Regulation of Na\textsubscript{v}1.6 and Na\textsubscript{v}1.8 peripheral nerve Na\textsuperscript{+} channels by auxiliary β-subunits

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Zhao J, O’Leary ME, Chahine M. Regulation of Na\textsubscript{v}1.6 and Na\textsubscript{v}1.8 peripheral nerve Na\textsuperscript{+} channels by auxiliary β-subunits. J Neurophysiol 106: 608–619, 2011. First published May 11, 2011; doi:10.1152/jn.00107.2011.—Voltage-gated Na\textsuperscript{+} (Na\textsubscript{v}) channels are composed of a pore-forming α-subunit and one or more auxiliary β-subunits. The present study investigated the regulation by the β-subunit of two Na\textsuperscript{+} channels (Na\textsubscript{v}1.6 and Na\textsubscript{v}1.8) expressed in dorsal root ganglion (DRG) neurons. Single cell RT-PCR was used to show that Na\textsubscript{v}1.8, Na\textsubscript{v}1.6, and β\textsubscript{1}–β\textsubscript{4} subunits were widely expressed in individually harvested small-diameter DRG neurons. Coexpression experiments were used to assess the regulation of Na\textsubscript{v}1.6 and Na\textsubscript{v}1.8 by β-subunits. The β\textsubscript{1}-subunit induced a 2.3-fold increase in Na\textsuperscript{+} current density and hyperpolarizing shifts in the activation (−4 mV) and steady-state inactivation (−4.7 mV) of heterologously expressed Na\textsubscript{v}1.8 channels. The β\textsubscript{2}-subunit caused more pronounced shifts in activation (−16.7 mV) and inactivation (−9.3 mV) but did not alter the current density of cells expressing Na\textsubscript{v}1.8 channels. The β\textsubscript{3}-subunit did not alter Na\textsubscript{v}1.8 gating but significantly reduced the current density by 31%. This contrasted with Na\textsubscript{v}1.6, where the β-subunits were relatively weak regulators of channel function. One notable exception was the β\textsubscript{3}-subunit, which induced a hyperpolarizing shift in activation (−7.6 mV) but no change in the inactivation or current density of Na\textsubscript{v}1.6. The β-subunits differently regulated the expression and gating of Na\textsubscript{v}1.8 and Na\textsubscript{v}1.6. To further investigate the underlying regulatory mechanism, β-subunit chimeras containing portions of the strongly regulating β\textsubscript{1}-subunit and the weakly regulating β\textsubscript{2}-subunit were generated. Chimeras retaining the COOH-terminal domain of the β\textsubscript{1}-subunit produced hyperpolarizing shifts in gating and increased the current density of Na\textsubscript{v}1.8, similar to that observed for wild-type β\textsubscript{1}-subunits. The intracellular COOH-terminal domain of the β\textsubscript{1}-subunit appeared to play an essential role in the regulation of Na\textsubscript{v}1.8 expression and gating.

voltage-gated sodium channels; nociception

Voltage-gated Na\textsuperscript{+} (Na\textsubscript{v}) channels are responsible for the rising phase of action potentials in many excitable cells and consist of a pore-forming α-subunit and one or more auxiliary β-subunits. The TTX-resistant Na\textsubscript{v}1.8 channel is highly expressed in small-diameter dorsal root ganglion (DRG) neurons and trigeminal ganglia. It exhibits slow activation, slow inactivation, and rapid repriming kinetics (Sangameswaran et al. 1996; Vijayaravanag et al. 2001). The TTX-sensitive Na\textsubscript{v}1.6 channel is found in many different neuronal populations in the peripheral nervous system and central nervous system (CNS) and exhibits fast activation and inactivation kinetics (Chatelier et al. 2010). To date, at least five isoforms of auxiliary β-subunits (β\textsubscript{1}–β\textsubscript{4} subunits as well as the β\textsubscript{1A}–β\textsubscript{1C} subunit, a splice variant of the β\textsubscript{1}-subunit) have been identified (Chahine et al. 2005). They are transmembrane proteins containing an extracellular Ig domain, a single transmembrane segment, and a small intracellular COOH-terminal domain. The β\textsubscript{1}–β\textsubscript{4}-subunits interact noncovalently with the α-subunit, whereas the β\textsubscript{2}– and β\textsubscript{3}-subunits interact covalently with the α-subunit via a disulfide bond. The β-subunits form a family of cell adhesion molecules and modulate the channel gating, location, expression levels, and functional properties of α-subunits (Isom 2002b). The β-subunits also regulate cell migration and aggregation as well as interactions with the cytoskeleton (Malhotra et al. 2000).

The β\textsubscript{1}-subunit is expressed abundantly in intermediate- to large-diameter (≥25 μm) DRG neurons and at much lower levels in small-diameter (<25 μm) DRG neurons (Oh et al. 1995). Increased β\textsubscript{1}-subunit mRNA levels in the dorsal horn of the spinal cord after nerve injuries indicate that the β\textsubscript{1}-subunit may be involved in the generation of neuropathic pain (Blackburn-Munro and Fleetwood-Walker 1999) and may also regulate Na\textsuperscript{+} channel function. Coexpression of β\textsubscript{1}-subunits with Na\textsubscript{v}1.7 or Na\textsubscript{v}1.8 in Xenopus oocytes accelerates current decay kinetics, negatively shifts steady-state curves, and significantly enhances the expression of Na\textsubscript{v}1.8. In addition, Na\textsubscript{v}1.8 + β channels rapidly enter into slow inactivation states at hyperpolarized voltages, causing a frequency-dependent reduction of current amplitudes and modulating the firing frequency in tsA201 cells and Xenopus oocytes (Zhao et al. 2007; Vijayaraganav et al. 2004). The β\textsubscript{1}-subunit promotes neurite outgrowth in cerebellar granule neurons and plays a critical role in neuronal development (Davis et al. 2004). β\textsubscript{1}-Subunit-null mice exhibit a hypereexcitable phenotype, including epilepsy, ataxia, abnormal neuronal pathfinding, and a prolonged QT interval (Lopez-Santiago et al. 2007; Chen et al. 2004).

The β\textsubscript{2}-subunit is expressed at low levels in small- to large-diameter DRG neurons (Takahashi et al. 2003) but is strongly expressed throughout the CNS (Gastaldi et al. 1998). So far, information on the expression of the β\textsubscript{2}-subunit in neuropathic pain models is contradictory. Immunohistochemistry and Western blot analyses have revealed that β\textsubscript{2}-subunit protein levels are markedly upregulated for at least 4 wk in DRG neurons after spared nerve injuries (Pertin et al. 2005) but are downregulated in cerebellar sensory ganglia after avulsion injuries (Coward et al. 2001). Other studies have found that the β\textsubscript{2}-subunit selectively increases TTX-sensitive Na\textsuperscript{+} channel mRNA and protein expression, particularly of Na\textsubscript{v}1.7, in small-fast DRG neurons (Lopez-Santiago et al. 2006). In addition, β\textsubscript{2}-subunit-null mice exhibit a reduced response to neuropathic and inflammatory pain (Pertin et al. 2005).

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The β3-subunit is expressed at high levels in small-diameter DRG neurons and in the II and X layers of the spinal cord. The distribution of β3-subunits in DRG neurons and the CNS exhibits a complementary pattern with that of the β2-subunit (Morgan et al. 2000). Unlike β1- and β2-subunits, the β3-subunit has a clearer role in neuropathic pain in that its β3-subunit mRNA and protein levels are upregulated in various neuropathic pain models (Shah et al. 2001; Takahashi et al. 2003). Furthermore, β3-subunit mutations are associated with early-onset lone atrial fibrillation, and β3-subunit-null mice exhibit cardiac ventricular electrophysiology abnormalities (Hakim et al. 2008; Olesen et al. 2010).

The β1- and β2-subunits share a similar expression pattern in the CNS, but the β3-subunit is more abundantly expressed in DRG neurons than the β2-subunit, with higher levels in large-diameter DRG neurons and lower levels in small- and intermediate-diameter neurons (Yu et al. 2003). The β3-subunit has been reported to induce negative shifts in the activation of several Na\(^+\) channel subtypes, including Na\(_{1.1}\), Na\(_{1.2}\), Na\(_{1.4}\), and Na\(_{1.6}\), indicating that the β3-subunit may modulate the electrical properties of neurons by allowing Na\(^+\) channels to activate at more negative voltages (Yu et al. 2003; Chen et al. 2008; Aman et al. 2009). Since a free peptide derived from its cytoplasmic tail replicates the action of the endogenous blocking protein, the β3-subunit may be indirectly involved in the generation of resurgent currents (Grieco et al. 2005). Furthermore, recent studies have shown that the β3-subunit plays a role in the pathophysiology of a cardiac disease, long QT syndrome type 3, and neurological Huntington’s disease (Oyama et al. 2006; Medeiros-Domingo et al. 2007).

Using single cell RT-PCR techniques, we show that a large percentage of these small-diameter neurons (40–60%) express β1-, β2-, and β3-subunits, whereas only ~10% express the β2-subunit. We investigated how these β-subunits modulate the expression and gating properties of two different Na\(^+\) channel subtypes: Na\(_{1.6}\) and Na\(_{1.8}\). We demonstrate that the β1-subunit induces a significant increase in the current density of Na\(_{1.8}\) but has no effect on the current density of Na\(_{1.6}\). In addition, the COOH-terminal domain of the β1-subunit is involved in the modulation of the Na\(_{1.8}\) channel based on the results of experiments with a β1 COOH-terminal deletion variant and β1/β2-subunit chimeras harboring various regions of the β1-subunit together with the entire β2-subunit.

**MATERIALS AND METHODS**

Preparation of DRG neurons. Seven-day-old rat pups were anesthetized with isoflurane before decapitation. The rats were handled in accordance with the principles and guidelines of the local animal care committee, from which we received approval. DRGs were transferred to L-15 Leibovitz media supplemented with 1% FBS (GIBCO), 2 mM glutamine, 24 mM NaHCO\(_3\), 38 mM glucose, 2% penicillin-streptomycin (GIBCO), and 50 ng/ml nerve growth factor (Sigma-Aldrich). The ganglia were incubated for 30 min at 37°C in 2 ml of HBSS-HEPES containing 1.5 mg/ml collagenase (Sigma-Aldrich, St. Louis, MO) followed by 1 mg/ml trypsin (Sigma-Aldrich) for an additional 30 min. Trypsin was removed, and the ganglia were transferred to L-15 Leibovitz media supplemented with 1% FBS (GIBCO), 2 mM glutamine, 24 mM NaHCO\(_3\), 38 mM glucose, 2% penicillin-streptomycin (GIBCO), and 50 ng/ml nerve growth factor (Sigma-Aldrich). The ganglia were disrupted using fire-polished Pasteur pipettes, and dissociated neurons were placed in 35-mm dishes containing 2 ml of supplemented Leibovitz media.

Single cell RT-PCR. Intact neurons were harvested by drawing the cells into large-bore 20-μm-diameter pipettes containing 20 μl of RNase-free water and were rapidly frozen for further analysis. Random hexamer primers (65 ng, Invitrogen, Carlsbad, CA) were added to 10-μl aliquots of cell lysates, which were heated to 70°C for 3 min and rapidly cooled on ice. mRNA was reverse transcribed in a 25-μl reaction containing Moloney murine leukemia virus reverse transcriptase (200 units, Fisher Bioreagents), 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl\(_2\), 10 mM DTT, 0.5 mM dNTPs, and RNase inhibitor (1 U/μl, Promega). The remaining 10-μl aliquots of cell lysate were treated in an identical fashion except that water was substituted for reverse transcriptase in the reaction mixture. Thereafter, the first-strand cDNA synthesized in the reactions with or without reverse transcriptase (1–3 μl) was amplified in two successive rounds of a standard PCR protocol (30 cycles each) using nested gene-specific primers for Na\(^+\) channel β1-β2-subunits. Primer sets were designed to span one or more exon-intron borders to eliminate the possibility of contamination by genomic DNA. The PCR amplification was based on Taq polymerase (Roche Biochemicals) and used the following protocol: 94°C/1 min, 55°C/0.5 min, and 72°C/1 min (30 cycles). Additional controls included blanks in which the PCR amplification was performed in the absence of added reaction mixture with and without reverse transcriptase and a full RT-PCR analysis of the bath solution immediately surrounding the harvested neurons. Amplification of the reaction without reverse transcriptase, the PCR blank, and the bath solution immediately surrounding the harvested neurons routinely failed to produce amplicons. The sizes of the cDNA amplicons were estimated by running the samples on 2% agarose gels, after which the DNA was purified (QiAEx II, Qiagen) and sequenced.

Gene transfections and cell cultures. Two human embryonic kidney (HEK)-293 cell lines stably expressing human Na\(_{1.6}\) and rat Na\(_{1.8}\) were used. Both HEK-293 cell lines were grown under standard tissue culture conditions (5% CO\(_2\), 37°C) in high-glucose DMEM supplemented with FBS (10%), L-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (10 mg/ml; GIBCO-BRL Life Technologies). Rat auxiliary β1-, β2-, and β3-subunits were cloned in our laboratory as previously described (Vijayaragavan et al. 2004). The rat β1-subunit was a gift from Dr. Lori L. Isom (University of Michigan, Ann Arbor, MI). The Na\(^+\) channel β1-β2-subunits and CD8 (empty vector) were constructed in the pIREs vector (Invitrogen), respectively (pIERS/CD8/β1, pIERS/CD8/β2, pIERS/CD8/β1-β2, and pIERS/CD8). HEK-293 cell lines stably expressing Na\(_{1.6}\) or Na\(_{1.8}\) were transiently transfected with the same amount of individual β1-β2-subunits or empty vector pIREs/CD8 DNA. Transient transfections were carried out using the calcium phosphate method as previously described (Zhao et al. 2007). Transfected cells were briefly preincubated with CD8 antibody-coated beads before currents were recorded (Dynabeads M450 CD8-a). HEK-293 cells expressing the pIREs/CD8/B bicistronic vector were decorated with CD8 beads, which were used to identify cells for recording currents (Zhao et al. 2007).

The β1/β2-subunit chimeras (β111, β221, and β112) and COOH-terminal deletion variant (β11) were generous gifts from Dr. Thomas Zimmer (Institute of Physiology II, Friedrich Schiller University, Jena, Germany). β111 contains the extracellular domain of the β2-subunit and the transmembrane and intracellular domains of the β1-subunit. β221 contains the extracellular domain and transmembrane domains of the β2-subunit and the intracellular domain of the β1-subunit. β112 contains the extracellular and transmembrane domains of the β1-subunit and the intracellular domain of the β2-subunit. β11 contains only the extracellular and transmembrane domains of the β1-subunit and the intracellular domain of the β2-subunit. β221 contains only the extracellular and transmembrane domains of the β2-subunit. Transient transfections were performed using the calcium phosphate method as previously described (Zhao et al. 2007). Transfected cells were identified for patch-clamp recordings using a phase-contrast microscope.
analysis by preincubation with CD8 antibody-coated beads, as mentioned above.

