Common Mechanisms of Drug Interactions with Sodium and T-Type Calcium Channels

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ABSTRACT
Voltage-gated sodium (Nav) and calcium (Cav) channels play important roles in physiological processes, including neuronal and cardiac pacemaker activity, vascular smooth muscle contraction, and nociception. They are thought to share a common ancestry, and, in particular, T-type calcium (T-type) channels share structural similarities with Nav channels, both with regard to membrane topology and with regard to gating kinetics, including rapid inactivation. We thus reasoned that certain drugs acting on Nav channels may also modulate the activities of T-type channels. Here we show that the specific Nav1.8 blocker 5-(4-chlorophenyl-N-(3,5-dimethoxyphenyl)furan-2-carboxamide (A803467) tonically blocks T-type channels in the low micromolar range. Similarly to Nav1.8, this compound causes a significant hyperpolarizing shift in the voltage dependence of inactivation and seems to promote a slow inactivation-like phenotype. We further hypothesized that the structural similarity between T-type and Nav channels may extend to structurally similar drug-binding sites. Sequence alignment revealed several highly conserved regions between T-type and Nav channels that corresponded to drug-binding sites known to alter voltage-dependent gating kinetics. Mutation of amino acid residues in this region within human Cav3.2 T-type channels altered A803467 blocking affinity severalfold, suggesting that these sites may be exploited for the design of mixed T-type and Nav channel blockers that could potentially act synergistically to normalize aberrant neuronal activity.

Introduction

Nav channels mediate the induction and propagation of action potentials in most electrically excitable cells (Yu and Catterall, 2004). The mammalian genome encodes nine different types of Nav subunits that are functionally classified as either tetrodotoxin (TTX)-sensitive or TTX-resistant, with the latter exhibiting slower inactivation kinetics than other Nav channel subtypes (Waxman et al., 1999; Blair and Bean, 2002). The various Nav channel α subunits share a common transmembrane topology of four homologous domains that each contain six membrane-spanning helices plus a p-loop. Whereas the α subunits define the Nav channel isoform and contain all of the machinery to form a sodium-selective voltage-activated channel, their functional properties are modulated by association with ancillary β1 and β2 subunits (for review, see Isom, 2001). Mutations in various Nav channel subunits have been linked to disorders such as paramyotonia congenita, cardiac arrhythmias, epilepsy, and both hypersensitivity and insensitivity to pain, thus underscoring their importance for nerve, muscle, and heart function (Catterall et al., 2008; Jarecki et al., 2010).

Low-voltage-activated (LVA), i.e., T-type, channels, trigger low-threshold depolarizations that in turn lead to the initiation of action potentials (Cain and Snutch, 2010; Bender et al., 2012). These channels can be activated by small membrane depolarizations and display a small single-channel conductance, and compared with other Cav channel subtypes, they display rapid activation and inactivation kinetics (Perrez-Reyes, 2003). T-type channels are encoded by one of three different types of Cav3 α1 subunits (Ca v3.1, Ca v3.2, or Ca v3.3), whose membrane topology is similar to that of Nav channels (Catterall et al., 2005). Mutations in Ca v3.2 T-type channels have been linked to absence seizures (Khosravani and Zamponi, 2006; Heron et al., 2007). Moreover, up-regulation of Ca v3.2 T-type channel activity in primary afferent fibers has been linked to the development of chronic pain (for reviews, see Altier and Zamponi, 2004; Park and Luo, 2010), and T-type channel dysfunction contributes to cardiac hypertrophy (Cribbs, 2010; David et al., 2010). Both Nav channels and T-type channels contribute to neuronal excitability and to similar disorders such as epilepsy and pain. Indeed, knock-
out of Na<sub>1.8</sub> and Ca<sub>3.2</sub> results in hypersensitivity to pain (for reviews, see Wang et al., 2011; Cregg et al., 2010), suggesting the possibility that mixed Na<sub>1</sub>/T-type channel blockers may be a possible strategy for the development of new analgesics (Hildebrand et al., 2010). 5-(4-Chlorophenyl-N-(3,5-dimethoxyphenyl)furan-2-carboxamide (A803467), a new inhibitor of Na<sub>1.8</sub> channels, has been shown to be efficacious in animal pain models (McGaraughty et al., 2008). The interaction site of A803467 on the Na<sub>1</sub> channel complex is unknown, but its mode of action seems to be preferential binding to the slow inactivated state of this channel (Jarvis et al., 2007).

