i; 550 μM free [Ni2+]i; 650 μM total [Ni2+]i; 750 μM free [Ni2+]i; 850 μM total [Ni2+]i. 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): 135 CsCl, 10 EGTA, 2 CaCl2, 10 HEPES, and 1 MgCl2; pH 7.2 (adjusted with CsOH). Series resistance was compensated to 80% of the initial value. Steps of 250 ms duration to ~40 mV (5 s interval)
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_{CaT} 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_{CaT} amplitudes obtained in control, Ni^{2+}-treated, and Ni^{2+} plus TTX-treated groups over voltage range from −40 to +60 mV, consistent with I_{CaT} 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: −99 ± 6 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.

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 Ca_{V}3.1–Ca_{V}3.3 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_{v3} 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
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\(_{\text{CaT}}\)) and high threshold L-type (I\(_{\text{CaL}}\)) 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\(_{\text{CaT}}\) by TTX**

While examining the pharmacological profile of the LVA in our preparation, we found that TTX does in fact interact with I\(_{\text{CaT}}\) 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 HP = \(-90\) mV evoked a typical I\(_{\text{CaT}}\), 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
observed in all 16 myocytes studied and took place regardless of the order of application of Ni\(^{2+}\) or TTX. Fig. 3 B illustrates that the effect of TTX on Ni\(^{2+}\)-induced block of \(I_{CaT}\) was not shared by the structurally unrelated Na\(^{+}\) channel antagonist lidocaine. Moreover, the inhibition of \(I_{CaT}\) by an 8–10-min exposure to 5 μM mibefradil could not be reversed by 30 μM TTX; the amplitude of \(I_{CaT}\) 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\(^{2+}\), and in the combined presence of Ni\(^{2+}\) and 30 μM TTX. Nickel abolished \(I_{CaT}\) and partially suppressed \(I_{CaL}\); for example, the inward current recorded at +20 mV, which mainly consists of \(I_{CaL}\) (see Fig. 2 B), was inhibited 46% by Ni\(^{2+}\), 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\(^{2+}\) block by TTX was mainly apparent between −40 and +10 mV, which supports the idea that TTX interacts with \(I_{CaT}\) but not \(I_{CaL}\). Two-way ANOVA analysis revealed a significant difference (\(P < 0.05\)) of \(I_{CaT}\) densities obtained in control, Ni\(^{2+}\)-treated, and Ni\(^{2+}\) plus TTX-treated conditions over the voltage range from −40 to −10 mV. It also suggests that the relief of the Ni\(^{2+}\) block of \(I_{CaT}\) 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 \(I_{CaT}\) induced by 50 μM Ni\(^{2+}\) (Fig. 4 A). Data pooled from several experiments showed that TTX relieved the block produced by Ni\(^{2+}\) with an IC\(_{50}\) of 33 μM (Fig. 4 B). We next explored the concentration dependence of the block exerted by Ni\(^{2+}\) 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\(^{2+}\) ranging from 1 to 200 μM, with (righthand side) or without 30 μM TTX (lefthand side) throughout. These experiments clearly show that Ni\(^{2+}\) was more effective at inhibiting \(I_{CaT}\) in the absence than in the presence of TTX. Fig. 4 D shows mean data from such similar experiments. The Na\(^{+}\) channel toxin induced a rightward shift of the dose–response curve without affecting the slope of the relationship; the IC\(_{50}\) was 7.6 and 30 μM in the absence and presence of TTX, respectively. These results support the notion that TTX interferes with Ni\(^{2+}\) block of the \(I_{CaT}\) 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. 5 C; 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). Fig. 6 A 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 (Fig. 5 A; P < 0.05), and dose dependently reduced this current in the absence of this divalent cation (Fig. 5 B). Fig. 5 C 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_{CaT} in mouse ventricular myocytes (K_{d} ~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. 5 C; ~83% block) but similar to that produced by STX from the Institute for Marine Biosciences on CaV3.2 (~51% block; see Fig. 7).