Whole cell patch-clamp recordings. Macroscopic Na⁺ currents from rat DRG neurons and HEK-293 stable cells were recorded using the whole cell configuration of the patch-clamp technique. For whole cell patch-clamp recordings of DRG neurons, the pipette solution was composed of (in mM) 100 CsF, 25 CsCl, 10 NaCl, 1 EGTA, and 10 HEPES (pH 7.4). The bath solution was composed of (in mM) 140 NaCl, 2 KCl, 1.5 CaCl₂, 1 MgCl₂, and 10 HEPES (pH 7.4). TTX was bath applied at a final concentration of 300 nM. For HEK-293 stable cells, the pipette solution was composed of (in mM) 5 NaCl, 135 CsF, 10 EGTA, and 10 Cs-HEPES. The pH was adjusted to 7.4 using 1 N NaOH. The bath solution was composed of (in mM) 150 NaCl, 2 KCl, 1.5 CaCl₂, 1 MgCl₂, 10 glucose, and 10 Na-HEPES. The pH was adjusted to 7.4 with 1 N NaOH.

The liquid junction potential was measured as described by Neher (1992) (+7 mV) and was consistent to the one calculated using pCLAMP (+7.1 mV, Molecular Devices, Union City, CA). To correct for this junction potential, the pipette voltage was held at −7 mV, and the pipette offset was zeroed before making a giga seal. After that, no additional correction was necessary, and the applied voltages are the reported voltages.

The recordings were taken exactly 10 min after the whole cell configuration was obtained to allow the current to stabilize and fully dialyze the cell with pipette solution. Na⁺ currents were recorded at room temperature (22–23°C). Command pulses were generated, and currents were recorded using pCLAMP software (version 8.0) and an Axopatch 200 amplifier (Molecular Devices). Patch electrodes were fashioned from borosilicate glass (Corning 8161) and coated with silicone elastomer (Sylgard, Dow-Corning, Midland, MI) to minimize stray capacitance. Current recordings were taken using low-resistance electrodes (<1 MΩ), and the series resistance was compensated at values of ≥80% to minimize patch-clamp errors. Whole cell currents were filtered at 5 kHz, digitized at 10 kHz, and stored on a microcomputer equipped with an analog-to-digital converter (Digidata 1300, Molecular Devices).

Average current-voltage curves were obtained by plotting the current density (in pA/pF) versus the voltage. For the construction of activation curves, Na⁺ conductance (GNa) was calculated from the peak current (Ipeak) using the following equation: $G_{Na} = I_{peak}(V - E_{Na})$, where $V$ is the test potential and $E_{Na}$ is the reversal potential. Normalized $G_{Na}$ was plotted against the test potentials. For the construction of inactivation curves, the peak current was normalized relative to the maximal value and plotted against the conditioning pulse potential. Steady-state activation and inactivation curves were fit to a Boltzmann equation of the following form: $G/G_{max} = 1/[1 + \exp(V_{1/2} - V)/k_r]$, where $G$ is conductance, $G_{max}$ is maximal conductance, $I$ is peak current, $I_{max}$ is maximal current, $V_{1/2}$ is the voltage at which the channels are half-maximally activated or inactivated, and $k_r$ is the slope factor. The window current results from the overlap of voltage-dependent activation and inactivation that determines a range of potentials (window) at which Na⁺ channels are nonactivated and available for activation. Using the $V_{1/2}$ and $k_r$ values of voltage-dependent activation and inactivation, the probability of Na⁺ channels being within the window was calculated using the following equation: $(1/[1 + \exp(V_{1/2} activation - V)/k_r activation)] \times (1/[1 + \exp(V - V_{1/2} inactivation)/k_r inactivation])$.

Analysis of electrophysiological data. Data were analyzed using a combination of pCLAMP software (version 9.0, Molecular Devices), Microsoft Excel, and SigmaPlot (version 11.0, SPS, Chicago, IL). Data are expressed as means ± SE.

RESULTS

Single cell analysis of Na⁺ channel and β-subunit expression in DRG sensory neurons. Figure 1A shows whole cell Na⁺ currents of a typical small-diameter (<25 μm) DRG neuron before (control) and after bath application of 300 nM TTX. The slowly inactivating TTX-resistant Na⁺ current observed in this neuron is characteristic of Naᵥ1.8 channels, which are known to be preferentially expressed in small-diameter sensory neurons (Sangameswaran et al. 1996). Single cell RT-PCR was used to investigate the expression of Naᵥ1.8 channels and auxiliary β-subunits in this population. Figure 1B shows the analysis of 53 individually harvested neurons. A high percentage of these neurons (80–85%) expressed Naᵥ1.7, Naᵥ1.8, and Naᵥ1.9 channels, consistent with what has recently been reported for small-diameter sensory neurons (Ho and O’Leary 2010). Between 40% and 60% of these neurons expressed at least one β₁, β₂, or β₃ subunit. Only a small percentage (17%) expressed the β₃ subunit, suggesting that this subunit is not widely expressed in these neurons. These data also pro-
vided insights into the overlap of β-subunit expression. The β2-β4 combination (39% neurons) was most frequently observed followed by β1-β3 (28%), β1-β2 (22%), β2-β4 (13%), β3-β4 (9%), and β1-β4 (7%). Overall, the data indicate that β-subunits are differentially expressed in subpopulations of small-diameter neurons, where they may regulate the expression and gating properties of Na1.7, Na1.8, and Na1.9 channels present in these neurons.

**Regulation of expression levels of Na1.8 by β1–4 subunits of HEK-293 stable cells.** Western blots showed no detectable endogenous expression of β1–β4-subunits in HEK-293 cells (Aman et al. 2009). HEK-293 cells are thus well suited for assessing the effect of auxiliary β-subunits on the expression levels and properties of heterologously expressed Na1.6 and Na1.8 channels.

Transient transfections of Na1.8 in HEK-293 cells only achieved partial cell surface expression of the channel (John et al. 2004). To increase the expression of Na1.8, we constructed HEK-293 stable cells. Current amplitudes ranged between 500 and 1,500 pA, and currents resisted a high concentration (10 μM) of TTX, as previously reported (Zhao et al. 2007).

To test the impact of Na+ channel β-subunits on the expression of Na1.8, we transiently transfected individual β1–β4-subunits or empty vector into HEK-293 cells stably expressing Na1.8. Figure 2 shows representative Na1.8 currents recorded from the HEK-293 stable cell line transiently transfected with individual β1–β4-subunits or empty vector and normalized to membrane capacitance. For all groups except for the β4-subunit coexpression group, Na1.8 currents activated at approximately −45 mV, and the peak inward current occurred at approximately −5 to 5 mV. The β2-subunit coexpression group activated earlier at −55 mV and reached its maximum at −10 mV. The reversal potential for all groups was −80 mV, or 6.2 mV less than the calculated value (86.2 mV; Fig. 3A). When cells were depolarized to 0 mV, coexpression of the β1-subunit induced a 2.3-fold increase in current density compared with the empty vector control (Na1.8 + β1; −165.7 ± 13.6 pA/pF, n = 18, vs. control: 73.9 ± 5.7 pA/pF, n = 15). Coexpression of the β2- and β4-subunits in Na1.8-expressing HEK-293 stable cells did not alter the expression of Na1.8 (Na1.8 + β2; −73.3 ± 4.2 pA/pF, n = 21, and Na1.8 + β4; −86.4 ± 8.0 pA/pF, n = 14, P > 0.05). Interestingly, expression of the β3-subunit induced a 31% reduction in Na1.8 current amplitude (−51.0 ± 3.0 pA/pF, n = 19) compared with the empty vector control (Fig. 3B). The regulation of Na1.8 expression by β1–β3 subunits encouraged us to investigate the influence of these regulatory subunits on the gating properties of the Na1.8 channel.

**Effect of β-subunits on the gating properties of Na1.8 in HEK-293 stable cells.** Voltage-dependent activation of Na1.8 was assessed from the peak Na+ conductance and plotted versus test voltages (Fig. 3C and Table 1). Activation of Na1.8 current was modified after the transient transfection of β1-, β2-, β3-, or β4-subunits in HEK-293 cells. Current amplitude increased 13.6 pA/pF, n = 18, vs. control: 73.9 ± 5.7 pA/pF, n = 15). This was in good agreement with the value recorded using TTX-resistant DRG neurons (Zhao et al. 2007). The V1/2 of the activation curve was not sensitive to β2- or β3-subunit expression, and no shift in the steady-state activation curves was observed (P > 0.05). When the β4-subunit was expressed, the V1/2 value of the activation curve

![Fig. 2. Representative Na1.8 current traces in human embryonic kidney (HEK)-293 stable cells transiently coexpressed with individual β1 (A), β2 (B), β3 (C), or β4 (D) subunits or empty vector (E) and normalized to membrane capacitance. The inset in A shows the protocol. Currents were elicited by depolarizing steps between −100 and 90 mV in 5-mV increments for 50 ms. Cells were held at a holding potential of −140 mV.](image-url)
produced a significant $-16.7$-mV shift ($-29.2 \pm 1.7$ mV, $n = 14$, $P < 0.01$).

To investigate the effects of $\beta_1$-$\beta_4$ subunits on steady-state $\text{Nav}_{1.8}$ inactivation (Fig. 3C and Table 1), we applied a two-pulse voltage-clamp protocol composed of 500-ms prepulses to potentials between $-140$ mV and $5$ mV followed by test pulses to $15$ mV. Like steady-state activation, when individual $\beta_1$-$\beta_3$-subunits were coexpressed with $\text{Nav}_{1.8}$, the $\beta_1$- and $\beta_3$-subunits caused $-4.6$-mV ($P < 0.05$) and $-9.3$-mV ($P < 0.01$) shifts in steady-state inactivation, respectively. Little modulation of voltage-dependent inactivation was observed when $\text{Nav}_{1.8}$ was coexpressed with $\beta_2$- or $\beta_4$-subunits ($P > 0.05$).

The overlapping area of the steady-state activation and inactivation curves shown in Fig. 3C gives a range of potentials (window) at which some channels are in the open state but do not undergo fast inactivation. Na$^+$ channels activated in this way cause “window currents” (Hodgkin et al. 1952). The leftward shifts in steady-state activation and inactivation caused by the $\beta_1$- and $\beta_3$-subunits should induce alterations in window currents. Figure 3D shows that the $\text{Nav}_{1.8}$ channels were probably within this window, that is, were inactivated and were available for activation when coexpressed with $\beta_1$- or $\beta_3$-subunits. Compared with the empty vector control, the small but significant hyperpolarization caused by the $\beta_1$-subunit shortened the window, indicating a lower probability of $\text{Nav}_{1.8}$ being activated. The peak probability shifted to a more depolarized potential, whereas the $\beta_3$-subunit slightly increased the window current of $\text{Nav}_{1.8}$.
Effect of individual β-subunits on the expression and gating properties of Nav1.6 in HEK-293 cells.

To study the specificity of β-subunit regulation, we investigated the effect of β1–β4 subunits on the expression of Nav1.6 in the HEK-293 stable cell line. We transiently transfected individual β1–β4 subunits or empty vector in HEK-293 cells stably expressing Nav1.6. Figure 4 shows representative current traces of Nav1.6 recorded from HEK-293 cells and normalized to membrane capacitance. Figure 5A shows the current-voltage relations of Nav1.6 currents. The activation threshold for these Na\textsubscript{v}1.6

Table 1. Effect of individual β-subunits on the activation and inactivation of Na\textsubscript{v}1.8 and Na\textsubscript{v}1.6 channels in HEK-293 cells

<table>
<thead>
<tr>
<th>Subunit Combination</th>
<th>Activation</th>
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<th>Steady-State Inactivation</th>
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<tr>
<td></td>
<td>(V_{1/2}), mV</td>
<td>(n)</td>
<td>(k_v), mV</td>
<td>(n)</td>
<td>(V_{1/2}), mV</td>
<td>(n)</td>
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<td>Na\textsubscript{v}1.8</td>
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<tr>
<td>+β\textsubscript{1}</td>
<td>-16.5 ± 1.4(\dagger)</td>
<td>18</td>
<td>-7.5 ± 0.4(\dagger)</td>
<td>18</td>
<td>-47.8 ± 1.5(\ast)</td>
<td>18</td>
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<tr>
<td>+β\textsubscript{2}</td>
<td>-13.9 ± 1.0</td>
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<td>21</td>
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<tr>
<td>+β\textsubscript{3}</td>
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<td>19</td>
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<td>19</td>
<td>-45.9 ± 1.4</td>
<td>15</td>
</tr>
<tr>
<td>+β\textsubscript{4}</td>
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<td>14</td>
<td>-7.2 ± 0.5(\dagger)</td>
<td>14</td>
<td>-52.5 ± 2.0(\dagger)</td>
<td>14</td>
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<tr>
<td>+Empty vector</td>
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<td>-10.5 ± 0.7</td>
<td>15</td>
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<tr>
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<td>-5.8 ± 0.2</td>
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<td>-37.3 ± 1.1</td>
<td>8</td>
<td>-5.9 ± 0.2</td>
<td>8</td>
<td>-75.8 ± 1.3</td>
<td>8</td>
</tr>
<tr>
<td>+β\textsubscript{4}</td>
<td>-44.3 ± 0.5(\dagger)</td>
<td>11</td>
<td>-5.6 ± 0.2</td>
<td>11</td>
<td>-77.1 ± 1.2</td>
<td>11</td>
</tr>
<tr>
<td>+Empty vector</td>
<td>-36.7 ± 1.1</td>
<td>9</td>
<td>-5.6 ± 0.3</td>
<td>9</td>
<td>-74.3 ± 2.3</td>
<td>9</td>
</tr>
</tbody>
</table>

Values are means ± SE; \(n\), number of cells. HEK-293 cells, human embryonic kidney-293 cells; \(V_{1/2}\), voltage at which Na\textsubscript{v}1.6 channels are half-maximally activated or inactivated; \(k_v\), slope factor. \(\ast P < 0.05 and \dagger P < 0.01\) compared with the control (+empty vector) group.

(0.81%) of Na\textsubscript{v}1.8 channels that would be activated near the resting membrane potential.

Effect of β\textsubscript{1}–β\textsubscript{4}-subunits on the expression and gating properties of Na\textsubscript{v}1.6 in HEK-293 stable cells. To study the specificity of β-subunit regulation, we investigated the effect of β\textsubscript{1}–β\textsubscript{4} subunits on the expression of Na\textsubscript{v}1.6 in the HEK-293 stable cell line. We transiently transfected individual β\textsubscript{1}–β\textsubscript{4} subunits or empty vector in HEK-293 cells stably expressing Na\textsubscript{v}1.6. Figure 4 shows representative current traces of Na\textsubscript{v}1.6 recorded from HEK-293 cells and normalized to membrane capacitance. Figure 5A shows the current-voltage relations of Na\textsubscript{v}1.6 currents. The activation threshold for these Na\textsubscript{v}1.6 currents was evoked with depolarizing voltage steps from −100 to 90 mV in 5-mV increments for 50 ms at a holding potential of −140 mV.
activation and inactivation curves of Nav1.6. Coexpression of

\( \beta_1 \)- and \( \beta_2 \)-subunits or empty vector. For clarity, we show only the symbols for the \( \beta_1 \) and control groups and have removed the symbols for the other groups. The activation curves were generated using the same protocol as in Fig. 4A. Inactivation was measured using 500-ms prepulses to potentials between \(-140 \) and \( 5 \) mV. The fraction of available current was determined using test pulses to \(-20 \) mV at a holding potential of \(-140 \) mV (see the inset under the inactivation curve for the protocol). The smooth lines of activation and inactivation are fits to a Boltzmann function (see data analysis in MATERIALS AND METHODS). Coexpression of the \( \beta_4 \)-subunit caused a negative shift in the activation curve from \(-65 \) to \(-20 \) mV (\( P < 0.01 \) or \( P < 0.05 \)). However, coexpression of \( \beta_1 \)- and \( \beta_2 \)-subunits did not affect the voltage-dependent inactivation of Nav1.6 in HEK-293 cells. The values of \( V_{1/2} \) and \( k \) are shown in Table 1.

