Use-Dependent Block by Lidocaine but Not Amitriptyline Is More Pronounced in Tetrodotoxin (TTX)-Resistant Na$_v$1.8 Than in TTX-Sensitive Na$^+$ Channels

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ABSTRACT

The excitability of sensory neurons depends on the expression of various voltage-gated Na$^+$ channel isoforms. The tetrodotoxin-resistant (TTXr) Na$_v$ channel Na$_v$1.8 accounts for the electroresponsiveness of nociceptive neurons and contributes to inflammatory and neuropathic pain. Na$^+$ channel blockers are clinically employed for chronic pain management, but side effects limit their use. There is conflicting information whether their potency to block tetrodotoxin-sensitive (TTXs) and TTXr Na$_v$ channels differs. We analyzed the action of lidocaine and amitriptyline on TTXr Na$_v$1.8 heterologously expressed in ND7/23 cells in comparison with TTXs Na$_v$ channels endogenously expressed in ND7/23 cells. TTXs Na$_v$1.8 and TTXs currents were investigated under whole-cell voltage-clamp. At a holding potential of −80 mV, lidocaine was 5-fold and amitriptyline 8-fold more potent to tonically block TTXs than Na$_v$1.8 currents. This was due to a higher percentage of TTXs channels residing in the inactivated, high-affinity state at this potential. Tonic block of either resting or inactivated channels by lidocaine or amitriptyline revealed little differences between TTXs and Na$_v$1.8 channels. Use-dependent block by amitriptyline was similar in TTXs and Na$_v$1.8 channels. Surprisingly, use-dependent block by lidocaine was more pronounced in Na$_v$1.8 than in TTXs channels. This result was confirmed in dorsal root ganglion neurons and is associated with the greater tendency of Na$_v$1.8 to enter a slow inactivated state. Our data suggest that amitriptyline could selectively block Na$_v$1.8-mediated action potential firing. It is conceivable that the expression pattern of Na$_v$ channels in sensory neurons might influence the efficiency of Na$^+$ channel blockers used for chronic pain management.

Voltage-gated sodium channels play a central role in excitable membranes by initiating and propagating action potentials. Sensory neurons express a unique ensemble of Na$^+$ channel α-subunits, including both tetrodotoxin (TTX)-resistant (TTXr) (Na$_v$1.8 and Na$_v$1.9) and TTX-sensitive (TTXs) (Na$_v$1.1, Na$_v$1.6, and Na$_v$1.7) subunits (Waxman et al., 1999; Lai et al., 2003). Patch-clamp studies on dorsal root ganglion (DRG) neurons have identified two distinct TTXr sodium currents, a slowly inactivating and a persistent current (Roy and Narahashi, 1992; Cummins et al., 1999). Generation of transgenic mice lacking specific subunits revealed that the slowly inactivating current is produced by Na$_v$1.8, whereas the persistent current is produced by Na$_v$1.9 (Akopian et al., 1999; Cummins et al., 1999; Priest et al., 2005). Na$_v$1.8 is expressed mainly by C-type DRG neurons, corresponding to nociceptive C- and Aδ-fibers (Akopian et al., 1996; Djouhri et al., 2003). Several studies indicate that Na$_v$1.8 is important for the excitability of nociceptive neurons (Renganathan et al., 2001; Blair and Bean, 2002) and, more importantly, for the hyperexcitability of sensory neurons associated with conditions of chronic pain (Akopian et al., 1999; Lai et al., 2002; Roza et al., 2003). Following injury of peripheral nerves, Na$_v$1.8 is strongly down-regulated in DRG neurons (Cummins and Waxman, 1997; Gold et al., 2003). Studies using antisense oligonucleotides against Na$_v$1.8 have suggested that it may contribute to neuropathic pain by an up-regulation in uninjured afferents (Lai et al., 2002; Gold et al., 2003). In contrast, neuropathic pain was demonstrated to develop normally in mice lacking both Na$_v$1.7 and Na$_v$1.8 (Nassar et al., 2005). Proinflammatory mediators modulate the function of Na$_v$1.8 in a protein kinase C- and protein kinase A-dependent manner, resulting in a hyperexcitability of sensory neurons observed in conditions of inflammatory pain (England et al., 1996; Gold et al., 1998). Based on these properties, Na$_v$1.8 is considered to be an attractive target for the development of new analgesic drugs. Various Na$^+$ channel blockers are already employed for pain treatment, albeit with variable suc-

ABBREVIATIONS: TTX, tetrodotoxin; TTXr, tetrodotoxin-resistant; TTXs, tetrodotoxin-sensitive; DRG, dorsal root ganglion.
cess. These compounds comprise local anesthetics, antiar-rhythmics, anticonvulsants, and antidepressants. Na\(^+\) currents in sensory neurons are potently blocked by Na\(^+\) channel blockers; however, TTXs and TTXr Na\(^+\) currents in DRG neurons seem to exhibit different sensitivities (Roy and Narahashi, 1992; Cummins and Waxman, 1997; Scholz et al., 1998). Local anesthetics and the tricyclic antidepressant amitriptyline are known to block voltage-gated Na\(^+\) channels by binding to specific amino acid residues in the α-subunit S6 segments, which are located within the ion-conducting pore of the channel (Ragsdale et al., 1994; Nau and Wang, 2004). Block is strongly state-dependent, with a preference for open and inactivated rather than for resting channel states. The nature of the local anesthetic binding site has led to the presumption that it is highly unlikely to find isoform-specific blockers interacting with this site.

