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 Na\textsubscript{v} channels may also modulate the activities of T-type channels. Here we show that the specific Na\textsubscript{v}1.8 blocker 5-[(4-chlorophenyl-N-(3,5-dimethoxyphenyl)furan-2-carboxamide (A803467) tonically blocks T-type channels in the low micromolar range. Similarly to Na\textsubscript{v}1.8, 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 Na\textsubscript{v} channels may extend to structurally similar drug-binding sites. Sequence alignment revealed several highly conserved regions between T-type and Na\textsubscript{v} 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 Na\textsubscript{v} channel blockers that could potentially act synergistically to normalize aberrant neuronal activity.

Introduction
Na\textsubscript{v} 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 Na\textsubscript{v} α subunits that are functionally classified as either tetrodotoxin (TTX)-sensitive or TTX-resistant, with the latter exhibiting slower inactivation kinetics than other Na\textsubscript{v} channel subtypes (Waxman et al., 1999; Blair and Bean, 2002). The various Na\textsubscript{v} 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 Na\textsubscript{v} channel isofrom 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 Na\textsubscript{v} 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 (Cav3.1, Cav3.2, or Cav3.3), whose membrane topology is similar to that of Na\textsubscript{v} channels (Catterall et al., 2005). Mutations in Cav3.2 T-type channels have been linked to absence seizures (Khosravani and Zamponi, 2006; Heron et al., 2007). Moreover, up-regulation of Cav3.2 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 (Cribs, 2010; David et al., 2010). Both Na\textsubscript{v} channels and T-type channels contribute to neuronal excitability and to similar disorders such as epilepsy and pain. Indeed, knock-
out of Na\textsubscript{1.8} and Ca\textsubscript{3.2} results in hyposensitivity to pain (for reviews, see Wang et al., 2011; Gregg et al., 2010), suggesting the possibility that mixed Na\textsubscript{1}/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\textsubscript{1.8} channels, has been shown to be efficacious in animal pain models (McGaraughty et al., 2008). The interaction site of A803467 on the Na\textsubscript{1} 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\textsubscript{v} 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\textsubscript{3.2}, rat Ca\textsubscript{1.2}, and rat Ca\textsubscript{2.1} cDNA constructs, as well as ancillary Ca\textsubscript{v} channel subunit cDNAs were kindly provided by Dr. Terrance Snutch (University of British Columbia, Vancouver, BC, Canada). Human Ca\textsubscript{3.3} was obtained from Dr. Arnaud Monteil (Centre National de la Recherche Scientifique, Montpellier, France), and human Ca\textsubscript{3.1} 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\textsubscript{v} 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 (Altieri 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\textsubscript{1.2} or rCa\textsubscript{2.1}, α1 subunits (3 μg), were each cotransfected with rat β1b (3 μg) or rat β4 (3 μg). For experiments involving T-type channels, hCa\textsubscript{3.1}, hCa\textsubscript{3.2}, and hCa\textsubscript{3.3}, 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\textsubscript{2}.

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 CsCl, 2 mM CaCl\textsubscript{2}, 1 mM MgCl\textsubscript{2}, 10 mM HEPES, and 10 mM glucose, adjusted to pH 7.4 with CsOH. The external recording solution for hCa\textsubscript{3.2} Q1848 mutants and their hCa\textsubscript{3.2} wild-type controls contained 124 mM CsCl, 20 mM BaCl\textsubscript{2}, 1 mM MgCl\textsubscript{2}, 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\textsubscript{4}, 2 mM MgCl\textsubscript{2}, 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 micropipet 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 205B 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 <10 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 p/4 online leak-subtraction protocol was used (the p/4 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: 

$$I = \frac{I_{\text{max}} \cdot (V - V_{\text{m}})}{1 + \exp\left(V_{\text{m},\text{act}} - V_{\text{m}}\right)/k}$$

where $V_{\text{m}}$ is the test potential, $V_{\text{m},\text{act}}$ is the half-activation potential, $E_{\text{rev}}$ is the reversal potential, $G_{\text{max}}$ is the maximum slope conductance, and $k$ reflects the slope of the activation curve. Data from concentration dependence studies were fitted with the equation $y = A_1 + (A_2 - A_1)\exp\left((C/C_{\text{IC}_{50}})^n\right)$, 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: 

$$I = \frac{1}{1 + \exp(V_{\text{h}} - V_{\text{h},\text{act}})/S}$$

where $V_{\text{h}}$ 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\textsubscript{1.8} channels (Jarvis et al., 2007). Given that T-type channels share structural similarities with Na\textsubscript{v} 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\textsubscript{3.2} 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
calcium channels, transient receptor potential vanilloid-1, KCNQ2/3 potassium channels, and other channels or receptors found in peripheral sensory neurons (Jarvis et al., 2007).

Unlike what has been reported for hNa1.8 channels (Jarvis et al., 2007), we observed a significant hyperpolarizing shift in the half-activation voltages of both Ca3.1 and Ca3.2 channels (Fig. 2). There was also a trend toward more hyperpolarized voltages for Ca3.3; however, these did not reach statistical significance (Table 1). Application of A803467 to hCav3.1 and hCav3.2 (Fig. 2, C and D) similar to that described previously for hNa1.8 channels (Jarvis et al., 2007). The leftward shift in the steady-state inactivation curve is consistent with inactivated channel block (Hille, 1977).

Taken together, these data indicate that A803467 mediates both resting channel inhibition and inactivated channel block of T-type channels.