C: effect of the \( \beta_4 \)-subunit on the Nav1.6 window current. The \( \gamma \)-axis shows the probability of Nav1.6 channels being within the window, which was measured using the equation given in MATERIALS AND METHODS. Coexpression of the \( \beta_4 \)-subunit increased the probability of Nav1.6 opening within the window and shifted the peak probability in a more hyperpolarizing direction.

D: representative resurgent Na\(^+\) current in HEK-293 cells coexpressing Nav1.6 and the \( \beta_4 \)-subunit. The voltage protocol used to elicit the currents is shown under the current trace. HEK-293 cells coexpressing Nav1.6 and the \( \beta_4 \)-subunit were depolarized from a holding potential of \(-120 \) mV by a prepulse to \( 30 \) mV for 40 ms to activate and inactivate Na\(^+\) channels. Each prepulse was followed by a single 100-ms test pulse to potentials ranging from \(-60 \) to \( 5 \) mV in \( 5 \)-mV increments. Resurgent currents were not detected in all HEK-293 cells coexpressing Nav1.6 and the \( \beta_4 \)-subunit.

Figure 5B is a composite figure showing the steady-state activation and inactivation curves of Nav1.6. Coexpression of \( \beta_1 \)-, \( \beta_2 \)-, or \( \beta_3 \)-subunits with Nav1.6 did not cause a shift in the activation and inactivation curves compared with the empty vector control group. However, the \( \beta_2 \)-subunit induced a 7.6-mV hyperpolarized shift in the \( V_{1/2} \) value of Nav1.6 activation but did not appreciably alter the voltage dependence of inactivation. As such, only the \( \beta_2 \)-subunit appeared to regulate Na\(^+\) voltage dependence in HEK-293 cells. The leftward shift of steady-state activation of Nav1.6 caused by the \( \beta_2 \)-

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Differential regulation of the expression and gating properties of Na\textsubscript{a}1.8 in HEK-293 stable cells by β\textsubscript{1}/β\textsubscript{2}-subunit chimeras and a β\textsubscript{1} COOH-terminal deletion variant. As indicated by our above results, only the β\textsubscript{1}-subunit significantly increased the current density of Na\textsubscript{a}1.8 (2.3-fold) in HEK-293 cell stable cells. The β\textsubscript{2}-subunit did not regulate the expression and gating properties of Na\textsubscript{a}1.8. We took advantage of the nonmodulating β\textsubscript{2}-subunit to investigate the molecular basis of β\textsubscript{1}-subunit-mediated enhancement of Na\textsubscript{a}1.8 expression. Auxiliary β-subunits are transmembrane proteins composed of a NH\textsubscript{2}-terminal extracellular domain, a single transmembrane domain, and an intracellular COOH-terminal domain (Isom 2002a) (Fig. 6A, top). In the present study, we used β\textsubscript{1}/β\textsubscript{2}-subunit chimeras (β\textsubscript{211}, β\textsubscript{221} and β\textsubscript{112}) and a β\textsubscript{1} COOH-terminal deletion variant (β\textsubscript{11A}) to identify the molecular regions of the β\textsubscript{1}-subunit that are involved in the modulation of Na\textsubscript{a}1.8 (Fig. 6A, bottom).

Coexpression of chimera β\textsubscript{211}, which was composed of the β\textsubscript{2}-subunit extracellular domain and the β\textsubscript{1}-subunit transmembrane and intracellular domains, increased the current density (−155.8 ± 12.2 pA/pF, n = 14) compared with control cells (−73.9 ± 5.7 pA/pF, n = 15, P < 0.01). To determine whether

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Fig. 6: A: schematic representation of the construction of β\textsubscript{1}/β\textsubscript{2}-subunit chimeras and the deletion mutant. Top, typical structure of wild-type β\textsubscript{1}- and β\textsubscript{2}-subunits; bottom, the NH\textsubscript{2}-terminal (N), COOH-terminal (C), and transmembrane-spanning segments of the β\textsubscript{1}-subunit were systematically replaced by corresponding segments of the β\textsubscript{2}-subunit. β\textsubscript{211}, β\textsubscript{221}, and β\textsubscript{112} are β\textsubscript{1}/β\textsubscript{2}-subunit chimeras constructed by site-directed mutagenesis; β\textsubscript{11A} is a COOH-terminal deletion mutant of the β\textsubscript{1}-subunit. B: current-voltage relationship showing the average current densities of Na\textsubscript{a}1.8 currents in HEK-293 stable cells transiently transfected with β\textsubscript{1} (n = 14), β\textsubscript{2} (n = 13), β\textsubscript{211} (n = 14), β\textsubscript{221} (n = 9), β\textsubscript{112} (n = 14), or β\textsubscript{11A} (n = 16). The protocol is the same as in Fig. 2A. C: histogram showing the current densities of Na\textsubscript{a}1.8 channels transiently coexpressing wild-type, chimera, and mutant β-subunits. The current densities of the β\textsubscript{1}, β\textsubscript{211}, and β\textsubscript{221} groups were significant larger than that of the empty vector control group (**P < 0.01) but similar to each other. D: composite figure showing steady-state activation (right) and inactivation (left). The activation curves were generated using the same protocol as in Fig. 2A. Inactivation was measured using the same protocol as in Fig. 3C. The smooth lines of activation and inactivation are fits to a Boltzmann function (see data analysis in MATERIALS AND METHODS). For clarity, we show only the symbols for the β\textsubscript{1}, β\textsubscript{211}, and β\textsubscript{221} groups, which exhibited significant differences compared with the empty vector group, and removed the symbols for other groups (β\textsubscript{2}, β\textsubscript{112}, and β\textsubscript{11A}). When β\textsubscript{1}, β\textsubscript{211}, or β\textsubscript{221}, were coexpressed with Na\textsubscript{a}1.8, the steady-state activation curves exhibited a negative shift compared with the empty vector group (P < 0.01). When β\textsubscript{2} or β\textsubscript{221} were coexpressed with Na\textsubscript{a}1.8, the steady-state inactivation curves exhibited a negative shift compared with the empty vector control group (P < 0.05), whereas coexpression of β\textsubscript{2}, β\textsubscript{211}, β\textsubscript{112}, and β\textsubscript{11A} with Na\textsubscript{a}1.8 did not have a significant effect on voltage-dependent inactivation (P > 0.05). The values of V\textsubscript{1/2} and k\textsubscript{i} are shown in Table 2.
the β1-subunit intracellular domain was sufficient to modulate the current density of Na1.8, chimera β221, which was composed of the β2-subunit extracellular and transmembrane domains and the β1-subunit intracellular domain, was coexpressed with Na1.8. β221 increased the current density of Na1.8 (−152.7 ± 16.0 pA/pF, n = 9, P < 0.01). Current densities of the β211 and β221 groups were similar to the value observed with the β1-subunit group (−164.8 ± 15.7 pA/pF, n = 14, P > 0.05). No increase in the current density of Na1.8 was observed in the β121 and β112 groups, which contain the NH2-terminus and transmembrane domain of the β1-subunit but lack the COOH-terminus of the β1-subunit (β121: −81.5 ± 9 pA/pF, n = 14, and β112: −87.2 ± 7 pA/pF, n = 16, P > 0.05; Fig. 6, B and C). This suggested that the increase in the peak current densities of Na1.8 observed in the wild-type β1, β211, and β221 groups is due to the COOH-terminal region of the β1-subunit.

To determine whether the increase in the peak current densities of Na1.8 caused by COOH-terminal of the β1-subunit was accompanied by changes in the biophysical properties of Na1.8, the voltage dependence of activation and inactivation of wild-type channels, chimeras, and the deletion variant were studied (Fig. 6D). Compared with the empty vector control group, the β1, β211, and β221 groups produced significant negative shifts in the V1/2 values of the activation curves by 5.2, 4.8, and 6.6 mV (P < 0.01 or P < 0.05), respectively, and in the V1/2 values of the inactivation curves by 4.5, 4.9, and 5.8 mV (P < 0.05), respectively. No significant differences in the voltage dependence of activation and inactivation were observed among the β1, β211, and β221 groups.

**DISCUSSION**

The main goal of the present study was to investigate the regulation of Na1.6 and Na1.8 channels by auxiliary β-subunits. These Na+ channels are widely expressed in primary sensory neurons, where they contribute to the rapid rising phase of action potentials (Chahine et al. 2005). We used a combination of single cell RT-PCR of acutely dissociated DRG neurons and heterologous expression experiments to identify the β-subunits expressed in small-diameter sensory neurons and to investigate their regulation of functional Na+ channels, which are known to be expressed in these neurons. Our results indicated that small-diameter DRG neurons widely express Na1.6 and Na1.8 channels and auxiliary β1–β3-subunits. These findings are in agreement with previous work using RT-PCR, in situ hybridization, and double labeling coupled with immunohistochemistry (Morgan et al. 2000; Takahashi et al. 2003; Yu et al. 2003).

**Regulation of Na1.6 by auxiliary β-subunits.** Na1.6 channels are mainly expressed in large-diameter (>30 μm) myelinated sensory neurons (Ho and O’Leary 2010), where they are predominantly located at the nodes of Ranvier (Krzemien et al. 2000; Caldwell et al. 2000). Heterologously expressed Na1.6 channels generate a rapidly inactivating TTX-sensitive current that is activated at a relatively hyperpolarized (−60 mV) voltage (Fig. 5A). The β1-subunit and Na1.6 have reciprocal functions, such that β1-subunit-mediated neurite outgrowth requires Na+ current carried by Na1.6, and the β1-subunit is required for normal expression/high-frequency action potential firing of Na1.6 at the axon initial segment (Brackenbury et al. 2010). Our results showed that coexpression of β-subunits (β1–β3) does not alter the peak current density or voltage-dependent gating of heterologously expressed Na1.6 channels (Fig. 5). The sole exception was the β1-subunit, which produced a hyperpolarizing shift (−7.6 mV) in Na1.6 activation. This may be significant because it resulted in a twofold increase in the window current and shifted Na1.6 activation into a range of voltages considered to be near the resting membrane potential of sensory neurons. These changes may increase the excitability of Na1.6 channels, leading to a reduction in the threshold for initiating action potentials in large-diameter, low-threshold sensory neurons.

**The COOH-terminal domain of the β1-subunit is critical for Na1.8 regulation.** Previous studies have used β-subunit chimeras to identify the structural domains of the auxiliary sub-
units required for regulating Na\(^+\) channel function. Early studies of the neuronal Na\(_{1.2}\) channel indicated that the NH\(_2\)-terminus of the \(\beta_1\)-subunit is sufficient to fully recapitulate the accelerated inactivation, increased expression, and shifts in voltage-dependent activation and inactivation produced by the full-length \(\beta_1\)-subunit (McCormick et al. 1998). A similar role for the NH\(_2\)-terminal domain of the \(\beta_1\)-subunit has been previously postulated for the skeletal muscle Na\(_{1.4}\) channel (Chen and Cannon 1995). These findings were further supported by studies showing that the regions important for \(\alpha\)-\(\beta_1\) interactions are located within the extracellular loops of Na\(_{1.2}\) and Na\(_{1.4}\) channels (Makita et al. 1996; Qu et al. 1999). Subsequent work has implicated the intracellular COOH-terminal domain of the \(\beta_1\)-subunit as another important determinant of \(\alpha\)-\(\beta_1\) interactions and of Na\(_{1.2}\) regulation (Meadows et al. 2001). Current evidence suggests that both NH\(_2\)- and COOH-terminals of the \(\beta_1\)-subunit contribute to \(\alpha\)-\(\beta_1\) interactions and the functional regulation of neuronal and skeletal muscle Na\(^+\) channels. These findings contrast sharply with the results of studies on the cardiac Na\(_{1.5}\) channel, where the membrane-spanning domain coupled with secondary interactions with either the NH\(_2\)- or COOH-terminal of the \(\beta_1\)-subunit were reported to be required for Na\(_{1.5}\) regulation (Zimmer and Benndorf 2002). These results point to substantial differences in the mechanisms of \(\beta_1\)-subunit regulation of neuronal, skeletal muscle, and cardiac Na\(^+\) channels.

We used chimeras of the strongly regulating \(\beta_1\)-subunit and weakly regulating \(\beta_2\)-subunit to identify the structural domains required for Na\(_{1.8}\) regulation. The intracellular COOH-terminal domain of the \(\beta_1\)-subunit appeared to be required for the increase in Na\(^+\) current density and the hyperpolarizing shifts in the activation and inactivation of Na\(_{1.8}\) channels. The observed changes in expression and gating were not altered by replacing the extracellular NH\(_2\)-terminus or membrane-spanning domains of the \(\beta_1\)-subunit with those of the \(\beta_2\)-subunit or by deleting the COOH-terminus of the \(\beta_1\)-subunit. These findings differ substantially from previous studies of neuronal and skeletal muscle Na\(^+\) channels, where the extracellular domain of the \(\beta_1\)-subunit appeared to play a more prominent role in Na\(^+\) channel regulation. Immunoprecipitation studies have identified sites in the COOH-terminus of the \(\beta_1\)-subunit and neuronal Na\(_{1.1}\) channels that directly contribute to \(\alpha\)-\(\beta_1\) interactions (Spampanato et al. 2004). A similar interaction between the intracellular COOH-terminus of the \(\beta_1\)-subunit and Na\(_{1.8}\) may contribute to the observed increase in the expression and functional regulation of these channels.

The \(\beta_1\)- and \(\beta_2\)-subunits are both cell adhesion molecules that interact in a trans-homophilic fashion, resulting in ankyrin recruitment to the plasma membrane at points of cell-cell contact. Only the \(\beta_1\)-subunit can heterophilically interact with contactin, leading to increased surface expression of Na\(_{1.2}\) in CHL cells. \(\beta_1/\beta_2\)-Subunit chimeric studies in which the various regions of the \(\beta_1\) Ig loop were exchanged showed that the \(\beta_1\) Ig loop is not enough to induce full \(\beta_1\)-subunit-mediated enhancement of the Na\(_{1.2}\) cell surface (McEwen et al. 2004; Malhotra et al. 2000). Further studies showed that ankyrin recruitment by the \(\beta_1\)-subunit depends on the phosphorylation of \(\beta_1\)Y181, an intracellular tyrosine residue. A mutant of this residue (\(\beta_1\)Y181E) inhibits \(\beta_1\)-subunit-mediated ankyrin recruitment in response to homophilic adhesion and enhancement of Na\(_{1.2}\) surface expression (Malhotra et al. 2002; McEwen et al. 2004). While the \(\beta_1\)-subunit extracellular domain is homologous to the \(\beta_1\)-subunit, the \(\beta_2\)-subunit does not mediate the trans-homophilic cell adhesion that results in ankyrin recruitment (McEwen et al. 2009). Taken together, these findings provide support for the hypothesis that the COOH-terminus of the \(\beta_1\)-subunit plays an essential role in the modulation of Na\(^+\) channel function (McEwen et al. 2009).