Here we show that A803467 inhibits T-type channels in the low micromolar range and mediates a hyperpolarizing shift in the voltage dependence of activation and inactivation. In addition, the compound promotes a slow inactivation-like phenotype. Sequence alignment between T-type and Na<sub>1</sub> channels and their local anesthetic interaction site identified key residues involved in the blocking action of this compound on T-type channels.

**Materials and Methods**

**cDNA Constructs.** Human Ca<sub>3.2</sub>, rat Ca<sub>1.2</sub>, and rat Ca<sub>2.1</sub> cDNA constructs, as well as ancillary Ca<sub>1</sub> channel subunit cDNAs were kindly provided by Dr. Terrance Snutch (University of British Columbia, Vancouver, BC, Canada). Human Ca<sub>3.3</sub> was obtained from Dr. Arnaud Monteil (Centre National de la Recherche Scientifique, Montpellier, France), and human Ca<sub>3.1</sub> was described previously by our laboratory (Beedle et al., 2002).

**Site-Directed Mutagenesis.** Site-directed mutagenesis was performed using the QuikChange II Site-Directed Mutagenesis Kit and protocols from Agilent Technologies, Inc. (Santa Clara, CA). Mutations were then verified by on-site sequencing of the full-length cDNA clone.

**Chemicals.** Unless stated otherwise, chemicals were purchased from Sigma-Aldrich (St. Louis, MO). A803467 was purchased from Tocris Bioscience (Ellisville, MO) and was dissolved in dimethyl sulfoxide (DMSO) at the stock concentration of 10 or 30 mM. Dilutions were made in external recording solutions so that the final concentration of DMSO was 0.1% or less. Ca<sub>1</sub> channel currents were not affected by 0.1% DMSO.

**tsA-201 Cell Culture and Transfection.** Human embryonic kidney tsA-201 cells were cultured and transfected using the calcium phosphate method as described previously (Altier et al., 2006). Enhanced green fluorescent protein DNA (0.5 µg of enhanced green fluorescent protein; Clontech, Mountain View, CA) was transfected as a marker. For experiments involving L- and P/Q-type channels, rCa<sub>1.2</sub> or rCa<sub>2.1</sub>, α1 subunits (3 µg), were each cotransfected with rat β1b (3 µg) or rat β4 (3 µg). For experiments involving T-type channels, hCa<sub>3.1</sub>, hCa<sub>3.2</sub>, and hCa<sub>3.3</sub>, α1 subunits were transfected alone (6 µg). Cells were resuspended with 0.25% (w/v) trypsin-EDTA (Invitrogen, Carlsbad, CA) and plated on glass coverslips a minimum of 3 to 4 h before patching and kept at 37°C and 5% CO<sub>2</sub>.

**Electrophysiology.** Whole-cell voltage-clamp recordings on tsA-201 cells were performed at room temperature 2 to 3 days after transfection. The external recording solution for calcium channel recordings contained 142 mM CaCl<sub>2</sub>, 2 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 10 mM HEPES, and 10 mM glucose, adjusted to pH 7.4 with CsOH. The external recording solution for hCa<sub>3.2</sub> Q1845 mutations and their hCa<sub>3.2</sub> wild-type controls contained 124 mM CsCl, 20 mM BaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 10 mM HEPES, and 10 mM glucose, adjusted to pH 7.4 with CsOH. For all recordings, the internal patch pipette solution contained 128.5 mM CsMeSO<sub>4</sub>, 2 mM MgCl<sub>2</sub>, 11 mM EGTA, and 10 mM HEPES adjusted to pH 7.3 with CsOH. The internal solution was supplemented with 0.6 mM GTP and 2 mM ATP, which were added directly to the internal solution immediately before use. Liquid junction potentials for the above solutions were left uncorrected.