Detailed pharmacological characterization of recombinant Na\(_{\text{v}1.8}\) is rare due to difficulties to functionally express this channel in mammalian cell lines. Recently, ND7/23 cells have been used successfully to express recombinant Na\(_{\text{v}1.8}\) channels (Choi et al., 2004; John et al., 2004). In the present study, we employed this expression system and asked whether Na\(_{\text{v}1.8}\) and TTXs channels indeed display different sensitivities toward two drugs used clinically for the treatment of neuropathic pain, the local anesthetic lidocaine and the tricyclic antidepressant amitriptyline.

Materials and Methods

**Transient Transfection.** Rat Na\(_{\text{v}1.8}\)-DNA was subcloned into the mammalian expression vector pcMV-Script (Stratagene, La Jolla, CA). The dorsal root ganglion neuroblastoma hybridoma cell line ND7/23 was purchased from the European Collection of Animal Cell Cultures (Porton Down, UK). ND7/23 cells were maintained in Dulbecco’s modified Eagle’s medium ( GibCO-Invitrogen, Karlsruhe, Germany), supplemented with 100 U/ml penicillin/streptomycin (GIBCO-Invitrogen), 25 mM HEPES (GIBCO-Invitrogen), 10% heat-inactivated fetal bovine serum (GIBCO-Invitrogen), and 3 mM taurine (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) at 37°C and 5% CO\(_2\). Cells were transiently transfected with rat Na\(_{\text{v}1.8}\)-pcMV-Script (5–10 μg), rat β1-pcDNA1/Amp (5 μg), and CD8-pH3m reporter plasmid (1 μg), or β1-pcDNA1/Amp and reporter plasmid alone (to investigate endogenously expressed TTXs channels) by the calcium phosphate precipitation method. After incubation for 12 to 15 h, cells were replated in 35-mm culture dishes. Transfected cells were used for experiments within 3 to 4 days. Transfection-positive cells were identified by immunobeads (anti-CD-8 Dynabeads; Dynal Biotech, Oslo, Norway). Transfection efficiency was about 20% on average for Na\(_{\text{v}1.8}\). Activation and inactivation kinetics were also studied in untransfected ND7/23 cells that were maintained as described above and replated in 35-mm culture dishes the day preceding the experiments.

**Cell Culture.** Littermates of heterozygous C57/BL6 Na\(_{\text{v}1.8}\)-null mice were genotyped using commercially available primers. Animals were killed by CO\(_2\) asphyxiation. DRGs were removed from adult (20–30 g) C57/BL6 wild-type or Na\(_{\text{v}1.8}\)-null mice and collected in Dulbecco’s modified Eagle’s medium. The DRGs were treated with papain (20 U/ml; Sigma) for 20 min followed by collagenase (0.28 U/ml; Biochim, Berlin, Germany) for 30 min. Cells were dispersed by trituration in Ham’s F12 medium supplemented with 10% horse serum, penicillin (100 U/ml), streptomycin (100 μg/ml), and l-glutamine (3.0 mM). Cells were plated on glass coverslips coated with poly-l-lysine and kept at 37°C and 5% CO\(_2\). Cells were used for experiments within 24 h after plating.

**Electrophysiological Technique and Data Acquisition.** Whole-cell patch-clamp recordings were conducted at room temperature (−21°C). Patch pipettes were pulled from borosilicate glass tubes (TW150F-3; World Precision Instruments, Berlin, Germany) and heat-polished at the tip to give a resistance of 0.8 to 1.5 MΩ. Currents were acquired with an Axopatch 200B patch-clamp amplifier (Axon Instruments/Molecular Devices, Sunnyvale, CA), filtered at 5 kHz, and sampled at 20 kHz. The offset potential was zeroed before the electrode was attached to the cell. Voltage errors were minimized using 70 to 80% series resistance compensation, and the capacitance artifact was canceled by using the computer-controlled circuitry of the patch clamp amplifier. Linear leak subtraction, based on resistance estimates from four hyperpolarizing pulses applied before the test pulse, was used for all voltage-clamp recordings except for the experiments on use-dependent block. pCLAMP 8.2 software (Axon Instruments/Molecular Devices) was used for acquisition and analysis of currents. Microlab Origin 6.1/7 software (OriginLab Corp., Northampton, MA) was used to perform least-squares fitting and to create figures. Data are presented as mean ± S.E.M. or fitted value ± S.E. of the fit. An unpaired Student’s t test (SigmaStat; SPSS Science, Chicago, IL) was used to evaluate the significance of changes in mean values. p < 0.05 was considered statistically significant.