Previous studies of \(\beta\)-subunit regulation. Previous studies have investigated the regulation of Na\(_{1.8}\) channels by auxiliary \(\beta\)-subunits. Early work examining Na\(_{1.8}\) channels expressed in Xenopus oocytes failed to detect changes in current kinetics when the channels were coexpressed with the \(\beta_1\)-subunit (Akopian et al. 1996; Sangameswaran et al. 1996). However, subsequent studies found that coexpression of the \(\beta_1\)-subunit in oocytes accelerates inactivation kinetics, increases the current density, and produces hyperpolarizing shifts in the activation and steady-state inactivation of Na\(_{1.8}\) channels (Vijayaragavan et al. 2004). A similar \(\beta_1\)-subunit-induced shift in activation was reported for the heterologously expressed human Na\(_{1.8}\) channel (Rabert et al. 1998). These observations are consistent with both our results and recent work showing that the \(\beta_1\)-subunit increases the current density and produces hyperpolarizing shifts in the gating of Na\(_{1.8}\) channels expressed in mammalian cells (Zhao et al. 2007).

There are conflicting results concerning the regulation of Na\(_{1.8}\) channels by the \(\beta_2\)-subunit. Coexpression of the \(\beta_2\)-subunit in oocytes results in an increase in Na\(_{1.8}\) current and a hyperpolarizing shift in activation (Shah et al. 2000). This contrasts with previous work showing that coexpression of the \(\beta_2\)-subunit in oocytes produces a depolarizing shift in Na\(_{1.8}\) inactivation but no change in current density (Vijayaragavan et al. 2004). \(\beta_2\)-Subunits have been reported to directly bind to

Table 2. Effect of \(\beta_1/\beta_2\)-subunit chimeras and \(\beta_1\) COOH-terminal deletion mutant on the activation and inactivation of Na\(_{1.8}\) channels in HEK-293 cells

<table>
<thead>
<tr>
<th>Subunit Combination</th>
<th>Activation</th>
<th></th>
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<th>Steady-State Inactivation</th>
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</tr>
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<tbody>
<tr>
<td></td>
<td>(V_{1/2}) mV</td>
<td>(k_e) mV</td>
<td>(n)</td>
<td>(V_{1/2}) mV</td>
<td>(k_e) mV</td>
<td>(n)</td>
</tr>
<tr>
<td>Na(_{1.8}) + (\beta_1)</td>
<td>-17.7 ± 1.8*</td>
<td>-8.1 ± 0.6†</td>
<td>14</td>
<td>-47.7 ± 1.5*</td>
<td>7.8 ± 0.0.5†</td>
<td>13</td>
</tr>
<tr>
<td>Na(_{1.8}) + (\beta_2)</td>
<td>-14.4 ± 1.2</td>
<td>-9.4 ± 0.7</td>
<td>13</td>
<td>-45.9 ± 0.9</td>
<td>6.5 ± 0.5</td>
<td>11</td>
</tr>
<tr>
<td>Na(<em>{1.8}) + (\beta</em>{111})</td>
<td>-17.3 ± 1.5*</td>
<td>-7.9 ± 0.3†</td>
<td>14</td>
<td>-48.1 ± 2.2*</td>
<td>7.1 ± 0.3*</td>
<td>14</td>
</tr>
<tr>
<td>Na(<em>{1.8}) + (\beta</em>{212})</td>
<td>-19.1 ± 1.1†</td>
<td>-8.2 ± 0.5†</td>
<td>9</td>
<td>-49.0 ± 1.2*</td>
<td>7.0 ± 0.4*</td>
<td>12</td>
</tr>
<tr>
<td>Na(<em>{1.8}) + (\beta</em>{122})</td>
<td>-14.5 ± 2.0</td>
<td>-9.0 ± 0.5*</td>
<td>14</td>
<td>-44.7 ± 1.4</td>
<td>6.6 ± 0.6</td>
<td>12</td>
</tr>
<tr>
<td>Na(<em>{1.8}) + (\beta</em>{112})</td>
<td>-15.7 ± 2.1</td>
<td>-8.9 ± 0.5†</td>
<td>16</td>
<td>-47.3 ± 1.3</td>
<td>7.3 ± 0.6*</td>
<td>15</td>
</tr>
</tbody>
</table>

Values are means ± SE; \(n\), number of cells. *\(P < 0.05\) and †\(P < 0.01\) compared with the Na\(_{1.8}\) + empty vector group (values are shown in Table 1).
Na\textsubscript{1.8} channels via the COOH-terminus of the \(\beta_3\)-subunit and to help translocate Na\textsubscript{1.8} from the endoplasmic reticulum to the plasma membrane (Zhang et al. 2008). However, in the present study, coexpression of the \(\beta_3\)-subunit in mammalian cells produced a significant 31% decrease in Na\textsubscript{1.8} current density (Fig. 3B). This result is in agreement with a previous study showing that \(\beta_3\)-subunits do not improve the functional expression of Na\textsubscript{1.8} in COS-7 cells (Swanwick et al. 2010). Collectively, these findings suggest that coexpression of the \(\beta_3\)-subunit either has no effect or reduces Na\textsubscript{1.8} current without altering voltage dependence or gating kinetics. The role of the \(\beta_4\)-subunit in neuropathic pain is closely associated with Na\textsubscript{1.3}, both of which are upregulated after axotomy in a coordinated fashion. They have also been shown to be highly coexpressed in DRG neurons using the double-labeling method (Takahashi et al. 2003). The \(\beta_3\)-subunit depolarizes the voltage-dependent activation and inactivation of Na\textsubscript{1.3} when expressed in HEK-293 cells and induces biphasic components of the inactivation curves, increasing the proportion of channels with slower inactivation kinetics (Cusdin et al. 2010).

The \(\beta_2\) - and \(\beta_3\)-subunits share 35% sequence similarity, and both subunits covalently associate with Na\textsuperscript{+} channels via disulfide bonds (Yu et al. 2003). In the present study, the \(\beta_2\)-subunit did not alter the expression, kinetics, or voltage dependence of Na\textsubscript{1.8} channels (Table 1). This is in good agreement with previous work showing that the \(\beta_2\)-subunit does not regulate Na\textsubscript{1.8} channels expressed in Xenopus oocytes (Vijayaragavan et al. 2004). In contrast, the \(\beta_3\)-subunit produced hyperpolarizing shifts in the activation of Na\textsubscript{1.8} and Na\textsubscript{1.6} channels. Similar changes in activation have been reported for Na\textsubscript{1.1}, Na\textsubscript{1.2}, Na\textsubscript{1.4}, and Na\textsubscript{1.6} channels coexpressed with \(\beta_2\)-subunits (Chen et al. 2008; Yu et al. 2003; Aman et al. 2009). The hyperpolarizing shift in activation produced by the \(\beta_3\)-subunit expanded the predicted Na\textsubscript{1.8} window current (Fig. 3D) and increased the likelihood of Na\textsubscript{1.8} activation and persistent TTX-resistant Na\textsuperscript{+} currents at hyperpolarized voltages. While the COOH-terminal domain of the \(\beta_3\)-subunit has been proposed to act as the endogenous open channel blocker of Na\textsuperscript{+} channels (Grieco et al. 2005), we could not detect a reduction in current density or resurgent currents when Na\textsubscript{1.6} and Na\textsubscript{1.8} channels were coexpressed with the \(\beta_3\)-subunit.

**Conclusions.** The present study examined the functional regulation of neuronal Na\textsubscript{1.6} and sensory neuron-specific Na\textsubscript{1.8} channels by auxiliary \(\beta\)-subunits. Single cell RT-PCR revealed that the Na\textsubscript{1.8} channel and several \(\beta\)-subunits (\(\beta_1\), \(\beta_2\), and \(\beta_3\)) were coexpressed in the same population of small-diameter neurons. The high level expression of TTX-resistant Na\textsuperscript{+} currents and the preferential expression of Na\textsubscript{1.7}, Na\textsubscript{1.8}, and Na\textsubscript{1.9} transcripts in these neurons were consistent with what has been reported for unmyelinated C-fibers. Association with the \(\beta_1\)-subunit increased Na\textsuperscript{+} current density and produced shifts in gating, leading to Na\textsubscript{1.8} activation at voltages near the resting membrane potential of sensory neurons. The predicted increase in TTX-resistant Na\textsuperscript{+} currents could have important implications for the electrical excitability of sensory neurons. Previous work has shown that \(\beta_1\)-subunits are predominately expressed in medium- and large-diameter DRG neurons, a pattern that is not altered in animal models of nerve injury (Oh et al. 1995; Takahashi et al. 2003). While Na\textsubscript{1.8} channels and \(\beta_1\)-subunits are differentially expressed in small- and large-diameter sensory neurons, these subunits may overlap in subpopulations of these neurons (Ho and O’Leary 2010; Oh et al. 1995; Takahashi et al. 2003). Our single cell analysis showed that 44% of the neurons expressing Na\textsubscript{1.8} also express transcripts coding for the \(\beta_1\)-subunit, which provides support for this possibility. Further studies are required to determine whether these \(\beta_1\)-subunits are associated with Na\textsubscript{1.8} channels and whether they regulate the expression and gating of Na\textsubscript{1.8} channels in these neurons. (Table 2).

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**REFERENCES**


Brackenbury WJ, Calhoun JD, Chen C, Miyazaki H, Nukina N, Oyama F, Ranscht B, Isom LL. Functional reciprocity between Na\textsubscript{1.6} channel and \(\beta_1\) subunits in the coordinated regulation of excitability and neurite outgrowth. *Proc Natl Acad Sci USA* 107: 2283–2288, 2010.


Grieco TM, Malhotra JD, Chen C, Isom LL, Raman IM. Open-channel block by the cytoplasmic tail of sodium channel \(\beta_4\) as a mechanism for resurgent sodium current. *Neuron* 45: 233–244, 2005.


Isom LL. The role of sodium channels in cell adhesion. Front Biosci 7: 12–23, 2002a.


Biophysical characterization of M1476I, a founder mutation associated with cold-induced myotonia in French Canadians

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Running Title: Biophysical characterization of a novel myotonia mutation

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Abstract

M1476I, a French Canadian founder mutation of Na⁺ channel Na,1.4 causes potassium-aggravated myotonia, with cold-induced myotonia as the most distinctive clinical feature. Mexiletine, a class 1B local anesthetic, relieves the myotonic symptoms of patients carrying the M1476I mutation. We used the patch-clamp method to investigate the functional characteristics of this mutation by heterologous expression in tsA201 cells. The M1476I mutation caused an increased persistent Na⁺ current, a 2- to 3-fold slower fast inactivation, a 6.4 mV depolarizing shift in the midpoint of steady-state inactivation, and an accelerated recovery from fast inactivation compared to the wild-type (WT) channel. Cooling slowed the kinetics of both channel types and increased the amplitude of the persistent current in M1476I channels. Mexiletine suppressed the persistent Na⁺ current generated by the M1476I mutation and blocked both WT and M1476I channels in a use-dependent manner. The inactivation-deficient M1476I channels were less susceptible to mexiletine during repetitive pulses. The decreased use-dependent block of M1476I channels might have resulted from the slower onset of mexiletine block, and/or the faster recovery from mexiletine block, given that the affinity of mexiletine for the inactivated state of the WT and mutant channels was similar. Increased extracellular concentrations of potassium had no effect on either M1476I or WT currents. These results indicated that cooling can augment the disruption of the voltage-dependence of fast inactivation by M1476I channels. The therapeutic efficacy of mexiletine in M1476I carriers may be partly due to the open-channel block targeting the persistent Na⁺ currents generated by M1476I channels.
Non-technical summary

Na^+ channels are pores present at the surface of every muscle cell. The initiation of muscle contraction requires the opening of a large number of Na^+ channels. Na_v1.4 channels are encoded by the SCN4A gene and represent over 90% of Na^+ channels in adult skeletal muscle cells. The M1476I mutation of Na_v1.4 causes potassium-aggravated myotonia in a French Canadian population of the Saguenay-Lac-Saint-Jean region of Quebec. Individuals carrying this mutation exhibit typical features ranging from asymptomatic myotonic discharges on electromyography to severe diffuse myotonia, as well as unusual cold-induced, painful myotonia. Our study provides a detailed characterization of the underlying biophysical defect of the M1476I mutation, including an increased persistent Na^+ current, a disruption of fast inactivation and an accelerated recovery from inactivation. Cooling further enhances the abnormalities of fast inactivation of the mutant channels. Our data suggest that mexiletine could be used as a therapeutic for patients carrying this mutation.

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Introduction

Na,1.4 channels, which are encoded by the SCN4A gene, comprise over 90% of voltage-gated Na+ channels in postnatal skeletal muscle. Like other Na+ channel subtypes, Na,1.4 channels are transmembrane glycoprotein complexes composed of a pore-forming α subunit and one or more regulatory β subunits. The α subunit is a large protein with four internally homologous domains (DI-IV), each containing six α-helical transmembrane segments (S1-S6) (Catterall, 2000; Chahine et al., 2005). These Na,1.4 channels are located in the sarcolemma and T-tubular membranes, with a high density near the endplate of the muscle cell.

Disturbances in the function of Na,1.4 channels cause autosomal dominant Na+ channelopathies, including hypokalemic periodic paralysis (HypoPP), hyperkalemic periodic paralysis (HyperPP), paramyotonia congenita (PMC), potassium-aggravated myotonia (PAM, or sodium channel myotonia), and congenital myasthenic syndrome (CMS) (Jurkat-Rott et al., 2010). Myotonia is the predominant feature of PMC and PAM (Lehmann-Horn & Rudel, 1996; Ebers et al., 1991). More than 10 SCN4A mutations have been identified in association with PAM. The three most common mutations, Gly1306 Ala / Val / Glu, occur at the same residue of the DIII–DIV linker (Hayward et al., 1996), which acts as a ‘hinged lid’ to block the permeation pathway after activation to inactivate the Na+ channel (Yu & Catterall, 2003). Other PAM mutations such as V445M (Rosenfeld et al., 1997), S804F (McClatchey et al., 1992), and V1589M (Heine et al., 1993) are situated in the S5 and S6 segments of four domains of the Na,1.4 channel. These regions form part of the inner vestibule of the central pore to which the inactivation gate binds. PAM mutations thus produce a pattern of Na,1.4 channel dysfunction that includes disruption of entry into fast inactivation, an increase in the persistent Na+ current, and accelerated recovery from fast inactivation (Lerche et al., 1996; Hayward et al., 1996; Mitrovic et al., 1995; Green et al., 1998; Richmond et al., 1997). PAM is exacerbated by K+ ingestion (e.g., fruit juices) or strenuous work. Myotonia of this phenotype does not worsen significantly after exposure to cold and is not associated with attacks of weakness. Among PAM mutations, only V1589M has been reported to cause cold-aggravated myotonia (Heine et al., 1993). The severity of PAM often fluctuates temporally and ranges from mild to severe episodes of muscle stiffness.