Drugs were prepared daily in external solution and were applied locally to cells with the use of a custom-built gravity-driven micropressure system that allows solution exchanges in approximately 1 s (Feng et al., 2003). A series of vehicle-only control experiments was performed to ensure that there were no time-dependent shifts in half-activation and half-inactivation potentials, and no such changes were observed (data not shown). Currents were elicited from a holding potential of −110 mV and were measured by a conventional whole-cell patch clamp using an Axopatch 200B amplifier in combination with Clampex 9.2 software (Molecular Devices, Sunnyvale, CA). After establishment of the whole-cell configuration, cellular capacitance was minimized using the analog compensation available on the amplifier. Series resistance was <30 MΩ and was compensated >85% in all experiments. Data were filtered at 1 kHz (8-pole Bessel) and digitized at 10 kHz with a Digidata 1320 interface (Molecular Devices). In addition to collecting the raw data, a pulse/number p4 online leak-subtraction protocol was used (the p4 protocol involved four very brief hyperpolarizing pulses, which should have a negligible effect on drug interactions). Non–leak-subtracted currents were acquired in parallel for quality control purposes. For current-voltage relation studies, the membrane potential was held at −110 mV, and cells were depolarized from −90 to 20 mV in 10-mV increments. For steady-state inactivation studies, the membrane potential was depolarized by test pulses to −30 mV after 3.6-s conditioning prepulses ranging from −110 to −20 mV. Individual sweeps were separated by 10 s to allow for complete recovery from inactivation between conditioning pulses. The current amplitude obtained from each test pulse was then normalized to that observed at a holding potential of −110 mV. For slow inactivation studies channels were assessed using a test pulse (P2) that followed a 10-s conditioning prepulse of between −70 and −80 mV to elicit a peak current amplitude that was approximately 50 to 60% of the initial test pulse (P1).

**Data Analysis and Statistics.** Data were analyzed using Clampfit 9.2 (Molecular Devices). Preparation of figures and curve fitting were performed with Origin 7.5 software (Origin Lab Corp., Northampton, MA). Current-voltage relationships were fitted with the modified Boltzmann equation: $I = \frac{I_{\text{max}} \cdot (V_m - E_{\text{rev}})/[1 + \exp(V_m - E_{\text{rev}})/S]}{[1 + \exp(V_m - E_{\text{rev}})/S]}$, where $V_m$ is the test potential, $V_{0.5\text{act}}$ is the half-activation potential, $E_{\text{rev}}$ is the reversal potential, $G_{\text{max}}$ is the maximum slope conductance, and $h$ reflects the slope of the activation curve. Data from concentration dependence studies were fitted with the equation $y = A_1 + (A_2 - A_1)/[1 + ([C]/K_{\text{IC}})^n]$, where $A_1$ is initial current amplitude and $A_2$ is the current amplitude at saturating drug concentrations, $[C]$ is the drug concentration, and $n$ is the Hill coefficient. Statistical significance was determined by paired or unpaired Student's t tests and one-way or repeated-measures analysis of variance followed by Tukey multiple comparison tests, and significant values were set as indicated in the text and figure legends. All data are given as means ± S.E. Steady-state inactivation curves were fitted using the Boltzmann equation: $I = I_1/[1 + \exp(V_m - V_h)/S]$, where $V_h$ is the half-inactivation potential and $S$ is the slope factor.

**Results**

**A803467 Blocks T-Type Channels.** A recent study identified a novel compound (A803467) as a specific and potent blocker of TTX-resistant hNa<sub>1.8</sub> channels (Jarvis et al., 2007). Given that T-type channels share structural similarities with Na<sub>1</sub> channels, we examined whether this compound may affect T-type channels at both therapeutic plasma and brain tissue concentrations (10–17 and 3–5 µM, respectively) (Jarvis et al., 2007). Figure 1, A and B, illustrates the effect of 5 µM A803467 on human Ca<sub>3.2</sub> channels expressed tran-
siently in tsA-201 cells at a holding potential of −110 mV. As evident from the figure, this compound mediated robust peak current inhibition that could be partially reversed upon washout. The concentration dependence of this type of tonic (i.e., resting state) block could be well described by a Hill coefficient close to 1 (Fig. 1C), suggesting a bimolecular interaction between the channel and the blocker. We then examined the calcium channel subtype selectivity of A803467 (Fig. 1D) and found that all LVA subtypes were blocked with IC_{50} values in the low micromolar range, whereas two representative members of the high-voltage-activated channel family (i.e., L- and P/Q-type channels) exhibited lower affinities (i.e., higher IC_{50} values). A previous study has shown that this drug does not show significant block of N-type

![Fig. 1. Tonic block of voltage-gated calcium channels by A803467. A, representative current trace recorded from Ca_{3.2} channels transiently expressed in tsA-201 cells before and after application of 5 μM A803467. Currents were elicited by stepping from a holding potential of −110 mV to a test potential of −30 mV. B, representative time course of development of and recovery from A803467 block. C, ensemble dose-response curve for tonic Ca_{3.2} channel inhibition by A803467. The solid line is a fit via the Hill equation. D, IC_{50} values for tonic A803467 block of rat high-voltage-activated and human LVA calcium channels. Data were obtained by fitting dose-response curves for each channel subtype (n = 6 per channel). Error bars reflect S.E.s. Con, control.](image1)