**Chemicals and Solutions.** Experiments on ND7/23 cells were performed with an external solution containing 65 mM NaCl, 85 mM choline Cl, 2 mM CaCl\(_2\), and 10 mM HEPES (adjusted to pH 7.4 with tetramethylammonium hydroxide) and a pipette solution containing 100 mM NaF, 30 mM NaCl, 10 mM EGTA, and 10 mM HEPES (adjusted to pH 7.2 with CsOH). The reversed Na\(^+\) gradient was used to minimize the series resistance artifact, which is less serious with outward currents. In some experiments, more physiological ionic conditions were employed that generate inward currents at depolarizing pulses more negative than 60 to 70 mV. In these experiments, the external solution contained 140 mM NaCl, 3 mM KCl, 1 mM MgCl\(_2\), 1 mM CaCl\(_2\), and 10 mM HEPES (adjusted to pH 7.4 with tetramethylammonium hydroxide), and the pipette solution contained 140 mM CsF, 1 mM EGTA, 10 mM NaCl, and 10 mM HEPES (adjusted to pH 7.2 with CsOH). Experiments on DRG neurons were performed with an external solution containing 40 mM NaCl, 100 mM choline Cl, 3 mM KCl, 1 mM MgCl\(_2\), 1 mM CaCl\(_2\), and 10 mM HEPES (adjusted to pH 7.4 with tetramethylammonium hydroxide) and a pipette solution containing 140 mM CsF, 1 mM EGTA, 10 mM NaCl, and 10 mM HEPES (adjusted to pH 7.2 with CsOH). The osmolarity of all solutions was adjusted to 300 to 310 mOsm. Lidocaine hydrochloride and amitriptyline hydrochloride (both Sigma-Aldrich) were dissolved in dimethyl sulfoxide to give stock solutions of 100 mM. The highest dimethyl sulfoxide concentration obtained was 1% and had no effect on Na\(^+\) currents. TTX (Tocris, Ellisville, MO) was dissolved in water to give a stock solution of 5 mM. Control and test solutions were applied through a gravity-driven polytetrafluoroethylene-glass multiple-barrel perfusion system allowing superperfusion of the cell under investigation.

**Results**

**Biophysical Properties of Heterologously Expressed Na\(_{\text{v}1.8}\) and Endogenously Expressed TTXs Channels in ND7/23 Cells.** As an expression system for this study, we chose the ND7/23 hybrid cell line derived from neonatal rat DRG neurons fused with the mouse neuroblastoma N18Tg2, that exhibit sensory neuron-like properties (Wood et al., 1990). ND7/23 cells endogenously express sodium currents with rapid kinetics that are completely blocked by 250 nM TTX. The molecular identities of these TTXs currents are unknown; however, they exhibit similar kinetics and activation and inactivation properties to the TTXs currents in DRGs (John et al., 2004).
We transiently transfected ND7/23 cells with Na\textsubscript{v}1.8-pCMV-Script, β1-pcDNA1/Amp, and CD8-phi3m reporter plasmid. As negative controls, we transfected ND7/23 cells with β1-pcDNA1/Amp and reporter plasmid alone or left the cells untransfected. TTXr Na\textsubscript{v}1.8 currents were investigated in the presence of 250 nM TTX and compared with TTXs currents endogenously expressed in β1-transfected or untransfected ND7/23 cells.

Activation and inactivation kinetics were characterized by standard pulse protocols. Holding potential (V\textsubscript{h}) was −140 mV. The standard ionic conditions used in this study generated outward currents at depolarizing pulses more positive than −20 to −10 mV (calculated E\textsubscript{Na} = −17.5 mV) (A and B). In the second line of traces, TTXr-Na\textsubscript{v}1.8 currents (C) and TTXs Na\textsuperscript{+} currents (D) are shown recorded at V\textsubscript{h} = −80 mV and under ionic conditions that generate inward currents at depolarizing pulses more negative than 60 to 70 mV (calculated E\textsubscript{Na} = 67 mV). Voltage dependence of activation (E) and steady-state inactivation (F) of Na\textsubscript{v}1.8 (□) and TTXs currents in cells transfected with β1 unit (○) or untransfected cells (□) at V\textsubscript{h} = −140 mV. For activation, currents were evoked by 5-ms-long test pulses from −120 to 60 mV in steps of 10 mV. Conductance was determined at a given voltage step from the equation g\textsubscript{m} = I\textsubscript{Na}/(E\textsubscript{m} − E\textsubscript{rev}), where I\textsubscript{Na} is the peak current, E\textsubscript{m} is the amplitude of the voltage step, and E\textsubscript{rev} is the estimated reversal potential. The data were fitted with a Boltzmann function. Note the drop in the normalized peak Na\textsuperscript{+} conductance in TTXs currents at potentials >+30 mV. The average midpoint voltages and slope factors are given in Table 1. The h\textsubscript{m} curves were determined by a two-pulse protocol: 100-ms-long prepulses from −120 to 40 mV in steps of 5 mV were followed by a test pulse to 50 mV. Peak currents were measured at the test pulse, normalized, and plotted against the prepulse potential. The data were fitted with a Boltzmann function.

![Diagram](image.png)
TABLE 1
Biophysical and pharmacological properties of heterologously expressed Na<sub>1.8</sub> and endogenously expressed TTXs currents in ND7/23 cells

Data for activation and steady-state inactivation were determined as described in Fig. 1 and IC<sub>50</sub> values as described in Figs. 2 and 3B. Hill coefficients are given in brackets.