M1476I, a new dominant missense SCN4A mutation in exon 24, was found in a French Canadian population of the Saguenay–Lac-Saint-Jean region of Quebec. This mutation (substitution of methionine for isoleucine) is located in the cytoplasmic S4-S5 loop of DIV. Individuals carrying this mutation exhibit a PAM phenotype with typical features ranging from asymptomatic myotonic discharges on electromyography (EMG) to severe diffuse myotonia, as well as unusual cold-induced (41% of patients), painful myotonia (18% of patients). Mexiletine is an effective treatment for M1476I carriers, especially compared to other class 1B antiarrhythmic agents and local anesthetics (Rossignol et al., 2007). To elucidate the mechanism underlying the M1476I phenotype, we utilized a whole-cell
patch-clamp technique to study tsA201 cells expressing WT or M1476I channels. The mutation exhibited biophysical defects similar to other PAM-causing mutations, in particular impaired fast inactivation and accelerated recovery from inactivation. To better understand the unusual cold sensitivity of patients carrying the M1476I mutation, we compared the biophysical properties of the mutant channels at different temperatures (10°C, 18°C, and 23°C). We also studied the effect of higher extracellular K⁺ concentrations and the action of mexiletine on M1476I channels.

Materials and Methods

Na⁺ channel mutagenesis
Mutant human Naᵥ1.4/M1476I was generated using QuickChange TM site-directed mutagenesis kits according to the manufacturer's instructions (Stratagene, La Jolla, CA, USA). Oligonucleotide primers containing the corresponding M1476I mutation were synthesized using following sequences:

5′- ctg ctg ttc gcc ctc ata atg tcg ctg cct gcc-3′ (forward primer) and
5′- ggc agg cag cga cat tat gag ggc gaa cag cag-3′ (reverse primer).

The mutated site is underlined. Mutant and WT Naᵥ1.4 channels were inserted in the pcDNA1 plasmid, amplified in E. coli DH5α, and purified using Qiagen columns (Qiagen Inc., Chatsworth, CA, USA).

Gene transfection and cell culture
TsA201 cells are derived from HEK 293 cells by stable transfection with SV40 large T antigen (Margolskee et al., 1993). The cells were grown using standard tissue culture conditions (5% CO₂, 37°C) in high glucose DMEM supplemented with FBS (10%), L-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (10 mg/ml) (Gibco BRL Life Technologies, Burlington, ON, Canada). The cells were transiently transfected with WT or mutant Naᵥ1.4 cDNA and the human β₁ subunit by calcium phosphate precipitation as previous described (Zhao et al., 2007). The β₁ subunit and CD8 were constructed in the piRES vector (piERS/CD8/β₁) (Invitrogen Corporation, Carlsbad, CA, USA). After preincubation with CD8 antibody-coated beads (Dynabeads M-450 CD8-a) prior to recording, cells expressing the piRES/CD8/β₁ bicistronic vector were decorated with CD8 beads. The transfected cells were identified under a light microscope for patch-clamp analyses (Margolskee et al., 1993).

Whole-cell voltage-clamp recordings
Macroscopic Na⁺ currents from transfected tA201 cells were recorded using the whole-cell configuration of the patch-clamp technique. A correction for the liquid junction potential between the patch pipette and bath solutions (−7 mV) was applied to command pulses before seal formation. Unless indicated otherwise, Na⁺ current recordings were initiated after an equilibration period of 10 min once the whole-cell configuration was established.
Command pulses were generated and currents were recorded using pCLAMP software 10.2 and an Axopatch 200B amplifier with a CV 203BU headstage (Molecular Devices, Sunnyvale, CA, USA). Patch electrodes were fashioned from borosilicate glass (Corning 8161) and coated with silicone elastomer (Sylgard, Dow-Corning, Midland, MI, USA) to minimize stray capacitance. Current recordings were made using low-resistance electrodes (<1MΩ), and the series resistance was compensated at values ≥ 80% to minimize voltage-clamp errors. Whole-cell currents were filtered at 5 kHz, digitized at 10 kHz, and stored on a microcomputer equipped with an AD converter (Axon Digidata 1440A, Molecular Devices, Sunnyvale, CA, USA). The current signal was low-pass filtered at 5 kHz and digitalized at a sampling rate of 100 µs during acquisition. Na⁺ currents were recorded at 10°C, 18°C, and 23°C (room temperature). The temperature was controlled using a water bath equipped with a bipolar temperature controller (Model TC-202, Medical Systems Corp., Greenvale, NY, USA) and was measured as close as possible to the cells using a bath sensor (Thermistor BSC-T3, Harvard Apparatus, Holliston, MA, USA). Except where indicated, currents were recorded at 23°C. 

Peak currents were measured during a current-voltage protocol. Na⁺ current densities (pA/pF) were obtained by dividing the peak current by the cell capacitance. Average I/V curves were obtained by plotting normalized peak currents versus the voltage. For the construction of activation curves, the Na⁺ conductance (G\text{Na}) was calculated from the peak current (I\text{Na}) using the following equation: 
\[ G\text{Na} = \frac{I\text{Na}}{V-E\text{Na}} \]
where V is the test potential and E\text{Na} is the reversal potential. The normalized G\text{Na} was plotted against the test potential. Steady state activation was fitted to a Boltzmann equation of the following form: 
\[ G/G\text{max} = \frac{1}{1+\exp (-\frac{V-V_{1/2}}{k_v})} \]
where V_{1/2} is the voltage at which channels are half-maximal activated and k\text{v} is the slope factor. For the construction of inactivation curves, the peak current (I) was normalized relative to the maximal value (I\text{max}) and was plotted against the conditioning pulse potential. Steady-state activation and inactivation curves were fitted to a Boltzmann equation of the following form: 
\[ I/I\text{max} = \frac{1}{1+\exp (-\frac{V-V_{1/2}}{k_v})} \text{ or } I/I\text{max} = (1-C) \frac{1}{1+\exp (-\frac{V_{1/2}-V}{k_v})}+C \]
where V_{1/2} is the voltage at which channels are half-maximal inactivated, k\text{v} is the slope factor, and C is the nonzero pedestal. Time constants of fast inactivation were assessed by fitting a single exponential curve to the current decay. For the recovery from inactivation or mexiletine block, the test pulse peak current (I\text{test}) was normalized to the corresponding prepulse current (I\text{cont}). \[ I\text{test}/I\text{cont} \] was plotted against the interpulse interval and was fitted to a single or double exponential function with a single time constant (τ), or two time constants (τ\text{fast} and τ\text{slow} for recovery from fast inactivation, τ_1 and τ_2 for recovery from mexiletine block).

The overlap between steady-state inactivation and activation has been called the window current. Na⁺ channels are not inactivated by the mechanism of steady-state inactivation and are available for opening over this window of voltages where the two curves overlap. Using the V_{1/2} and K\text{v} values of voltage-dependent activation and inactivation, the probability of a Na⁺ channel being within the window was calculated using the following equation: 
\[ (1/(1+\exp((V_{1/2 \text{ activation}}-V)/k_v \text{ activation}))) \ast (1/(1+\exp((V-V_{1/2 \text{ inactivation}})/k_v \text{ inactivation}))) \]
The probability of a Na$^+$ channel being a mixture of persistent and window currents was calculated using the following equation: 

\[
\frac{1}{1+\exp((V_{1/2 \text{ activation}}-V)/k_v \text{ activation})})*\left((1-C)/(1+\exp((V-V_{1/2 \text{ inactivation}})/k_v \text{ inactivation})\right)+C),
\]

where C is a constant representing the fraction of the persistent Na$^+$ current.

**Solutions and reagents**

For whole-cell patch-clamp recordings of tsA201 cells, the pipette solution contained (in mM) 35 NaCl, 105 CsF, 10 EGTA, and 10 Cs-HEPES. The pH was adjusted to 7.4 using 1 N CsOH. The standard extracellular solution generally contained (in mM) 150 NaCl, 2 KCl, 1.5 CaCl$_2$, 1 MgCl$_2$, 10 glucose, and 10 Na-HEPES (pH 7.4). The pH was adjusted to 7.4 with 1 N NaOH. To evaluate the effect of variable extracellular concentrations of K$^+$ on the gating properties of WT and M1476I channels, the concentration of KCl in the extracellular solution was increased to 4, 6, or 9 mM. To maintain constant external osmolarity, the concentrations of NaCl were correspondingly decreased to 148, 146, or 143 mM. Stock solutions of 10 mM mexiletine and 10 µM TTX were prepared and were diluted in bath solution. The effects of these drugs were recorded 10 min after application to allow for drug equilibration.

**Data analysis and statistics**

Data was analyzed using a combination of pCLAMP software 10.2 (Molecular Devices), Microsoft Excel, and SigmaPlot for Windows version 11.0 (SPS, Chicago, IL, USA). Data were expressed as means±SE. Statistical significance was determined by comparing control and mutant groups using unpaired Student’s t-tests, and the level of statistical significance was set at p<0.05.

**Results**

*M1476I mutation induces a persistent Na$^+$ current*

A common feature of most naturally occurring Na$_{1.4}$ mutations causing PAM is a persistent or non-inactivating Na$^+$ current. To verify the presence of the non-inactivating component in M1476I channels, we measured whole-cell Na$^+$ currents from tsA201 cells transiently expressing WT or M1476I mutant Na$_{1.4}$ channels. Representative Na$^+$ currents are shown in Figure 1A. Compared to WT channels, only a small fraction of M1476I channels were resistant to inactivation (persistent current) during prolonged depolarization (>20 ms). Cells expressing WT and M1476I channels were studied by first constructing normalized peak current versus voltage (I-V) curves (Fig. 1B). Both channel types activated around –70 mV and peaked around –30 mV. On average, we observed no difference in peak current densities for cells expressing WT (523.9±49.2 pA/pF; n=21) or M1476I channels (567.1±42.5 pA/pF; n=35). The ratio of the persistent current was determined by comparing the current that persisted during the last 20 ms of depolarization to the peak transient current recorded in the same cell. This ratio gave an estimate of the
fraction of channels that failed to inactivate. The peak persistent current measured at a –30 mV test potential was 1.5% of the peak current, as shown in the inset of Figure 1B.

**M1476I channels exhibit a defective steady-state inactivation**

The kinetics of fast inactivation were assessed by fitting the decay of Na⁺ whole-cell currents elicited at voltages of –50 to +30 mV with a single exponential equation. A defect in the fast inactivation of M1476I channels was seen as a 2– to 3-fold slower time constant than that of WT channels (Fig. 2A) (p<0.05 or p<0.01).

The I-V curves shown in Figure 1B were converted to conductance versus voltage (G–V) curves and were fitted using the Boltzmann function described in Materials and Methods. The voltage dependence of activation was unaffected by the M1476I mutation (WT: V_{1/2} = –40.3±1.2 mV, K_v = –5.0±0.2 mV, n=21; M1476I: V_{1/2} = –41.7±1.2 mV, K_v = –5.2±0.1 mV, n=35) (p>0.05) (Fig. 2B and Table 1). Fast inactivation is a process by which Na⁺ channels switch to a non-conducting state milliseconds after the onset of depolarization. This process contributes to the repolarization of the action potential. Figure 2B shows that the M1476I mutation caused a 6.4 mV shift in the midpoint (V_{1/2}) of steady-state inactivation in a depolarizing direction (WT: V_{1/2} = –80.6±0.5 mV, K_v = 4.2±0.1 mV, n=23; M1476I: V_{1/2} = –74.2±0.5 mV, K_v = 4.5±0.04 mV, n=35) (p<0.01). Unlike WT currents, the fast inactivation of mutant currents was incomplete, and the fraction of non-inactivated (residual) M1476I currents was approximately 1.5% (Fig. 2C). This finding was consistent with the observed persistent Na⁺ current shown in Figure 1AB.

**M1476I increased the Na⁺ window current**

The window current results from the overlapping area of the activation and inactivation curves that identifies a voltage range (window) in which Na⁺ channels are not inactivated by the mechanism of steady-state inactivation and are available for opening by depolarization (Cummins et al., 1993). In the present study, the shift of the inactivation curve of M1476I channels along the voltage axis resulted in an increased overlap between steady-state activation and steady-state inactivation. This increased window current, plus the residual Na⁺ current during steady-state inactivation, predicted that a larger fraction of M1476I channels would be available to open in the voltage range of –80 to –10 mV (Fig. 2B). Figure 3A shows a plot of the probability of Na⁺ channels opening at the window or at more depolarized voltages calculated from the V_{1/2} and K_v values of the fitted activation and inactivation curves. The resulting probability was a biphasic function of voltage, with a combination of two components: a relatively small component with a peak near –70 mV and a larger component at depolarized voltages greater than –50 mV. This biphasic function could be separated into a window component and a persistent component, as shown in Figures 3B and 3C. The window component was defined by the overlap of the Na⁺ channel activation and inactivation curves. Compared to the WT, the M1476I mutation shifted the peak toward a more depolarized voltage (WT: –74 mV vs. M1476I: –66mV) and enlarged the window, as indicated by a 6-fold increase in the probability of channel
opening at the peak current (WT: 0.02% vs. M1476I: 0.13%). To characterize a “pure” persistent component, we subtracted the window component shown in Figure 3B from the biphasic function shown in Figure 3A. The persistent component occurred over a more depolarized range of voltages (> –50 mV). For WT channels, the persistent component increased over the range of voltages where Na⁺ channels activate and reached a peak probability of 0.19% at voltages more depolarized than –30 mV. The M1476I mutation increased the peak probability of channel opening 8-fold. The increased window and persistent current caused by the mutation would tend to depolarize skeletal muscle cells in PAM patients.

**M1476I accelerated recovery from fast inactivation**

Recovery from fast inactivation determines the time required for inactivated Na⁺ channels to move back to the active state. It thus limits the maximal firing rate and permits repetitive firing of muscle cells. Recovery from fast inactivation was assessed using a double-pulse protocol (see inset of Fig. 4). Cells were held at –140 mV, depolarized by a 40 ms or 500 ms conditioning pulse to –30 mV, and then repolarized to the recovery potential for increasing durations. With a 40 ms conditioning pulse, a single-exponential function was sufficient for fitting WT and M1476I currents, yielding only a single time constant (τ). In contrast, a double exponential equation was required to obtain an accurate fit with a 500 ms conditioning pulse and yielded two time constants (τ<sub>fast</sub> and τ<sub>slow</sub>). The τ<sub>fast</sub> had a relatively large weight (~90%) compared with the τ<sub>slow</sub> (~10%). We thus used τ<sub>fast</sub> to compare WT and M1476I channels. The time constants of M1476I channels under these two conditioning pulses were significantly lower (τ or τ<sub>fast</sub>) (Fig. 4 and Table 1), indicating that the M1476I mutation accelerated recovery from inactivation, as is the case for other PAM and PC-causing mutations (Mitrovic et al., 1994; Mitrovic et al., 1995; Chahine et al., 1994).