![Fig. 2. A and B, current-voltage relations recorded before and after application of 5 μM A803467 to hCa_{3.1} (A) or hCa_{3.2} (B) channels. Data from multiple paired experiments are included in each figure. C and D, steady-state inactivation curves for hCa_{3.1} (C) and hCa_{3.2} (D) channels in the absence (control) and presence of 5 μM A803467. Note the negative shift in the half-inactivation potential in the presence of A803467. The data were fitted with the Boltzmann equation; half-inactivation potentials obtained from the fits were as follows: hCa_{3.1}: control −74 mV, drug −84 mV; and hCa_{3.2}: control −64 mV, drug −71 mV.](image2)
钙通道，瞬时受体电位vanilloid-1，KCNQ2/3钾通道，以及其他通道或受体在周围感觉神经元中被检测到（Jarvis et al., 2007）。

不同以往的报告对hNa,1.8通道（Jarvis et al., 2007），我们观察到显著的去极化作用的转换，这在两种Ca,3.1和Ca,3.2的转换中（Fig. 2）。那里也有一个趋势，就是更加去极化的电压对于Ca,3.3；然而，这些都没有达到统计学意义（Table 1）。应用A803467到T型通道也导致了一个显著的（～10 mV）去极化作用的转换在非药物结合的Ca,3.1和Ca,3.2（Fig. 2, C和D）相似于先前描述的hNa,1.8通道（Jarvis et al., 2007）。左边的转变在慢态-静息转换曲线上与静息状态的转换一致。

考虑到这些数据，我们发现A803467调节两个静息通道抑制和去极化转换通道的T型通道。

**A803467 Block Promotes a Slow Inactivation-Like State of hCa,3.2**。先前的研究已经识别到慢态抑制通道抑制器的Na,s（Sheets et al., 2008），以及混合的钠/钙通道和钠通道抑制器，这些稳定化了慢态抑制的转换（这些通道（Hillebrand et al., 2010）。为了确定A803467是否可以在T型通道上起作用，我们使用了一个慢态抑制转换过程来诱导一个部分慢态抑制的T型通道。（Fig. 4）。

![Fig. 3. Summary of IC50 values for A803467 block of Ca,3 channel subtypes in a partial slow inactivated state. The inset shows the whole-cell voltage command protocol used to induce slow inactivation (i.e., a test pulse P1, followed by a 10-s conditioning pulse to −70 mV, a brief hyperpolarization to remove fast inactivation, and a second test pulse P2 to determine the fraction of slow inactivated channels). Note that only Ca,3.2 channels display a decrease in IC50 for A803467 inhibition during P2, indicating a selective increase of A803467 affinity for slow inactivated Ca,3.2 channels (*, p < 0.05; Student's t test).](image)

![Fig. 4. Sequence alignment of the local anesthetic binding regions in Na, channels with the analogous regions in hCa,3.2. Residues involved in local anesthetic binding to Na, channels are indicated in bold. Note the overall degree of sequence similarity between T-type channels, sodium channels, and the Na,Ab bacterial sodium channel whose crystal structure has recently been resolved.](image)
The authors revealed that Tyr1771 and Phe1764 in Nav1.2 substitutions resulted in a significant decrease in A803467 Nav1.2 channels and that mutagenesis of these two residues were important determinants of local anesthetic block of and mutant Cav3.2 channels. Currents were elicited by stepping to a test potential of −30 mV from a holding potential of −110 mV. Significance was assessed using analysis of variance (**, p < 0.01).

hypothesized that the drug-binding site on these two types of channels may be conserved. Sodium channels possess multiple drug interaction sites, including a receptor site for binding of local anesthetics and anticonvulsive drugs (Lipkind and Fozzard, 2005). A key locus for local anesthetic block of Naᵫ channels was first identified by Ragsdale et al. (1994). The authors revealed that Tyr1771 and Phe1764 in Naᵫ1.2 were important determinants of local anesthetic block of Naᵫ1.2 channels and that mutagenesis of these two residues (in particular residue 1764) resulted in a reduction in etidocaine blocking affinity for the channel. In hCav3.2 channels, the residues equivalent to these two residues are valine and glutamine (Fig. 4), indicating that one key component of the local anesthetic-binding site is absent in T-type channels. However, Yarov-Yaroveny et al. (2001, 2002) identified several amino acid residues in the IS6 and IIIIs6 segments of the Naᵫ 1.2 α subunit that also affected drug interactions. These authors showed that in addition to residues Tyr1771 and Phe1764, mutagenesis of Ile409 and Ile1469 reduced local anesthetic interactions. ClustalW2 multiple sequence alignment of hNaᵫ1.8 versus hNaᵫ1.2 and hCav3.2 revealed that these regions were highly conserved in these channels (Fig. 4). The corresponding loci of these residues in Cav3.2 are Ile403 and Val1551, indicating that Cav3.2 channels may possess some of the structures needed for local anesthetic interactions.