<table>
<thead>
<tr>
<th>Current</th>
<th>[Na]&lt;sub&gt;i&lt;/sub&gt;/[Na]&lt;sub&gt;i0&lt;/sub&gt;</th>
<th>V&lt;sub&gt;h&lt;/sub&gt;</th>
<th>Activation</th>
<th>Inactivation</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt;</th>
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<tr>
<td></td>
<td>mM/mM</td>
<td>mV</td>
<td>E&lt;sub&gt;0.5&lt;/sub&gt;, mV/e-fold</td>
<td>V&lt;sub&gt;0.5&lt;/sub&gt;, mV/e-fold</td>
<td>µM</td>
</tr>
<tr>
<td>Na&lt;sub&gt;1.8&lt;/sub&gt; + β1</td>
<td>65/130</td>
<td>−140</td>
<td>4.0 ± 1.7, (n = 16)</td>
<td>−46.6 ± 0.4, (n = 7)</td>
<td>319 ± 6, (n = 7)</td>
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<tr>
<td></td>
<td>65/130</td>
<td>−80</td>
<td>−3.8 ± 2.1, (n = 6)</td>
<td>−40.2 ± 0.3, (n = 7)</td>
<td>164 ± 8, (n = 5)</td>
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<tr>
<td></td>
<td>140/10</td>
<td>−80</td>
<td>9.0 ± 0.9, (n = 13)</td>
<td>−40.1 ± 0.5, (n = 10)</td>
<td>[1.11 ± 0.06, (n = 5)]</td>
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<tr>
<td></td>
<td>65/130</td>
<td>E&lt;sub&gt;pp&lt;/sub&gt; = −40</td>
<td>0.63 ± 0.05, (n = 6)</td>
<td>[0.84 ± 0.05, (n = 6)]</td>
<td></td>
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<tr>
<td>TTXs + β1</td>
<td>65/130</td>
<td>−140</td>
<td>−25.3 ± 1.2, (n = 17)</td>
<td>−66.0 ± 0.4, (n = 7)</td>
<td>517 ± 23, (n = 8)</td>
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<tr>
<td></td>
<td>65/130</td>
<td>−80</td>
<td>−25.8 ± 1.6, (n = 7)</td>
<td>−69.6 ± 0.3, (n = 5)</td>
<td>[0.96 ± 0.04, (n = 7)]</td>
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<tr>
<td></td>
<td>140/10</td>
<td>−80</td>
<td>−25.6 ± 1.7, (n = 9)</td>
<td>−69.7 ± 0.2, (n = 4)</td>
<td>30 ± 2, (n = 5)</td>
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<tr>
<td></td>
<td>65/130</td>
<td>E&lt;sub&gt;pp&lt;/sub&gt; = −70</td>
<td>0.47 ± 0.02, (n = 6)</td>
<td>[0.81 ± 0.02, (n = 6)]</td>
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<tr>
<td>TTXs Ø β1</td>
<td>65/130</td>
<td>−140</td>
<td>−27.6 ± 1.7, (n = 7)</td>
<td>−68.0 ± 0.2, (n = 7)</td>
<td>91.7 ± 0.2, (n = 7)</td>
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<tr>
<td></td>
<td>65/130</td>
<td>−80</td>
<td>−24.4 ± 3.7, (n = 5)</td>
<td>−66.5 ± 0.2, (n = 7)</td>
<td>[0.84 ± 0.04, (n = 7)]</td>
</tr>
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</table>

Tonic Block of Na<sub>1.8</sub> and TTXs Na<sup>+</sup> Currents by Lidocaine and Amitriptyline. Spinal sensory neurons have a resting membrane potential between −60 and −80 mV. For this reason, many studies have chosen a V<sub>h</sub> of −80 mV to study local anesthetic block of sodium channels in sensory neurons. It is important to note that at V<sub>h</sub> = −80 mV, the fraction of channels residing in the resting, low-affinity state is higher for Na<sub>1.8</sub> compared with TTXs channels but similar at V<sub>h</sub> = −140 mV. We investigated block of Na<sub>1.8</sub> and TTXs channels, both coexpressed with the β1 subunit, by lidocaine and amitriptyline when cells were held at −80 or −140 mV. Currents were activated by 5-ms-long test pulses to +50 mV at 30-s intervals in the presence of increasing concentrations of the drugs (Fig. 2, A and B). The 50% inhibitory concentrations (IC<sub>50</sub>) are given in Table 1. At V<sub>h</sub> = −80 mV, TTXs currents displayed an approximately 5-fold higher sensitivity to lidocaine and an approximately 8-fold higher sensitivity to amitriptyline (Fig. 2, C and D). At V<sub>h</sub> = −140 mV, TTXs channels displayed an approximately 1.6-fold lower sensitivity (p < 0.05) to lidocaine compared with Na<sub>1.8</sub> (Fig. 2E) and an approximately 1.4-fold higher sensitivity to amitriptyline compared with Na<sub>1.8</sub> (Fig. 2F).

To assess in detail state-dependent block of Na<sub>1.8</sub> and TTXs channels by amitriptyline, a three-step pulse protocol was applied as described previously (Wright et al., 1997; Nau et al., 1999). Cells were held at −140 mV, and 10-s-long conditioning prepulses between −160 and −30 mV were applied to allow amitriptyline binding to Na<sup>+</sup> channels to reach steady state. After a 100-ms-long interval at −140 mV, inserted to allow drug-free channels to recover from fast inactivation, drug-free channels were activated by test pulses to 50 mV. In Fig. 3A, normalized peak currents of Na<sub>1.8</sub> and TTXs channels in control and in the presence of 1 µM amitriptyline are plotted as a function of the conditioning prepulse potentials. In control solution, a small fraction of Na<sup>+</sup> channels exhibited slow inactivation after prepulses between −90 and −30 mV. Block by 1 µM amitriptyline was absent or small at prepulses < −100 mV for Na<sub>1.8</sub> and < −110 mV for TTXs channels, respectively. This block is deemed to correspond to block of resting channels. Block by 1 µM amitriptyline reached a second plateau after prepulse −40 mV for Na<sub>1.8</sub> and −70 mV for TTXs channels. This block is deemed to correspond to high-affinity binding and block of inactivated channels (Fig. 3A). State-dependent block by amitriptyline could be fitted by a Boltzmann equation with midpoint (millivolt) and slope factor (millivolt per e-fold) values, respectively, of −75.9 ± 0.8 and 11.3 ± 0.7 for Na<sub>1.8</sub> and −91.7 ± 0.8 for TTXs.
0.9 and 7.7 ± 0.8 for TTXs currents, respectively. Note that the midpoint values critically depend on the fractional distributions of resting and inactivated channels at a given conditioning potential (Wright et al., 1999). To more accurately determine the sensitivities of inactivated channels to amitriptyline, we measured the concentration dependence of block with conditioning prepulses to −40 mV for Nav1.8 and to −70 mV for TTXs channels and determined the IC_{50} values (Fig. 3B). As demonstrated in Fig. 3B, inactivated TTXs currents displayed an approximately 1.3-fold higher sensitivity (p < 0.05) to amitriptyline compared with Nav1.8 (Table 1).