**Elevating extracellular K⁺ concentrations had no effect on either M1476I or WT currents**

In PAM patients, myotonia attacks are precipitated by elevated serum K⁺ concentrations (Jurkat-Rott et al., 2010). We thus examined the effects of different extracellular K⁺ concentrations on the expression levels and gating properties of WT and M1476I channels. Elevating extracellular K⁺ concentrations from 2 mM to 4 mM, 6 mM, or 9 mM had no detectable effect on the peak current amplitudes of WT or M1476I currents. In addition, the ratio of persistent current, voltage dependence of activation and fast inactivation, and the time course of recovery from fast inactivation were similar at various concentrations of extracellular K⁺ (Table 1).

**Effects of temperature**

One of the most distinctive clinical features of patients carrying the M1476I mutation is the exacerbation of muscle stiffness by cold, while it is not a cardinal symptom of PAM (Lehmann-Horn & Jurkat-Rott, 2007). We thus investigated the effect of cooling on the biophysical properties of WT and M1476I channels. Compared to current traces recorded
at 23°C (Fig. 1A), lowering the temperature to 10°C or 18°C slowed the kinetics of both WT and M1476I channels (Fig. 6A). As soon as the whole-cell configuration was established, WT and M1476I channels both exhibited a significant increase in the persistent Na⁺ current at lower temperatures, which was more marked with the mutant channels and reached a maximum at 10°C (data not shown). Interestingly, persistent Na⁺ currents “ran down” over time. For example, for M1476I channels at 10°C, the persistent current measured at a –30 mV test potential 1 min after the whole-cell configuration was established was 13.6±1.1% (n=12), then dropped to 6.7±1.1% (n=11) after 5 min, and remained stable at around 3.5% after 10 min (10 min: 3.7±0.6% n=11; 20 min: 3.1±0.3%, n=8). The same significant alterations in persistent currents were also observed at 18°C, but were less prominent than at 10°C (Fig. 5 A).

At room temperature, M1476I channels activated at the same negative potentials as WT channels, as reflected in the absence of a shift in V_{1/2} of steady-state activation (Fig. 2B). Similarly, no significant shift was observed in the V_{1/2} of activation for M1476I channels compared to WT channels at 10°C or 18°C, suggesting that the opening of channels was unaffected by the cooling or the M1476I mutation (Table 1).

The effects of cooling on two main characteristics of fast inactivation were also investigated, that is, the voltage dependence of steady-state inactivation and the time constant of inactivation. The V_{1/2} of steady-state inactivation was shifted in a similar manner for both WT and M1476I channels in a hyperpolarized direction by cooling from 23°C to 18°C and 10°C: for WT from –80.6±0.5 mV (23°C, n=23) to –84.1±0.7 mV (18°C, n=9) and –91.5±1.4 mV (10°C, n=8), and for M1476I from –74.2±0.5 mV (23°C, n=35) to –79.6±0.9 mV (18°C, n=12) and –85.6±1.6 mV (10°C, n=9) (Fig. 5B and Table 1). Cooling thus caused a similar hyperpolarizing shift in steady-state inactivation for both channel types. The inactivation time constants were significantly slower for both channel types when the recording solution was cooled (Fig. 5C). At a test potential of –30mV, for example, WT channels inactivated with time constants of 0.6±0.03 ms (23°C, n=14), 1.0±0.1 ms (18°C, n=11), and 2.1±0.4 ms (10°C, n=8), while M1476I channels inactivated with time constants of 1.3±0.1 ms (23°C, n=12), ms 1.9± 0.3 (18°C, n=16), and 4.2±1.3 ms (10°C, n=11).

Figure 5D shows a plot of the time course of recovery from inactivation for WT and M1476I channels at 10°C, 18°C, and 23°C with a 40 ms conditioning pulse. Both channel types exhibited slower recovery kinetics at lower temperatures. Time constants of recovery from inactivation at 10°C were significantly larger than those at 18°C, while the time constants at 18°C were larger than those at 23°C. Similar alterations were also observed for recovery from inactivation with a 500 ms conditioning pulse at 10°C, 18°C, and 23°C (Table 1).

**Effects of mexiletine**

As mentioned above, the persistent current and the rapid recovery from inactivation caused by the M1476I mutation appeared to underlie the PAM phenotype. Mexiletine showed
promising potential for the treatment of patients carrying this mutation. We were interested in the therapeutic mechanism of mexiletine. We first assessed the ability of mexiletine to reduce the amplitude of the persistent current exhibited by M1476I channels, which may be the basis for its therapeutic efficacy (Wang et al., 1997). The persistent current recorded from M1476I channels was significantly inhibited after a 100 μM mexiletine treatment for 10 min (Fig. 6, bottom panel). In general, the persistent current was reduced by approximately 90% (Fig. 6B). At the same time, the block of the peak current in M1476I channels was minimal, unlike the total block of the current caused by a 10 min treatment with 50 nM TTX (Fig. 6A, top panel).

Many local anesthetics and antiarrhythmic drugs act by preferentially binding to the inactivated state of Na+ channels, which is characterized by the use-dependent block elicited by repetitive stimulation (Hille, 2001). We measured the use-dependent block of WT and M1476I currents by mexiletine with a series of 50 depolarizing pulses to –30 mV at different frequencies (from 2 to 50 Hz) for 10 ms. Peak currents were measured, normalized to the amplitude of the first pulse, and plotted against the pulse number. In the absence of mexiletine, there was no decrease in the current amplitude for the two channel types at frequencies up to 50 Hz. In the presence of mexiletine, the use-dependent block increased with higher frequencies. The steady-state level of block was significantly lower for M1476I channels than for WT channels (Fig. 7B). Figure 7A shows that the use-dependent block by mexiletine reached a steady-state level after a few depolarizing pulses at 50 Hz. The peak current amplitude of the last pulse normalized to the first pulse (P50/P1) indicated a use-dependent block of 49.6±1.9% for WT (n=13) and 55.2±1.6% for M1476I (n=14). We next investigated whether the reduced use-dependent block in M1476I channels was due to: (1) a lower affinity of mexiletine for the inactivated state; (2) a faster recovery from mexiletine block; or (3) the slower onset of mexiletine block.

The affinity of mexiletine for the inactivated state was estimated by observing the effect of mexiletine on steady-state inactivation. Figure 8A is a composite figure showing the voltage dependence of steady-state activation and inactivation of WT and M1476I channels expressed in tsA201 cells. Adding 100 μM mexiletine did not affect the peak current density (data not shown), but significantly shifted the voltage-dependent activation and inactivation curves of both WT and M1476I channels in a hyperpolarizing direction. The extent of shifts in the activation and inactivation curves induced by mexiletine was similar for WT and M1476I channels, indicating that the M1476I mutation does not interfere with the affinity of mexiletine for the inactivation state.

In terms of recovery from inactivated-state block after a treatment with 100 μM mexiletine, double exponentials were required to describe the recovery time courses and yielded two time constants (τ1 and τ2). With a brief conditioning pulse (40 ms) at –30 mV, the fast time constants (τ1) remained similar to those (τ in Table 1) measured in the absence of mexiletine. With this conditioning pulse, the recovery from fast inactivation had fully developed, and the contribution of slow inactivation was minimal (Fig. 4). The additional slow time constant (τ2) thus represented the recovery from mexiletine block. Compared to
WT channels, M1476I channels exhibited both accelerated recovery from mexiletine block and lower levels of mexiletine block. The level of block was approximately 60% for WT channels and only about 30% for M1476I channels in the same conditions (Fig. 8B and Table 2). With a 500 ms conditioning pulse at –30 mV, the level of mexiletine block was similar for both WT and M1476I channels (75%). The fast time constants (τ₁) had a relatively small weight (<18%), corresponding to a mixture of the fast and slow components of recovery from inactivation (τ_fast and τ_slow), as shown in Figure 4. The slow time constant (τ₂) showing the recovery from mexiletine block was not significantly different between WT and mutant channels with a 500 ms conditioning pulse (Fig. 8C and Table 2). With shorter conditioning pulse durations (i.e. 40 ms), M1476I channels had less use-dependent block (i.e. showed greater recovery within 20 ms) compared to WT channels. The use-dependent block was similar for both channels types with longer conditioning pulse (i.e. 500 ms), suggesting a slower onset of mexiletine block for M1476I channels compared WT channels. Since the affinity of mexiletine for the inactivated state was the same for WT and M1476I channels (Fig. 5A), the decreased use-dependent block (Fig. 7AB) of M1476I channels may result from the slower onset of mexiletine block, and/or the faster recovery from mexiletine block of mutant channels.

**Discussion**

**Genotype-phenotype relationships**

M1476I currents measured in tsA201 cells revealed an increased persistent current, a slower current decay, a more positive midpoint voltage of fast inactivation, and an accelerated recovery from fast inactivation. A depolarizing shift in steady-state inactivation produces an enhanced overlap between activation and inactivation curves, which leads to an increased window current. The accelerated recovery from fast inactivation in M1476I channels indicated that they had a shorter refractory period after the action potential than WT channels. These gain-of-function defects of M1476I channels observed in our study were consistent with those of other PAM mutations (Lerche et al., 1996; Hayward et al., 1996; Mitrovic et al., 1995; Green et al., 1998; Richmond et al., 1997). The functional disruption of M1476I channels enhances the excitability of the cell membrane, which increases the movement of Na⁺ ions into skeletal muscle cells. This extra Na⁺ influx is enough to change the membrane potential and trigger prolonged muscle contractions, which underlie episodes of myotonia characteristic of PAM (Cannon, 1996). Both the PAM mutation M1476I (Rossignol et al., 2007) and the adjacent PMC mutation F1473S (Fleischhauer et al., 1998) reside in the S4-5 cytoplasmic loop of domain IV, a docking site of the inactivation gate according to the “hinged lid” mechanism (Goldin, 2003). These two mutations induce an increase in the number of non-inactivating Na⁺ channels and a depolarizing shift in the fast inactivation, indicating that the DIV/S4-5 cytoplasmic loop is involved in the inactivation gating of Na⁺ channels.
The varying extents of depolarization caused by defective inactivation may explain how these mutations produce different clinical phenotypes. The F1473S mutation exhibits a more pronounced disruption of fast inactivation than the M1476I mutation (M1476I: 1.6% persistent current, 6.4 mV rightward shift of fast inactivation vs. F1473S: 1.9% persistent current, 18 mV rightward shift of inactivation). A slight depolarization caused by PAM mutations (e.g., M1476I in our study) results in a long-lasting hyperexcitability of the membrane, which causes muscle stiffness, an intermediate depolarization caused by PMC mutations (e.g., F1473S) results in muscle stiffness followed by weakness (Fleischhauer et al., 1998), and a strong depolarization induced by HyperPP mutations (e.g., T698M) results in membrane hypoexcitability and paralysis (Cummins & Sigworth, 1996).

**Role of extracellular K⁺ in PAM**

Myotonia is provoked or aggravated by K⁺ loading in patients with PAM mutations (Heine et al., 1993; Ricker et al., 1994; Kubota et al., 2009). While a K⁺-loading test was not performed during the diagnosis of patients carrying the M1476I mutation (Rossignol et al., 2007), we examined the effects of elevated extracellular K⁺ concentrations on M1476I channels to determine whether the biophysical defects of this mutation are also K⁺ sensitive. Increasing extracellular K⁺ from 2 mM to 4 mM, 6 mM, or 9 mM had no effect on the gating properties of either WT or M1476I channels. Over 10 Naᵥ1.4 mutations associated with PAM have been reported to date, and muscle stiffness is aggravated by K⁺ in patients with these mutations. Of these mutations, only G1306E has been reported to be sensitive to the elevation of extracellular K⁺ concentrations, with a 4-fold increase in extracellular K⁺ from 4 mM to 16 mM causing a 10-20% slowing of current decay and an increase in the amplitude of the persistent current (Hayward et al., 1996). However, a study by Mitrovic et al. using the same mutation and the same expression system failed to detect any effect of high extracellular K⁺ concentrations on WT and M1476I channels (Mitrovic et al., 1995). The mechanisms by which elevated serum K⁺ concentrations trigger myotonia attacks remain to be determined. High extracellular K⁺ concentrations (7-9 mM) have been shown to enhance depolarization in intact muscle fibers by decreasing the resting membrane potential from –80 to –63 mV (Lehmann-Horn et al., 1987). The depolarized voltage induced by the elevation in extracellular K⁺ concentrations in T-tubules incorporates the non-inactivated component exhibited by mutant Naᵥ1.4 channels and may trigger repetitive after-discharges following driven action potentials. After-discharges cause stiffness or delayed relaxation of muscles in patients with myotonia. This hypothesis is supported by mathematical models of muscle cells. For example, S804F is a PAM mutation that causes fast inactivation abnormalities. A model muscle cell containing this mutation exhibits enhanced excitability but does not display after-discharges unless the extracellular K⁺ concentration is raised to 5.5 mM (Green et al., 1998; Hayward et al., 1996; Cannon, 2000).

**Cold-induced myotonia**
Typically, myotonia in patients carrying the M1476I mutation is aggravated when the patients are exposed to cold temperatures. However, these patients exhibit a PAM phenotype, which is not very sensitive to cold (Lehmann-Horn & Jurkat-Rott, 2007). How then does this Na\(^+\) channel mutation cause a peculiar phenotype of PAM with unusual sensitivity to cold? Cold-induced stiffness and subsequent weakness are in fact characteristic features of PMC. PMC mutations lead to biophysical defects that are clearly pathogenic at low temperatures (Jurkat-Rott et al., 2010). Experimental cooling of mutant PMC channels results in a slowing of the kinetics, a hyperpolarizing shift in the steady-state inactivation, and an increase in the amplitude of persistent currents (Fleischhauer et al., 1998; Lerche et al., 1996; Carle et al., 2009). The same changes in fast inactivation and persistent currents were also observed with our cold-sensitive M1476I mutation as well as with other PAM mutations that are not sensitive to cold (Hayward et al., 1996). These changes are thus not sufficient to explain why myotonias in M1476I carriers are aggravated by cold. One possible mechanism might be that the both the slowing of fast inactivation and the increased persistent current have to reach a certain threshold to trigger myotonia at low temperatures (Hayward et al., 1996; Lerche et al., 1996). In the present study, cooling slowed the kinetics for both WT and M1476I currents (Fig. 5C). However, M1476I channels displayed a slower inactivation than WT channels at all temperatures investigated, as is the case for other PAM-causing mutations (Green et al., 1998; Hayward et al., 1996; Mitrovic et al., 1994). While cooling markedly increased the persistent Na\(^+\) current of the M1476I channel, it “ran down” over time, reaching 13.6 to 3.1% at the lowest temperature (10°C) 1 to 20 min after the whole-cell configuration was established. This “run down” of the persistent current has also been reported for some Na\(_{\text{1.4}}\) mutations associated with the HyperPP phenotype such as I1495F and T698M (Bendahhou et al., 1999; Cummins & Sigworth, 1996). A persistent current up to 2% is sufficient to elicit repetitive spikes in muscles (myotonia) in the computer model of Cannon et al., whereas a state of depolarization block (paralysis) occurs when the amplitude of the persistent current exceeds a certain level, as in HyperPP mutations (Cannon et al., 1993). Based on this model, the large persistent current observed in our study at 23°C (1.1%-1.9%) was close to the “borderline” of myotonia.