To determine whether these residues may be involved in A803467 interactions with hCav3.2, we replaced Ile403 and Val1551 with alanines and then assessed A803467 block of these mutant channels. As shown in Fig. 5, these amino acid substitutions resulted in a significant decrease in A803467 blocking affinity for hCav3.2 (by more than 1 order of magnitude for the IIIIs6 substitution). This effect was not secondarily due to reduced slow inactivation, because the only gating parameter that was significantly altered was a shift in half-activation voltage of the I403A mutant (Table 2).

Next, we tested whether these mutations affected the ability of A803467 to enhance slow inactivation of hCav3.2. As shown in Fig. 6, the A803467-induced stabilization of a slow inactivated-like state of the wild-type channel was abolished in the two mutant channels.

To further support the hypothesis that a locus analogous to the sodium local anesthetic-binding site controls A803467 block of Caᵥ3.2, we introduced a tyrosine residue in position 1855 of Caᵥ3.2 (corresponding to Tyr1771 in Naᵫ1.2) and determined the consequences on the A803467 block of Caᵥ3.2 (Fig. 7). The introduction of Tyr1855 significantly increased A803467 resting state blocking affinity by approximately 4-fold (Fig. 7A) and preserved the ability of the compound to stabilize the fast inactivated state (Fig. 7B). Furthermore, we observed a significant increase in blocking activity in response to the slow inactivation pulse paradigm, as evident from a reduction in the IC₅₀ during P2 (Fig. 7C).

Unlike substitutions in positions 403 and 1551, tyrosine substitution of residue 1855 induced a −10 mV depolarizing shift in the voltage dependences of activation (not shown) and inactivation (Fig. 7B), indicating that this locus is important for channel gating.

Finally, we mutated the glutamine residue in position 1848 of hCav3.2 to the phenylalanine found in the corresponding position in Naᵫ1.2 channels (Ragsdale et al., 1994) (Fig. 4). In addition, we also substituted this residue with another hydrophobic residue, leucine. When expressed in tsA-201 cells, both mutants yielded T-type currents, albeit with much reduced whole-cell current amplitudes compared with those seen with wild-type channels. In fact, reliable current recordings required us to increase the ionic strength of the extracellular recording solution to 20 mM barium. Under these conditions, we were at least able to test the ability of A803467 to mediate tonic (resting) blocking affinity. As shown in Fig. 8, the compound exhibited a 20-fold increase in affinity for the phenylalanine mutant and a 10-fold increase for the leucine mutant compared with wild-type channels bathed in the same barium solution. In both mutants, block was completely reversible upon washout (not shown).

Taken together, these data indicate that both tonic and slow inactivated-like channel block of Caᵥ3.2 channels by A803467 is mediated by interactions of this compound with residues that are analogous to those comprising the local anesthetic receptor site in Naᵫ channels.

**TABLE 2**

| Calcium Channel | Vₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉぇeee |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| hCaᵥ3.2        | −44.3 ± 1.4 (n = 7) | 27.3 ± 0.7 (n = 7) | −1.9 ± 0.8 (n = 7), N.S. | 64.2 ± 0.3 (n = 5), N.S. | 7.0 ± 0.5 (n = 6) |
| hCaᵥ3.2 1403A  | −54.0 ± 1.8 (n = 11)* | 20.3 ± 1.8 (n = 11)* | −1.7 ± 0.9 (n = 11), N.S. | 63.8 ± 0.8 (n = 6), N.S. | 20.0 ± 1.5 (n = 6)** |
| hCaᵥ3.2 V1551A | −47.0 ± 1.1 (n = 13), N.S. | 23.8 ± 1.5 (n = 13), N.S. | −1.9 ± 0.6 (n = 13), N.S. | 67.0 ± 0.9 (n = 6), N.S. | 40.0 ± 3.1 (n = 6)** |
| hCaᵥ3.2 V1855Y | −29.64 ± 2.1 (n = 14) | 14.64 ± 2.1 (n = 14) | −3.9 ± 0.14 (n = 14) | 58.4 ± 0.56 (n = 6) | 1.8 ± 0.41 (n = 6)* |

N.S., not significant.