The recovery time course of Na^+ channels from the inactivated drug-bound state is reported to be slow in the presence of amitriptyline (Nau et al., 2000; Wang et al., 2004). Because recovery from block by lidocaine is much faster, significant amounts of lidocaine unbinding from inactivated channels would occur during the 100-ms-long interval at −140 mV between the conditioning prepulse and the test pulse (Wright et al., 1997), leading to an underestimation of block of inactivated channels when using the pulse protocol as described above. To confirm this prediction under our experimental conditions, we determined the recovery time of inactivated Na_{v}1.8 and TTXs channels from block by amitriptyline and lidocaine (Fig. 4) by applying a 5-ms test pulse to +50 mV from a holding potential of −140 mV at various times after a 10-s conditioning prepulse to −40 mV for Na_{v}1.8 and to −70 mV for TTXs channels (Fig. 4, inset). Figure 4, A and B, show normalized peak currents of Na_{v}1.8 and TTXs channels in control and in the presence of 1 μM amitriptyline (n = 5 and 9, respectively) and 50 μM lidocaine (n = 8 and 7, respectively) as a function of the interpulse duration. Currents in the absence of drugs and in the presence of 1 μM amitriptyline or 50 μM lidocaine recovered with two time constants (τ_1, τ_2). In the presence of a drug, τ_1 describes the fast recovery from inactivation of unblocked channels, and τ_2 describes the recovery of inactivated channels that were blocked during the conditioning prepulse, including rebinding and dissociation from resting channels and recovery from slow inactivation seen also under control conditions.

The time constants are given in Fig. 4, A and B. It is apparent that in the control, a greater fraction of TTXr...
Nav1.8 than TTXs channels recovers from inactivation with a second time constant. This indicates that TTXr Nav1.8 channels are more prone to enter a slow inactivated state than TTXs channels. The fractional amplitudes of the second phase of recovery of Nav1.8 currents under amitriptyline and lidocaine were 64 and 65%, respectively (Fig. 4A). The fractional amplitudes of the second phase of recovery of TTXs currents under amitriptyline and lidocaine were 81 and 24%, respectively.

These results clearly show that at 100-ms recovery time, little recovery of inactivated and blocked Nav1.8 or TTXs channels had occurred in the presence of amitriptyline. In the presence of lidocaine, fast and slow components of recovery could not be separated adequately. This confirms the presumed significant differences in kinetics of block by amitriptyline and lidocaine and validates the pulse protocol used to estimate block of inactivated channels by amitriptyline but not lidocaine. To estimate block of inactivated channels by lidocaine, we employed an indirect approach, which is based on the concentration-dependent shift of the steady-state inactivation curve (Bean et al., 1983; Leuwer et al., 2004) and can be expressed by the equation:

\[ \Delta V_{0.5} = k \ln\left[\frac{1 + ([L]/IC_{50L})}{1 + ([L]/K_i)}\right] \]

where \( \Delta V_{0.5} \) is the shift in midpoint of the steady-state inactivation curve, \( k \) is the slope factor of the steady-state inactivation curve derived from a Boltzmann fit, \([L]\) is the concentration of lidocaine applied, \( IC_{50L} \) is the IC50 value for resting channels, obtained in concentration-inhibition experi-
iments at $V_h = -140\,\text{mV}$, and $K_i$ is the dissociation constant for block of inactivated channels by lidocaine. We measured steady-state inactivation of $\text{Nav1.8}$ and TTXs channels at $V_h = -140\,\text{mV}$ as described in Fig. 1F in control and in the presence of $50\,\mu\text{M}$ lidocaine. Use-dependent block was examined at 2 and 10 Hz with 60 5- or 25-ms-long test pulses to $50\,\text{mV}$ from $V_h = -140\,\text{mV}$. Development of use-dependent block (25-ms-long test pulses) at 2 and 10 Hz in control solution (D), with $1\,\mu\text{M}$ amitriptyline (E), and with $50\,\mu\text{M}$ lidocaine (F). Peak currents were measured, normalized to the current of the first pulse, and plotted against the pulse number. Block at pulse number 60 with respect to the current of the first pulse (5 or 25 ms) in control solution (G), with $1\,\mu\text{M}$ amitriptyline (H), and with $50\,\mu\text{M}$ lidocaine (I). An unpaired Student's $t$ test (SigmaStat; SPSS Science) was used to evaluate the significance of changes in mean values. *$p < 0.05$ was considered statistically significant.