Other temperature-dependent processes, such as the activity of the Na\(^+\), K\(^+\)-ATPase, can also affect membrane excitability and trigger myotonia. Cooling decreased the activity of the Na\(^+\), K\(^+\)-ATPase, resulting in a decreased Na\(^+\) ion efflux (accumulation of Na\(^+\) ions inside the muscle fibers) and a decreased K\(^+\) ion influx. Cooling from 37 to 13°C induced up to 20mV depolarization in rat skeletal myotubes (Brodie et al., 1987; Dlouha et al., 1980). In addition, compared with PMC and PAM mutations, cooling induces different defects of Na\(^+\) channels gating in Na\(_{\text{1.4}}\) mutations associated with a paralytic phenotype. For example, the P1158S mutation responsible for cold-induced paralysis and myotonia is located in the DIII/S4-5 cytoplasmic loop (Sugiura et al., 2003). Lowering the temperature from 37°C to 25°C induced a hyperpolarizing shift in activation and a depolarizing shift in slow inactivation, but fast inactivation was not substantially affected. The destabilization
of slow inactivation in combination with a defective activation of P1158S channels is expected to increase the risk of prolonged membrane depolarization and leads to loss of excitability with paralysis (Webb & Cannon, 2008).

**Effects of mexiletine**

The electrophysiological basis of myotonia is repetitive action potentials following voluntary contraction. Multiple defects in channel gating caused by SCN4A mutations have been identified as important mechanisms. The therapeutic effect of antmyotonic drugs such as mexiletine is commonly attributed to their well-known use-dependent block of Na\(^+\) channels that prevents repetitive firing of action potentials (Lehmann-Horn & Jurkat-Rott, 1999). In the present study, mexiletine induced a pronounced use-dependent block in both WT and M1476I channels, albeit to a lesser extent in the mutant channels. Mexiletine stabilizes inactivation by causing a hyperpolarizing shift in the steady-state inactivation curves, which is similar for both WT and M1476I channels, suggesting that the M1476I mutation does not substantially alter the affinity of mexiletine in the inactivated state. Thus, the decreased use-dependent block of M1476I channels may due to two mechanisms: a slower onset of mexiletine block (Fig. 8BC), and/or a fast recovery from mexiletine block (Fig. 8B). The M1476I mutation may accelerate the recovery by facilitating the untrapping of mexiletine from the non-inactivating mutant, as previously described for flecainide in Na\(_{\text{v}1.5}\) channels (Ramos & O’Leary, 2004).

Since the use-dependent phenomenon is nonspecific for most class 1B local anesthetics (Lehmann-Horn & Jurkat-Rott, 1999), it is unclear why mexiletine is more efficacious than similar drugs for treating M1476I carriers (Rossignol et al., 2007). One possible explanation is that mexiletine has a 20-fold higher affinity for the open-state than for the inactivated-state of Na\(_{\text{v}1.4}\) channels, with an IC\(_{50}\) of 3.3 \(\mu\)M for the open-state vs. 67.8 \(\mu\)M for the inactivated-state. At therapeutic concentrations (2.8-11\(\mu\)M), mexiletine preferentially blocks persistent late open channels (Wang et al., 2004). This open-channel block of mexiletine by selectively targeting persistent Na\(^+\) currents may be the therapeutic basis of antiarrhythmic drugs (Huang et al., 2011; Wang et al., 1997) and may also play a dominant role in preventing the repetitive firing of action potentials that cause muscle stiffness. Further studies are needed to determine why mexiletine is more efficacious in treating patients carrying this mutation.

Altogether, the present study indicated that cold-induced myotonia, a phenotypic peculiarity of the M1476I mutation of Na\(_{\text{v}1.4}\) channels, may be explained by a defective inactivation that increases Na\(^+\) influx, triggers depolarizations of muscle fibers, and induces membrane hyperexcitability and muscle stiffness. Cooling further enhances the abnormalities of fast inactivation of the mutant channels. The clinical efficacy of mexiletine in treating M1476I carriers may be partly due to the open-channel block targeting the persistent Na\(^+\) currents generated by the M1476I mutation, with the exception of the use-dependent block.
Figure legends

Figure 1. Whole-cell properties of WT and M1476I channels.
A. Representative whole-cell traces recorded from tsA201 cells expressing either WT (top) or M1476I (bottom) Na⁺ channels. Currents were elicited from a holding potential of –140 mV and were depolarized to potentials ranging from –100 to 50 mV in 10 mV increments lasting 20 ms for each step (protocol shown in the inset above the WT trace). Insets under the WT and M1476I traces show a zoom-in of the persistent current of both channel types. The dashed line represents zero current. B. Normalized current/voltage relationships of the WT (▲, n=21), M1476I (●, n=35) channels, and a persistent current of the M1476I (○, n=28) channel. The transient WT and M1476I currents were normalized to their peak currents. The persistent currents were calculated as the mean amplitude of the last 20 ms of the test pulse and are expressed as a percentage of the peak transient current recorded from the same cell. The inset shows a zoom-in of the persistent current/voltage relationship of M1476I channels.

Figure 2. Gating properties of WT and M1476I channels and the enlarged window current induced by the M1476I mutation.
A. Voltage dependence of inactivation time constants of the WT (▲, n=14) and M1476I (●, n=12) currents. The time course of the current decay elicited at depolarized voltages was best fitted to a single exponential function, and the resulting time constants were plotted versus voltage. B. Voltage dependence of activation and inactivation of WT (▲) and M1476I (●) Na⁺ currents fitted with a Boltzmann equation. Activation curves were generated using the same protocol as shown in Fig. 1A. Steady-state inactivation was determined using a 20 ms test pulse to –30 mV after a 500 ms prepulse to potentials ranging from –140 mV to –10 mV (see inset under inactivation curves for the protocol). The V₁/₂ and kᵣ values of the activation and inactivation curves are shown in Table 1. C shows a zoom-in of the enlarged window current (the sum of the dark gray and of the gray areas) induced by the M1476I mutation, that is, the enlarged overlapping area between the voltage-dependent activation and inactivation curves. The gray area shows the window current induced by WT channels.

Figure 3. Effects of the M1476I mutation on the window current.
The overlap of the activation and inactivation curves defines a range of voltages or window where Na⁺ channels are non-inactivated and are available for activation. A depolarizing shift of the inactivation curve caused by the M1476I mutation increased the overlap, resulting in an increased window current. A shows the probability of a M1476I channel...
being non-inactivated and available for activation at depolarized voltages calculated using the equation described in Materials and methods. This probability is a biphasic function of voltage, with a peak at around –70 mV and at depolarized voltages greater than –50 mV. The contribution of the window component plotted in B was calculated using the equation described in Materials and Methods. The M1476I mutation enhanced the peak opening probability of the channel being in the window by 6-fold and shifted the peak in a more depolarizing direction. C is the contribution of the persistent component calculated by subtracting the window component shown in B from A. The M1476I mutation increased the amplitude of the persistent current by 7-fold but did not alter the onset of the persistent component.

Figure 4. Recovery from inactivation of WT and M1476I Na⁺ channels.
From a holding potential of –140 mV, the cells were depolarized to –30 mV for 40 ms (WT: ▲, n=11; M1476I: ●, n=27) or 500 ms (WT: △, n=11; M1476I: ○, n=26) to inactivate all the Na⁺ channels and then repolarized to various recovery potentials. The time course of recovery from inactivation with a 40 ms conditioning pulse was well fitted by a single exponential equation, yielding a single time constant (τ), whereas a double exponential equation was required to obtain a good fit with a 500 ms conditioning pulse, yielding two time constants (τ_fast and τ_slow). The values of the time constants are listed in Table 1.

Figure 5. Effects of temperature on WT and M1476I channels.
A. The histogram shows the amplitudes of the persistent currents of M1476I channels recorded at different time points (1 min, 5 min, 10 min, and 20 min) after the whole-cell configuration was established at 10°C, 18°C, and 23°C. The persistent currents of the M1476I channel appeared to run down and then become stable 10 min after the whole-cell configuration was established. The effects of cooling on steady-state inactivation, the decay kinetics of the Na⁺ current, and the recovery from inactivation are shown in (B), (C), and (D). The functional properties at room temperature (23°C) (WT: ●; M1476I: ○) are also shown in order to compare the changes caused by lowering the temperature to 10°C (WT: □; M1476I: □) or 18°C (WT: ▲; M1476I: ▲). The voltage dependence of steady-state fast inactivation of WT and M1476I channels at different temperatures was recorded using the protocol in Fig. 2B. V_{1/2} and k, inactivation values are listed in Table 1. C. Voltage-dependent time constants of inactivation of WT and M1476I channels at 10°C, 18°C and 23°C. The time course of the current decay elicited at depolarized voltages was best fitted to a single exponential function, and the resulting time constants were plotted versus voltage. D. Recovery from inactivation with a 40 ms conditioning pulse at 10°C, 18°C and 23°C. The protocol is shown in the inset, and values of time constants are listed in Table 1.
Figure 6. Effects of Na⁺ channel blockers on persistent Na⁺ currents.
A. Effects of 50 nM TTX (top), or 100 µM mexiletine (bottom) on persistent Na⁺ currents. The dashed line represents zero current. The persistent Na⁺ currents were induced by a depolarizing step to –30 mV for 400 ms from a holding potential of –140 mV (see protocol in inset). The blue current represents the blocking effect of TTX, and the red current represents the blocking effect of mexiletine. B. The histogram shows the fraction of persistent Na⁺ currents before and after the administration of Na⁺ channel blockers.

Figure 7. Use-dependent inhibition by mexiletine.
A. To record the use-dependent inhibition of WT and M1476I channels by 100 µM mexiletine, a 50-pulse train was applied at –30 mV for 10 ms from a holding potential of –140 mV at 50 Hz. Each normalized peak current was plotted against the pulse number. B. Relative amplitudes of the 50th sweep (P₅₀/P₁) with and without mexiletine at different pulse frequencies (2-50 Hz). Before the mexiletine treatment, there were no differences in normalized currents between WT (▲, n=11) and M1476I (●, n=19) channels. After the mexiletine treatment, the normalized currents of both WT (△, n=12) and M1476I (○, n=16) were inhibited. However, the steady-state level of inhibition was lower for M1476I at different frequency stimuli ranging from 2 Hz to 50 Hz (*p<0.05 compared to WT+mexiletine).

Figure 8. Effects of mexiletine on the gating properties of WT and M1476I channels.
The properties in the absence of 100 µM mexiletine (WT: ▲; M1476I: ●,) are also shown in order to compare the changes induced by mexiletine (WT: △; M1476I: ○). A. Voltage dependence of activation and inactivation of WT and M1476I channels in the absence or presence of 100 µM mexiletine. Currents were evoked using the same protocol as in Fig. 1A (activation) and Fig. 2B (inactivation). The grey curves representing the activation and inactivation curves of WT and M1476I channels in the absence of mexiletine are the same as those shown in Fig. 2B in order to compare the blocking effect of mexiletine. Recovery from inactivation and mexiletine block was assessed using standard double-pulse protocols with 40 ms (B) or 500 ms conditioning pulses (C). Protocols are given in the insets under the recovery vs time curves of B and C. The black smooth curves were fitted to a double exponential equation with two time constants (τ₁ and τ₂). The grey curves shown in B and C represent the recovery from inactivation in the absence of mexiletine in order to compare the blocking effect of this drug. The V₁/₂ and kₜ values of steady-state activation and inactivation, and the values of time constants of recovery from inactivation and mexiletine block are listed in Table 2.
References:


Cummins TR & Sigworth FJ (1996). Impaired slow inactivation in mutant sodium


Table 1. Effects of temperature and extracellular K+ on Nav1.4 WT and M1476I currents.

<table>
<thead>
<tr>
<th>Extracellular K+</th>
<th>2 mM</th>
<th>2 mM</th>
<th>2 mM</th>
<th>4 mM</th>
<th>6 mM</th>
<th>9 mM</th>
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<tr>
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<td>18°C</td>
<td>23°C</td>
<td>23°C</td>
<td>23°C</td>
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<tr>
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<td>n=12</td>
<td>n=9</td>
<td>n=35</td>
<td>n=21</td>
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<td>V1/2 (mV)</td>
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<td>-40.3±1.2</td>
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<td>Kv (mV)</td>
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<td>n=35</td>
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<td>V1/2 (mV)</td>
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<td>Kv (mV)</td>
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<td>4.8±0.1</td>
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<td>40 ms conditioning pulse τ (ms)</td>
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<td>100 ms conditioning pulse τ (ms)</td>
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<td>Fraction τslow (%)</td>
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*p<0.5, # p<0.01, data was significantly different for mutant channels when compared to WT.
Table 2. Effects of mexiletine on Na<sub>+,1.4</sub> WT and M1476I currents.

<table>
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<td>V&lt;sub&gt;1/2&lt;/sub&gt; (mV)</td>
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<td></td>
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<td>(n)</td>
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<tr>
<td>M1476I</td>
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<td>-83.1±1.3# (15)</td>
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<td>WT</td>
<td>-48.0±1.3 (9)</td>
<td>-4.6±0.2 (9)</td>
<td>-88.6±0.8 (14)</td>
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</table>

*p<0.5, # p<0.01, data was significantly different for mutant channels when compared to WT.
Biophysical characterisation of the persistent sodium current of the Na<sub>v</sub>1.6 neuronal sodium channel: a single-channel analysis

Aurélien Chatelier · Juan Zhao · Patrick Bois · Mohamed Chahine

Abstract Na<sub>v</sub>1.6 is the major voltage-gated sodium channel at nodes of Ranvier. This channel has been shown to produce a robust persistent inward current in whole-cell experiments. Na<sub>v</sub>1.6 plays an important role in axonal conduction and may significantly contribute to the pathophysiology of the injured nervous system through this persistent current. However, the underlying molecular mechanisms and regulation of the persistent current are not well understood. Using the whole-cell configuration of the patch-clamp technique, we investigated the Na<sub>v</sub>1.6 transient and persistent currents in HEK-293. Previous studies have shown that the persistent current depended on the content of the patch electrode. Therefore, we characterised the single-channel properties of the persistent current with an intact intracellular medium using the cell-attached configuration of the patch-clamp technique. In HEK-293 cells, the Na<sub>v</sub>1.6 persistent current recorded in the whole-cell configuration was 3–5% of the peak transient current. In single-channel recording, the ratio between peak and persistent open probability confirmed the magnitude of the persistent current observed in the whole-cell configuration. The cell-attached configuration revealed that the molecular mechanism of the whole-cell persistent current is a consequence of single Na<sub>v</sub>1.6 channels reopening.