Figure 7. Effects of TTX and STX on CaV3.2 expressed in HEK-293 cells. (A) Typical T-type Ca^{2+} current recordings showing the effects of 15 μM Ni^{2+} alone (left), the effects of 30 μM TTX in the presence of 15 μM Ni^{2+} (middle), and the effects of 1 μM STX in the absence or presence of 15 μM Ni^{2+} (right). Please note that the leftward and middle traces were obtained from the same cell while the ones on the right hand side were from a different experiment. As for the native T-type Ca^{2+} current, TTX attenuated the block of CaV3.2-induced current by Ni^{2+} and STX produced a significant inhibition of this current. (B) Bar graph showing pooled data from similar experiments to those shown in A. The data were expressed as mean ± SEM % block of peak inward current. The numbers in parentheses reflect the number of experiments. TTX and STX for all these experiments were respectively purchased from Alomone Laboratories and the Institute for Marine Biosciences, NRC-IMB.

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). Fig. 6 A 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 ~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. 2 C 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). Again, similar to native I_{CaT} (Fig. 5), 1 μM STX directly inhibited CaV3.1 and
addition of Ni\textsuperscript{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 Ca\textsubscript{v}3.2 and the results are displayed in Fig. 7 following an identical format to the results presented in Fig. 6. A reduced concentration of Ni\textsuperscript{2+} (15 μM) was used to probe Ca\textsubscript{v}3.2 channels because of the higher affinity of Ni\textsuperscript{2+} for Ca\textsubscript{v}3.2 (IC\textsubscript{50} = 12 μM; Perez-Reyes, 2003). A similar trend was observed for this Ca\textsuperscript{2+} channel isoform, including lack of effect of TTX, attenuation of Ni\textsuperscript{2+} blockade by TTX, direct inhibition by STX, and maximal block by the combined addition of Ni\textsuperscript{2+} and STX, which was not significantly different from the level of block achieved by Ni\textsuperscript{2+} alone (Fig. 7 B). In contrast to the lack of effect of TTX on Ca\textsubscript{v}3.1 and Ca\textsubscript{v}3.2, the toxin at a concentration of 30 μM inhibited I\textsubscript{CaT} mediated by Ca\textsubscript{v}3.3 by >60% (Fig. 8 A, middle set of traces). The addition of 750 μM Ni\textsuperscript{2+} in the presence of TTX led to further block of I\textsubscript{CaT}, which was similar to that produced by Ni\textsuperscript{2+} alone (Fig. 8 A, leftward set of traces). The higher concentration of Ni\textsuperscript{2+} was chosen to produce similar block of I\textsubscript{CaT} and is consistent, as for I\textsubscript{CaT} arising from the expression of Ca\textsubscript{v}3.1, with the low affinity of Ca\textsubscript{v}3.3-mediated I\textsubscript{CaT} for Ni\textsuperscript{2+} (IC\textsubscript{50} = 216 μM; Perez-Reyes, 2003). Finally, 1 μM STX produced similar inhibitory effects on Ca\textsubscript{v}3.3-elicited I\textsubscript{CaT} to those observed on currents arising from expressed Ca\textsubscript{v}3.1 (Fig. 6) or Ca\textsubscript{v}3.2 (Fig. 7), with a similar response to Ni\textsuperscript{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\textsuperscript{2+}, on Ca\textsubscript{v}3-induced I\textsubscript{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\textsuperscript{+} channel toxins TTX and STX interact with the α-subunit of native and cloned T-type Ca\textsuperscript{2+} channels.