**Use-Dependent Block of $\text{Nav1.8}$ and TTXs $\text{Na}^+$ Currents by Lidocaine and Amitriptyline.** To study use-dependent block of $\text{Nav1.8}$ and TTXs channel by lidocaine and amitriptyline, currents were activated at 2 or 10 Hz by 5- or 25-ms-long test pulses to $+50\,\text{mV}$ from a holding potential of $-140\,\text{mV}$ (Fig. 5). Without drug, a stronger use-dependent reduction was observed in $\text{Nav1.8}$ compared with TTXs currents. This reduction increased with a higher frequency and with a longer test pulse (Fig. 5, D and G). In the presence of $1\,\mu\text{M}$ amitriptyline, $\text{Nav1.8}$ and TTXs channels exhibited similar degree of use-dependent block at both frequencies and both durations of test pulses (Fig. 5, E and H). In contrast, in the presence of $50\,\mu\text{M}$ lidocaine, $\text{Nav1.8}$ channels exhibited a higher degree of use-dependent block compared with TTXs channels at both frequencies and both durations of test pulses (Fig. 5, F and I). The differences were more pronounced at higher frequencies than at longer test pulses. Interestingly, use-dependent block of $\text{Nav1.8}$ channels was more pronounced for lidocaine compared with amitriptyline, whereas use-dependent block of TTXs channels was more pronounced for amitriptyline compared with lidocaine. Lidocaine exhibited only little use-dependent block of TTXs channels, indicating that dissociation of lidocaine from inactivated TTXs channels is fast, much faster than its dissociation from inactivated $\text{Nav1.8}$ channels.

We additionally investigated use-dependent block by lidocaine or amitriptyline on both channel populations in the
absence of TTX (Fig. 5C). Currents clearly showed two components with a fast one due to TTXs channels and a slower one due to TTXr Nav1.8 channels. Quantifying use-dependent block by measuring peak currents revealed results similar to the results obtained with TTXs currents (Fig. 5, D–I, gray symbols and bars). Quantifying use-dependent block by measuring current amplitudes 0.8 ms after current activation, a time point at which TTXs channels are already inactivated, revealed results similar to the results obtained with TTXr Nav1.8 channels (data not shown). Apparently, lidocaine as well as amitriptyline affect TTXs and TTXr Nav1.8 channels independently.

To investigate the concentration dependence of use-dependent block, various concentrations of amitriptyline and lidocaine were applied, and Na$_{1.8}$ and TTXs currents were activated by 25-ms-long test pulses at 10 Hz (Fig. 6, A and B). Use-dependent block decreased with lower and increased with higher drug concentrations in both Na$_{1.8}$ and TTXs channels. Decaying normalized peak currents could be best fitted by a single-exponential function after subtracting respective control currents. Time constants of current decays are given in the figure. Block at pulse number 60 with respect to the current of the first pulse by various concentrations of amitriptyline (C) or lidocaine (D), respectively. An unpaired Student's t test (SigmaStat; SPSS Science) was used to evaluate the significance of changes in mean values. *p < 0.05 was considered statistically significant.

![Fig. 6. Concentration dependence of use-dependent block of Na$_{1.8}$ and TTXs channels. Development of use-dependent block (25-ms-long test pulses, 10 Hz) in control solution and with 0.2, 1, and 10 μM amitriptyline (A) or 10, 50, and 500 μM lidocaine (B). Decaying normalized peak currents were fitted by a single-exponential function after subtracting respective control currents. Time constants of current decays are given in the figure. Block at pulse number 60 with respect to the current of the first pulse by various concentrations of amitriptyline (C) or lidocaine (D), respectively. An unpaired Student’s t test (SigmaStat; SPSS Science) was used to evaluate the significance of changes in mean values. *p < 0.05 was considered statistically significant.](image)

In this study, we show that tonic block of either resting or inactivated channels by lidocaine and amitriptyline reveals little difference between TTXr Na$_{1.8}$ channels heterologously expressed in ND7/23 cells and TTXs channels endogenously expressed in ND7/23 cells. This suggests that any substantial differences in affinities demonstrated before might be due to state-dependent drug affinities rather than to binding site differences between channel isoforms. Surprisingly, use-dependent block by lidocaine but not by amitriptyline was more pronounced in TTXr Na$_{1.8}$ than in Na$_{1.8}$ channels exhibiting a higher degree of use-dependent block compared with TTXs channels in the presence of 10 and 500 μM lidocaine, respectively (Fig. 6D).

To further confirm disparate use-dependent blocking properties of lidocaine and amitriptyline toward TTXs and TTXr Na$_{1.8}$ channels under more native conditions, we also investigated use-dependent block of Na$^+$ currents in DRG wild-type neurons in the presence of TTX, in DRG neurons of Na$_{1.8}$ knockout mice, and in DRG wild-type neurons in the absence of TTX (Fig. 7). The results obtained by these experiments resemble those obtained by experiments in ND7/23 cells. Specifically, use-dependent block by lidocaine was more pronounced in TTXr channels of wild-type mice than in TTXs channels of Na$_{1.8}$ knockout mice, whereas use-dependent block by amitriptyline was similar in these channel populations.

Discussion

In this study, we show that tonic block of either resting or inactivated channels by lidocaine and amitriptyline reveals little difference between TTXr Na$_{1.8}$ channels heterologously expressed in ND7/23 cells and TTXs channels endogenously expressed in ND7/23 cells. This suggests that any substantial differences in affinities demonstrated before might be due to state-dependent drug affinities rather than to binding site differences between channel isoforms. Surprisingly, use-dependent block by lidocaine but not by amitriptyline was more pronounced in TTXr Na$_{1.8}$ than in Na$_{1.8}$ channels exhibiting a higher degree of use-dependent block compared with TTXs channels in the presence of 10 and 500 μM lidocaine, respectively (Fig. 6D).
TTXs channels. This result was confirmed in DRG neurons and is associated with the greater tendency of Nav1.8 to enter a slow inactivated state. In the absence of isoform-specific blockers, it is conceivable that the expression pattern of Nav1.8 and TTXs channels in sensory neurons might influence the efficiency of Na$^{+}$ channel blockers used for chronic pain management.