**A803467 Block Promotes a Slow Inactivation-Like State of hCa3.2.** Previous studies have identified slow inactivation channel blockers of Na3.1 (Sheets et al., 2008), as well as mixed sodium/T-type channel blockers, which stabilize the slow inactivated conformation of these channels (Hildebrand et al., 2010). To determine whether A803467 may mediate a similar action on T-type channels, we used a slow inactivation protocol to induce a partial slow inactivated-like state of the channel. Specifically, we applied a brief test depolarization (P1) before a 10-s conditioning pulse to −70 mV, which is expected to induce both fast and slow inactivation. This was followed by a brief hyperpolarizing step to induce recovery from fast inactivation of non-drug-bound channels. A second depolarizing test pulse (P2) allowed us to determine the fraction of channels in the slow inactivated-like state (under our experimental conditions; this amounted to approximately 30% slow inactivation in the absence of the drug). The dose-dependent effects of A803467 on the currents elicited by P1 and P2 were then compared in order to ascertain the amount of resting versus slow inactivated-like channel block. As shown in Fig. 3, in hCa3.2 channels, A803467 induced channel inhibition that resembled features of enhanced slow inactivation, as evident from a reduction in the IC50 of P2 current inhibition. In contrast, the hCav3.1 and hCav3.3 channels did not show stabilization of the slow inactivated-like channel conformation. The combined effects of A803467 on different kinetic states of hCa3.2 channels predict substantial total/combinated inhibition of Ca3.2 currents in the high nanomolar to the low micromolar range that is well within the therapeutic range of A803467.

**A Locus Analogous to the Na3 Local Anesthetic Binding Site Controls A803467 Block of Ca3.2.** Given the structural similarity between T-type and Na3 channels, we

![Fig. 3. Summary of IC50 values for A803467 block of Ca3 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 Ca3.2 channels display a decrease in IC50 for A803467 inhibition during P2, indicating a selective increase of A803467 affinity for slow inactivated Ca3.2 channels (+, p < 0.05; Student's t test).](image-url)

![Fig. 4. Sequence alignment of the local anesthetic binding regions in Na3 channels with the analogous regions in hCa3.2. Residues involved in local anesthetic binding to Na3 channels are indicated in bold. Note the overall degree of sequence similarity between T-type channels, sodium channels, and the Na3Ab bacterial sodium channel whose crystal structure has recently been resolved.](image-url)
The authors revealed that Tyr1771 and Phe1764 in Nav1.2 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 hCa3.2. 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 Ca3.2 (corresponding to Tyr1771 in Na1.2) and determined the consequences on the A803467 block of hCA3.2 (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 9-fold increase for the phenylalanine mutant and a 10-fold increase for the leucine mutant 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 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 Ca3.2 channels by A803467 is mediated by interactions of this compound with residues that are analogous to those comprising the local anesthetic receptor site in Na1.2 channels.

TABLE 2

Biophysical parameters of wild type and mutant hCav3.2 calcium channels

<table>
<thead>
<tr>
<th>Calcium Channel</th>
<th>V0.5 act (mV)</th>
<th>Erev (mV)</th>
<th>Gmax (nS)</th>
<th>Vm (mV)</th>
<th>IC50 Tonic (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>hCa3.2</td>
<td>−44.3 ± 1.4 (n = 7)</td>
<td>27.3 ± 0.7 (n = 7)</td>
<td>−19 ± 0.8 (n = 5), N.S.</td>
<td>−64.2 ± 0.3 (n = 5), N.S.</td>
<td>7.0 ± 0.5 (n = 6)</td>
</tr>
<tr>
<td>hCa3.2 I403A</td>
<td>−54.0 ± 1.8 (n = 11)*</td>
<td>20.3 ± 1.8 (n = 11)*</td>
<td>−17 ± 0.9 (n = 11), N.S.</td>
<td>−63.8 ± 0.7 (n = 6), N.S.</td>
<td>20.0 ± 1.5 (n = 6)**</td>
</tr>
<tr>
<td>hCa3.2 V1551A</td>
<td>−47.0 ± 1.1 (n = 13), N.S.</td>
<td>23.8 ± 1.5 (n = 13), N.S.</td>
<td>−19 ± 0.6 (n = 13), N.S.</td>
<td>−67.0 ± 0.9 (n = 6), N.S.</td>
<td>40.0 ± 3.1 (n = 6)**</td>
</tr>
<tr>
<td>hCa3.2 V1855Y</td>
<td>−29.64 ± 2.1 (n = 14)</td>
<td>14.64 ± 2.1 (n = 14)</td>
<td>−3.9 ± 0.14 (n = 14)</td>
<td>−58.4 ± 0.56 (n = 6)</td>
<td>1.8 ± 0.41 (n = 6)*</td>
</tr>
</tbody>
</table>

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<sub>v</sub> and LVA Ca<sub>v</sub> channels.

Both hNa<sub>1.8</sub> and T-type Ca<sub>3.2</sub> 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<sub>v</sub> channels mediates analgesia (Bourinet et al., 2005; Choi et al., 2007).

Given the potent blocking effects of A803467 on Ca<sub>3.2</sub> 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<sub>v</sub> and Ca<sub>v</sub> 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<sub>v</sub> and Ca<sub>v</sub> channels may provide an opportunity for the synthesis of more potent antagonists acting on both of these channel subtypes.

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**Fig. 6.** IC<sub>50</sub> values for A803467 block of slow inactivated Ca<sub>3.2</sub> 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, IC<sub>50</sub> 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.
recordings in this figure were performed in 20 mM barium because of the small current amplitudes of the mutants. $I_{\text{Cav}}$ 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.

**Conducted 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**


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