**DISCUSSION**

In the present study, we provide evidence that the Na\textsuperscript{+} channel antagonists tetrodotoxin and saxitoxin both interact with native and cloned T-type Ca\textsuperscript{2+} channels. Our data indicate that while TTX produced no effect on cardiac I\textsubscript{CaT}, and Ca\textsubscript{v}3.1 and Ca\textsubscript{v}3.2, the toxin significantly attenuated the block produced by Ni\textsuperscript{2+}. In contrast, STX exerted relatively high affinity block of cardiac I\textsubscript{CaT} and Ca\textsubscript{v}3.1–3.3, and did not affect Ni\textsuperscript{2+}-induced inhibition of I\textsubscript{CaT}; TTX produced similar effects on Ca\textsubscript{v}3.3. These results point to common toxin-binding sites on I\textsubscript{CaT} and I\textsubscript{Na} channels and support the hypothesis that voltage-dependent T-type Ca\textsuperscript{2+} and Na\textsuperscript{+} channels may have evolved from a common ancestor.
Low Threshold Inward Current in Dog Atrial Myocytes is a T-type Ca\(^{2+}\) 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\(^{2+}\) in the bathing medium, this current shared many properties with T-type Ca\(^{2+}\) 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 \(\alpha_{IC\text{a}}\) and \(\alpha_{I\text{H}}\) 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\(^{2+}\) current, and (3) was blocked by mibebradil or Ni\(^{2+}\). LVA was likely not the product of a TTX-sensitive Ca\(^{2+}\) 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 \(\mu\)M) of external Na\(^+\) (Cole et al., 1997; Alvarez et al., 2004), but is unaffected by Ni\(^{2+}\) concentrations up to 250 \(\mu\)M (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\(^{2+}\) channel that is most likely primarily encoded by Ca\(_{V}\)3.2 since the IC\(_{50}\) for the block of I\(_{CaT}\) by Ni\(^{2+}\) (7.6 \(\mu\)M) in our study is similar to the range of values measured for expressed Ca\(_{V}\)3.2 but more than 20-fold lower than Ca\(_{V}\)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\(^+\) Channel Toxins Interact with T-Type Ca\(^{2+}\) Channels

The most salient observation of the present study was the demonstration that TTX and STX interact with I\(_{CaT}\). 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\(_{CaT}\) but reduces the efficacy of Ni\(^{2+}\)-induced block of this current. With the exception of Ca\(_{V}\)3.3, which was blocked by TTX, the toxin produced similar effects on I\(_{CaT}\) arising from Ca\(_{V}\)3.1 or Ca\(_{V}\)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\(^{2+}\) 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\(_{CaT}\) 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\(^{2+}\) 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\(_{V}\)3.2, as opposed to Gln172 in Ca\(_{V}\)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\(^{2+}\) than Ca\(_{V}\)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\(^{2+}\) 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 this effect of Ni\(^{2+}\) on native I\(_{CaT}\), an effect that was also observed with Ca\(_{V}\)3.1 and Ca\(_{V}\)3.2. Such an interaction could potentially explain why the toxin did not exert any effect on native or these cloned I\(_{CaT}\) 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\(^{2+}\) by mediating block of the pore. However, histidine at that same position is also replaced by a glutamine (Gln172) in Ca\(_{V}\)3.3 and yet STX blocked Ca\(_{V}\)3.2 and Ca\(_{V}\)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\(^+\) channels, a region within the outer vestibule near the pore of I\(_{CaT}\) channels. TTX binding would not obstruct Ca\(^{2+}\) binding and flux through the pore but would partially occlude the binding of Ni\(^{2+}\) through a competitive interaction, or alternatively by a remote alteration of the structure of the Ni\(^{2+}\) 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\(^{2+}\) entry through the pore. The alignment of the pore region of the four domains of several Na\(^+\) channel subtypes and Ca\(_{V}\)3.1, Ca\(_{V}\)3.2, and Ca\(_{V}\)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\(^+\) 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\(_{V}\)1.4) Na\(^+\) 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 Cα3.1–Cα3.3 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 Cα3.3 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 Cα3.1 and Cα3.3-mediated T-type Ca²⁺ channels were unaffected by TTX in the absence of Ni²⁺. The fact that TTX blocks Cα3.3 but not Cα3.1 and Cα3.2 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 Cα3.1 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 I_CaT and the attenuated block by Ni²⁺ of Cα3.1 and Cα3.2 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 Iso 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\(^{2+}\) 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\(_3\)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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