Expression of Nav1.8 in the ND7/23 Cell Line and Coexpression of the β1 Subunit. The TTXr Nav1.8 channel is the molecular entity underlying the slowly inactivating TTXr current of DRG neurons. Unlike other sodium channels, recombinant Nav1.8 is poorly expressed in most mammalian cell lines, even in the presence of accessory β-subunits. Annexin II light chain (p11), among other interacting proteins, has been identified as an essential regulatory factor that directly binds to the N terminus of Nav1.8 and promotes translocation of the channel to the plasma membrane (Okuse et al., 2002). The hybrid cell line ND7/23 derived from neonatal rat DRG neurons fused with the mouse neuroblastoma N18Tg2 has proven to be a suitable heterologous host cell line for sufficient expression of recombinant Nav1.8 channels (Choi et al., 2004; John et al., 2004). This cell line might provide the necessary cellular neuronal environment for functional expression of recombinant Nav1.8.

Four β-subunits have been described to date that potentially associate with Na$^{+}$ channel α-subunits (Catterall et al., 2003; Yu et al., 2003). β-Subunits affect Na$^{+}$ channel gating and cell surface expression when expressed heterologously. However, the effect of coexpression of β-subunits depends critically on the cell type in which the expression experiments are performed. In contrast to results in Xenopus oocytes, expression of β1-subunits with Nav1.2 or Nav1.4 in human embryonic kidney cells have little or no effect on the kinetics of sodium currents and cause positive shifts in the voltage dependence of activation and inactivation (Wright et al., 1999; Qu et al., 2001). ND7/23 cells reportedly express β1- and β3-subunits (John et al., 2004). We obtained larger Nav1.8 and TTXs current amplitudes by coexpression of β1. Thus, additional recombinant β1-subunit expression can enhance recombinant or constitutive Na$^{+}$ currents. Expression of Nav1.8 without coexpression of β1 was rather poor, and detailed analysis of the effect of β1 on current kinetics and voltage dependence of activation and inactivation was hampered. Surprisingly, coexpression of β1 did not influence current kinetics or activation and inactivation properties of TTXs currents in ND7/23 cells.

It was demonstrated previously that tonic block of resting and inactivated channels by the local anesthetic cocaine is
not changed significantly by coexpression of the β1-subunit in mammalian cells (Wright et al., 1999). To our knowledge, no data are available on the effect of β1 on use-dependent block of recombinant Na⁺ currents in mammalian cells. Because the β1-subunit has the tendency to reduce the proportion of slow gating, use-dependent block might be enhanced in the absence of β1, especially in Na₈,1.8. Clearly, further studies are needed to investigate interaction and effect of β1 and β3 subunits on Na₈,1.8 channels.

Similarities in Affinities of Resting and Inactivated Channels. Block of voltage-gated Na⁺ channels by local anesthetics is highly state-dependent with a preference for open and inactivated rather than for resting channel states. In this study, we have demonstrated that tonic block of resting TTXs and TTXr Na₈,1.8 channels by lidocaine and amitriptyline, measured at a holding potential of \( V_h = -140 \) mV, reveal little differences. At \( V_h = -80 \) mV, the fraction of channels residing in the inactivated, high-affinity state is higher for TTXs compared with TTXr Na₈,1.8 channels. Consequently, Na⁺ channel blockers are more potent to tonically block TTXs than TTXr currents at a holding potential of \( V_h = -80 \) mV, as demonstrated before (Roy and Narahashi, 1992; Scholz et al., 1998). This difference is therefore due to state-dependent drug affinity rather than to binding site differences of TTXr Na₈,1.8 and TTXs channel isoforms.

It was demonstrated previously that the binding affinity of inactivated channels could be estimated directly for Na⁺ channel blockers that unbind slowly from inactivated channels with the three-step protocol shown in Fig. 3 (Wright et al., 1997). For amitriptyline, this pulse protocol was applicable because the recovery time course from inactivated drug-bound channel states was slow, as reported before (Nau et al., 2000; Wang et al., 2004). In this study, block of inactivated TTXs and TTXr Na₈,1.8 channels by amitriptyline was estimated with conditioning prepulses to −70 and −40 mV, respectively. These prepulses induced some amount of slow inactivated channels with a higher percentage in TTXr Na₈,1.8 than in TTXs channels. Consequently, slow inactivated TTXr Na₈,1.8 channels might have contributed to block by amitriptyline measured with this approach. Still, TTXs channels displayed approximately 1.3-fold higher sensitivity to amitriptyline than TTXr Na₈,1.8 channels.

A different approach was required to estimate block of inactivated channels by lidocaine due to the fast recovery of inactivated channels from block by lidocaine. This approach exploits the concentration-dependent shift of the steady-state inactivation curve by Na⁺ channel blockers (Bean et al., 1983; Leuwer et al., 2004). The \( K_i \) values of 41 and 46 \( \mu \)M calculated for Na₈,1.8 and TTXs channels, respectively, are higher than the IC₅₀ value measured for block of TTXs channels in concentration-inhibition experiments at \( V_h = -80 \) mV (30 ± 2 \( \mu \)M). The same phenomenon was observed when employing this approach for estimating block of inactivated channels by amitriptyline (data not shown) and might be explained by the fact that the model tends to overestimate the effect of low drug concentrations and to underestimate the effect of high drug concentrations. However, based on the evidence demonstrated, we conclude that sensitivities for lidocaine or amitriptyline reveal little differences between inactivated Na₈,1.8 and TTXs channels.