* P < 0.05, relative to wild type.

** P < 0.01.
Discussion

A803467 was originally identified as a potent inhibitor of hNav1.8 channels with a mode of action that seems to involve, at least in part, a stabilization of the slow inactivated state (Jarvis et al., 2007). Furthermore, this compound was shown to reduce neuropathic and inflammatory pain in animal models (McGaraughty et al., 2008). Here, we demonstrate that A803467 also blocks T-type channels with high affinity, with IC50 values that fall into the range of therapeutic concentrations and that block of the Cav3.2 channel subtype seems to stabilize slow inactivation. The similarities between Nav1.8 and Cav3.2 channel inhibition by this compound are striking and are underscored by the observation that mutations in Cav3.2 in regions corresponding to the local anesthetic interaction site in sodium channels antagonize A803467 action. Taken together, our findings suggest evolutionarily conserved interactions between A803467 and related drugs with Na+, LVA Ca2+, and T-type Ca2+ channels.

Both hNa1.8 and T-type Ca3.2 channels are functionally expressed in both nociceptive dorsal root ganglion and lamina I spinal cord neurons (Ikeda et al., 2003; Hildebrand et al., 2010) and are known to regulate their excitability. Hyperfunction of both channel subtypes has been linked to the development of hyperalgesia and allodynia in various animal models of pain (Ikeda et al., 2003; Barton et al., 2005; Cummins et al., 2007). Conversely, knockout or inhibition of T-type or Na+ channels mediates analgesia (Bourinet et al., 2005; Choi et al., 2007).

Given the potent blocking effects of A803467 on Ca3.2 channels, it is possible that the previously reported effects of this compound on action potential firing in dorsal root ganglion neurons and the associated analgesia may be, at least in part, mediated by T-type channel inhibition. Given that Na+ and Ca2+ work together to prolong subthreshold depolarizations within lamina I neurons (Prescott and De Koninck, 2002; Ikeda et al., 2003; Hildebrand et al., 2010), a dual action of A803467 may promote a synergistic inhibition of pain signaling. Slow inactivation is significantly enhanced during prolonged depolarizations or during neuronal burst firing. Promotion of a slow inactivated-like state of both channel subtypes may thus mediate frequency-dependent inhibition of channel activity and therefore reduce overall neuronal excitability. A reduction in neuronal excitability may make compounds such as A803467 ideally suited toward treatment of neuronal hyperexcitability disorders including pain and may perhaps be extended to conditions such as epilepsy. The apparent conservation in the drug receptor site between Na+ and Ca3.2 channels may provide an opportunity for the synthesis of more potent antagonists acting on both of these channel subtypes.

Fig. 6. IC50 values for A803467 block of slow inactivated Cav3.2 channels using the same protocol as that described in the legend to Fig. 3 except that the holding potential was stepped to −80 mV. Note that the mutant channels do not show an increase in blocking affinity for the slow inactivated state (*, p < 0.05, t test). wt, wild-type.

Fig. 7. Effect of a tyrosine substitution in position 1855 of hCav3.2. A, IC50 for tonic A803467 of wild-type (wt) and mutant V1855Y (*, p < 0.05, t test). B, steady-state inactivation curves recorded in the presence and the absence of 5 µM A803467. Note the hyperpolarizing shift from −58 to −64 mV. For comparison, the inactivation curve of the wild-type (wt) (control) channel is included. C, dose dependence of slow inactivated channel block of mutant hCav3.2 V1855Y expressed as the fractional current at various drug concentrations. Note that the tyrosine substitution produces submicromolar block by this compound.
Fig. 8. Summary of IC₅₀ values for tonic A803467 block of wild type (wt) and mutant Q1848L and Q1848F hCaV.3.2 channels. Currents were elicited by stepping to a test potential of −20 mV from a holding potential of −110 mV. Statistical significance relative to control: **, p < 0.01. All recordings in this figure were performed in 20 mM barium because of the small current amplitudes of the mutants. IC₅₀ values were obtained by fitting dose-response curves with the Hill equation as described in the legend to Fig. 1. Both substitutions in position 1848 produced submicromolar block by this compound.

Authorship Contributions
Participated in research design: Bladen and Zamponi.
Performed experiments: Bladen.
Contributed new reagents or analytic tools: Bladen.
Performed data analysis: Bladen.
Wrote or contributed to the writing of the manuscript: Bladen and Zamponi.

References