Stronger Use-Dependent Inhibition of Na₈,1.8 by Lidocaine. The most interesting finding in this study is the significant difference in use-dependent block of TTXr Na₈,1.8 and TTXs channels by lidocaine but not by amitriptyline. This phenomenon holds true for several drug concentrations and could be observed in ND7/23 cells expressing TTXs and TTXr Na₈,1.8 channels and in DRG neurons of wild-type and Nav1.8 knockout mice. A more pronounced use-dependent block by lidocaine has been observed before for TTXr compared with TTXs channels in native DRG neurons (Roy and Narahashi, 1992) and for TTXr Na₈,1.8 compared with TTXs Na₈,1.7 channels coexpressed with the β1 subunit in Xenopus oocytes (Chevrier et al., 2004). However, it has not been noticed explicitly that a more pronounced use-dependent block of TTXr Na₈,1.8 might be a feature of some but not all Na⁺ channel blockers.

Use-dependent block arises from binding of local anesthetics to inactivated channels recruited during repetitive pulses and from dissociation of local anesthetics from inactivated states with a time constant slower than the frequency of the pulses. Alternatively, it was suggested that use-dependent block results from slow recovery of drug-bound channels due to an interaction between local anesthetics and slow inactivated states.

Indeed, slow inactivation is more pronounced in TTXr compared with TTXs channels (Rush et al., 1998; Blair and Bean, 2003; Chevrier et al., 2004). Moreover, slow inactivation in TTXr channels was suggested to play a major role in controlling adaptation of action potential firing in nociceptive sensory neurons (Blair and Bean, 2003).

In the present study, slow inactivation in TTXr Na₈,1.8 channels is responsible for the greater fraction of TTXr Na₈,1.8 channels recovering from inactivation with a slow time constant in control (Fig. 4) and for the more pronounced use-dependent inhibition of TTXr Na₈,1.8 currents in the absence of blockers (Figs. 5, D and G, and 6, D and G). Preferential binding of lidocaine to slow inactivated states of TTXr Na₈,1.8 might indeed explain the more pronounced use-dependent block of TTXr Na₈,1.8 compared with TTXs channels.

In contrast to lidocaine, amitriptyline is a potent use-dependent blocker of both TTXs and TTXr Na₈,1.8 currents. Several possible mechanisms could account for this difference in use-dependent block by amitriptyline. The slow recovery time course of fast-inactivated drug-bound Na⁺ channels in the presence of amitriptyline might cover or overlap any interactions with slow inactivated states. Amitriptyline might inhibit any interactions with slow inactivated states. Amitriptyline might prevent Na₈,1.8 channels to enter slow inactivated states. Clearly, further studies are needed to support or reject these hypotheses.

Clinical Relevance of the Findings. Following injury of peripheral nerves, the composition of Na⁺ channel isoforms expressed in sensory neurons undergoes significant changes (Lai et al., 2003). Subsequent electrophysiological changes might poise cells to fire spontaneously or at inappropriately high frequencies. Among the changes following nerve injury is an increase in the expression of TTXs Na₈,1.3 in cell bodies of sensory neurons (Cummins and Waxman, 1997) and a redistribution of Na₈,1.8 and Na₈,1.9 from cell bodies of sensory neurons to the peripheral axon at the site of injury (Lai et al., 2002; Gold et al., 2003).

Contribution of Na₈,1.8 to alteration of pain threshold is unclear to date. Studies on Na₈,1.8 knockout mice indicated that Na₈,1.8 might not be involved (Kerr et al., 2001). In
addition, neuropathic pain develops normally in mice lacking both Na\textsubscript{v}1.7 and Na\textsubscript{v}1.8 (Nassar et al., 2005). In contrast, antisense oligonucleotides directed against Na\textsubscript{v}1.8 administered i.t. completely reversed neuropathic pain behavior (Lai et al., 2002). Furthermore, Na\textsubscript{v}1.8 might play a role in spontaneous neuropathic pain because neurones in Na\textsubscript{v}1.8 knockout mice display less ectopic discharges than in wild-type mice (Roza et al., 2003).

Altogether, it is generally conceived that isoform-specific Na\textsuperscript{+} channel blockers, especially those targeting Na\textsubscript{v}1.8 and Na\textsubscript{v}1.3, could be useful analogues. Our data, however, confirm the presumption that it is highly unlikely to find isoform-specific Na\textsuperscript{+} channel blockers interacting with the local anesthetic binding site. On the other hand, they demonstrate that there is significant functional selectivity by lidocaine but not amitryptiline toward slow inactivated Na\textsubscript{v}1.8 channel states. This phenomenon could explain the potential of lidocaine to inhibit ectopic activity in peripheral nerves after injury at concentrations that do not block nociception (Devor et al., 1992). Furthermore, the disparate use-dependent drug effects found in this study could be exploited to search for other compounds with more favorable therapeutic profiles. Finally, it is conceivable that the efficiency of Na\textsuperscript{+} channel blockers in chronic pain management might be determined by the distinct and individual expression pattern of Na\textsubscript{v}1.8 and TTX channels in sensory neurons.

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