Keywords Voltage-gated sodium channel · Na<sub>v</sub>1.6 · Persistent current · Single-channel recording

Introduction

Voltage-gated sodium channels (VGSCs) play a critical role in electrical signalling in the nervous system and striated muscle as they are responsible for the initiation and propagation of action potentials. VGSCs are composed of one α-subunit, which forms the core of the channel and is responsible for the voltage-dependent gating and ion permeation, and several auxiliary β-subunits [2, 8, 19]. Nine mammalian α-subunit isoforms have been identified: Na<sub>v</sub>1.1 to Na<sub>v</sub>1.9 and an atypical sodium channel known as Nax. They differ by their sensitivity to tetrodotoxin (TTX) and are differentially expressed in the nervous system and striated muscle. Na<sub>v</sub>1.1, Na<sub>v</sub>1.2, Na<sub>v</sub>1.3 and Na<sub>v</sub>1.6 are predominantly expressed in the central nervous system (CNS) while Na<sub>v</sub>1.7, Na<sub>v</sub>1.8 and Na<sub>v</sub>1.9 are principally found in the peripheral nervous system. Na<sub>v</sub>1.4 and Na<sub>v</sub>1.5 are expressed mostly in skeletal and cardiac muscle, respectively. Nax, also known as Na<sub>v</sub>2, is expressed in the CNS and is involved in the control of salt intake [41].

Whereas the β-subunits are small proteins (22–36 kDa) that span the cell membrane only once, the α-subunits are large proteins (~260 kDa) composed of four homologous domains (DI–DIV), with each domain containing of six α-helical transmembrane-spanning segments (S1–S6).

The persistent sodium current (IN<sub>ap</sub>) has been described for different sodium channels in many cell types such as cortical neurons and nodes of Ranvier of myelinated fibres [18, 25, 28, 29, 31]. In the nervous system, Na<sub>v</sub>1.6 is the major VGSC at nodes of Ranvier and is also present in axon initial segments and dendrites [4, 6, 21]. In humans,
the loss of Na\textsubscript{v1.6} function cause ataxia and cognitive deficits; furthermore, Na\textsubscript{v1.6} knockout in mice results in ataxia and other movement disorders [23, 38].

This channel has been shown to produce a robust IN\textsubscript{ap} in whole-cell experiments with heterologous cells, including *Xenopus* oocytes [34] and HEK-293 or tsA201 cells [5, 33]. It is thought that under physiological conditions this IN\textsubscript{ap} serves to amplify or spatially integrate synaptic potentials and facilitates repetitive action potential firing [17, 37]. On the other hand, IN\textsubscript{ap} in other Na channels has been implicated in pathologies such as the long QT syndrome [12] or epilepsy [40]. Na\textsubscript{v1.6}, through its ability to produce IN\textsubscript{ap}, could contribute to the pathophysiology of injured nervous systems. Indeed, Na\textsubscript{v1.6} has been shown to accumulate along degenerating axons in demyelinated regions of the CNS in mice with experimental autoimmune encephalitis, and in patients with multiple sclerosis [15, 16]. These authors showed that the Na/Ca exchanger is co-localised with Na\textsubscript{v1.6} along the pathologically demyelinated nerve. Consequently, they suggest that an increased Na influx through the Na\textsubscript{v1.6} persistent current could cause an increase in intracellular calcium concentration by activating the reverse mode of the Na/Ca exchanger. Thus, Na\textsubscript{v1.6} appears to play an important role in axonal conduction and may significantly contribute to the pathophysiology of injured nervous systems. Indeed, Na\textsubscript{v} has been shown to accumulate along degenerating axons in demyelinated regions of the CNS in mice with experimental autoimmune encephalitis, and in patients with multiple sclerosis [15, 16].

Macroscopic currents from the HEK-293 stable cell line were recorded using the whole-cell configuration of the patch-clamp technique. For these experiments, low resistance patch electrodes were used. Series resistance compensation was performed to values >80% to minimise voltage errors. Linear leak currents and capacitance artefacts were removed using P/N leak subtraction. Sodium currents were filtered at 5 kHz and digitised at 10 KHz. Cell capacitance was recorded as telegraph readout of cell capacitance compensation using Axopatch 200B and pCLAMP.

Single-channel recordings were obtained using the cell-attached configuration of the patch-clamp technique. Patch electrodes with a resistance of 4–5 MΩ were used. Single-channel currents were filtered at 5 KHz and sampled at 20 kHz. Openings shorter than 0.1 ms were excluded from the analysis. The number of channels in the patch was estimated as the maximum number of channels seen open at the same time in all pulses for all voltages tested.

Solutions and reagents

For whole-cell experiments, the pipette contained (in mM) 120 CsCl, 1 MgCl\textsubscript{2}, 1 Na-GTP, 4 Mg-ATP, 10 EGTA and 10 HEPES. The pH was adjusted to 7.4 using CsOH. The bath solution contained (in mM) 150 NaCl, 2 KCl, 1.5 CaCl\textsubscript{2}, 1 MgCl\textsubscript{2}, 10 glucose and 10 HEPES. The pH was adjusted to 7.4 using NaOH. A correction for the liquid junction potential between the patch pipette and the bath solutions (≈5.4 mV) was applied to the command pulse.

For single-channel recording, the patch pipettes were filled with the bath solution used in whole-cell experiments.
added with 10 mM TEA-Cl to block endogenous potassium channels. The bath solution contained a high concentration of potassium composed of (in mM) 100 K-aspartate, 50 KCl, 1.5 CaCl2, 1 MgCl2, 10 glucose and 10 HEPES. This solution is used to completely depolarise HEK cells; hence, the absolute value of the voltage command is equal to the voltage applied through the patch pipette. A correction for the liquid junction potential between the patch pipette and the bath solutions (11.8 mV) was applied to the command pulse.

Data analysis

Data analysis and presentation were performed using a combination of pCLAMP software v 9.0 (Molecular Devices), QuB program, Microsoft Excel and Sigmaplot (Systat Software, Point Richmond, CA, USA).

For single-channel recordings, linear leak and capacitive transients were subtracted using the average of blank traces. Single-channel amplitudes were estimated using the cursors in clampfit. For open time histogram generation, the first bin was unused for curve fitting. Single-channel traces were filtered at 2 kHz for display purposes.

Data are presented as means±SEM. Statistical analysis was performed using one-way ANOVA or Kruskal–Wallis test. *P*<0.05 values were considered significant.

Results

The whole-cell properties of Na\(_{v}1.6\) channels

The whole-cell configuration of the patch-clamp technique was first used to study the macroscopic currents of the Na\(_{v}1.6\) stable cell line (Fig. 1). Sodium currents were generated from a holding potential of −100 mV and depolarised to potentials ranging from −80 to 50 mV for 100 ms in 5 mV increments with 5 s stimulus intervals (Fig. 1a). Currents recorded during the current–voltage relationship displayed a TTX sensitive persistent component at the depolarised potential that is present all along the test pulse (Fig. 1a, right panel). Figure 1b shows the I–V curve of the normalised transient and persistent current obtained during the recording presented in Fig. 1a (n=10). The persistent current was measured as the current remaining during the last 20 ms of the test pulse. This current is normalised to the peak transient current recorded in the same cell. Sodium transient current activation began at a potential of approximately −50 mV with a peak current density of 50.26±9.8 pA/pF (n=10) observed at −5 mV. The persistent current produced by Na\(_{v}1.6\) presents a current–voltage relationship shifted by 5–10 mV towards positive potentials compared with the peak current. It began
to activate around −40 mV and reached a maximum for potentials close to 0 mV. At 0 mV, maximum persistent currents reach a percentage of transient current of 3.23±1.12% (n=10).

The voltage dependence of the activation of Nav1.6 was determined from the relative membrane conductance as a function of the potential using the formula 
\[ G_{Na} = I_{Na}/(V_m - V_{rev}), \]
where \( G_{Na} \) is the peak conductance, \( I_{Na} \) is the peak sodium current for the test potential \( V_m \), \( V_{rev} \) is the estimated reversal potential of the sodium current obtained by extrapolation of the current–voltage relationship. The resulting sodium channel conductance was normalised to the maximum response for each cell and plotted versus the potential (Fig. 1c). The potentials between −110 and 0 mV from cell-attached patches containing at least seven channels during 200 ms step durations to better perceive the persistent component of Nav1.6. There is a repeated opening of channels all along the 200 ms pulses that may underlie the persistent component of Nav1.6. Late openings were slightly present at −30 mV but were much more pronounced at 0 mV, which could explain the largest persistent current observed in whole-cell experiments for depolarised potential. Indeed, ensemble average currents obtained from 200 consecutive traces recorded in the same cell display a persistent current at 0 mV, which qualitatively resembles the persistent component obtained in the whole-cell configuration at the same potential.

**Na\(_{1.6}\) channels exhibit a persistent sodium current in cell-attached single-channel recording**

Figure 3a shows single-channel records from cell-attached patches containing at least seven channels during 200 ms step durations at 0 mV after activation and were constructed for voltages in the range of −30 to 0 mV. The plots were best fitted with a double exponential function (Fig. 4a), and average open times were plotted against the test potential (Fig. 4b). Two open times were calculated for each potential (n≥4). The fast open time constants were not significantly sensitive to potentials and were 0.27±0.05, 0.27±0.02, 0.23±0.01 and 0.21±0.03 ms for −30 (n=5), −20 (n=4), −10 (n=4) and 0 mV (n=6), respectively. Similarly, the slow open time constants were not significantly sensitive to potentials and were 0.83±0.27, 0.82±0.21, 0.57±0.04 and 0.98±0.32 ms for −30 (n=5), −20 (n=4), −10 (n=4) and 0 mV (n=6), respectively. To estimate the persistent current of Na\(_{1.6}\) in the cell-attached configuration, we calculated the peak and persistent open probability (Po) of the channel. The peak Po was obtained by dividing the peak current obtained from ensemble average currents by the single-channel amplitude and the number of channels in the patch (Po=I/i×n). In the voltage range tested (from −30 to 0 mV), the Po was significantly higher at −10 and 0 mV.
compared with $-30$ mV with values of $0.41 \pm 0.04$ ($n=4$), $0.45 \pm 0.03$ ($n=6$) and $0.24 \pm 0.06$ ($n=5$), respectively (Fig. 4c). The Po for the persistent current was calculated by dividing the mean current obtained from ensemble average currents at the end of the stimulation by the single-channel amplitude and the number of channels estimated in the patch (Fig. 4d). The Po was significantly higher ($P<0.01$) at $0$ mV ($0.0161 \pm 0.0046$, $n=6$) compared with $-30$ mV ($0.0008 \pm 0.0009$, $n=5$). These values gave a ratio between the peak and persistent Po at $0$ mV of $\approx 3.6\%$. To check if this increase in persistent Po was because of an increase in the number of late openings (measured for the last $180$ ms of the stimulation) of Na$\text{v}1.6$, we plotted the number of late openings (for one channel within one sweep) versus the test potential (Fig. 4e). The evolution of late openings with voltage had a similar shape to the persistent Po of Na$\text{v}1.6$ (Fig. 4d). The number of late openings increased with voltage and was significantly higher ($P<0.05$) at $0$ mV ($n=6$), $-10$ mV ($n=4$) and $-20$ mV ($n=4$) compared with $-30$ mV ($n=5$). Moreover, the evolution of late openings with voltage had a similar shape than the persistent Po of Na$\text{v}1.6$ (Fig. 4d). Consequently, the relationship between the number of late openings and the Po of the persistent current fits a linear regression with a $R^2$ of $0.978$ (Fig. 4f). For depolarised voltages, some single-channel records exhibited a gating mode shift of Na$\text{v}1.6$, a representative recording is shown in Fig. 5. At $0$ mV, the probability for one channel entering into another gating mode longer than $20$ ms during one sweep as
calculated by Patlak and Ortiz [30, 31] was 1.89±1.1‰ (n=6). This gating mode shift was voltage sensitive since it is significantly lower (P<0.01, Kruskal–Wallis test) at −30 mV with a probability of 0.09±0.10‰ (n=6).

Discussion

Na channels play an important role in cell excitability. They exhibit a fast activation and inactivation kinetics. However, a non-inactivating Na current through these channels has been proposed to determine the threshold for action potential firing and neuronal bursting. Several subtypes of VGSCs have been suggested to conduct such a current. Na,1.1 and Na,1.2 have been proposed to conduct a persistent Na current and are modulated by G-proteins [26]. Na,1.3 has also been shown to produce a persistent component [14]. The cloning and functional expression of Na,1.6 raise the prospect that this channel may also carry the delayed sodium current. Indeed, using the whole-cell configuration of the patch-clamp technique the cloned Na,1.6 has been shown to exhibit a substantial persistent Na current [5] that could be modulated by ankyrin-G [33]. In addition, these authors reported that the magnitude of the persistent current was dependent on the content of the patch electrode. Fluoride ions seemed to reduce the magnitude of the persistent Na current.
We took advantage of the Na,1.6 stable HEK-293 cell line to study for the first time its single-channel properties. The hypothesis was that the persistent single-channel recordings observed in cell-attached configuration underlay the persistent current observed in whole-cell configuration. The cell-attached configuration is the most appropriate way of quantifying the magnitude of the persistent current. Several reports have shown that Na,1.6 has the property of exhibiting a persistent sodium current that is thought to play an important role in cell excitability in which this channel is expressed [5, 32–34]. In whole-cell experiments, we recorded, after the peak transient current, a persistent current that was maximal at potentials between 0 and 10 mV. This result is in agreement with previous studies on Na,1.6 [5, 32, 33]. However, this is in contrast with results obtained in some native neurons where the peak INa,p is reached for more negative voltage levels, close to the transient window current [22, 24, 36]. Furthermore, this persistent current characterised in native neurons was not sensitive to intracellular fluoride [24] whereas the persistent current observed for Na,1.6 was described to be decreased by intracellular fluoride [5, 33]. Unfortunately, the nature of sodium channels responsible for INa,p recorded from native neurons was not studied. Furthermore, these discrepancies indicate that there are probably two different types of sodium persistent currents depending on their voltage dependence and fluoride sensitivities. One possible explanation to these dissimilarities in persistent currents de-

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**Fig. 4** Single channel properties of Na,1.6. **a** Open time histogram obtained at 0 mV. Channel openings were recorded during the first 20 ms following membrane depolarization. The data were best fitted with a double exponential function (black line). **b** Voltage dependence of single-channel slow (grey) and fast (black) open time constant in the −30 to 0 mV voltage range. **c** Voltage dependence of peak open probability (Po) in the −30 to 0 mV voltage range. **d** Voltage dependence of persistent open probability (Po) calculated at the end of the 200 ms pulse from −100 mV to the indicated voltage. **e** Voltage dependence of the number of late openings by sweep for one channel measured from 20 to 200 ms after depolarization from −100 mV to the indicated voltage. **f** Relationship between persistent open probability (presented in d) and late opening (presented in e). The straight line is a linear regression with a $R^2$ of 0.978. Data (n≥4 for each figure) are presented as mean±SEM. *Indicates significant difference ($P<0.05$) with values at −30 mV. **Indicates significant difference ($P<0.01$) with values at −30 mV.
scribed is the cellular context. Indeed, it is possible that the heterologous system used in our study and others is unable to fully reproduce the physiological properties of Na<sub>1.6</sub> channels since it lacks some yet to be discovered regulatory proteins. For example, the influence of beta subunits on Na<sub>1.6</sub> INa<sub>p</sub> was tested in our laboratory (unpublished observations). These experiments revealed that β<sub>1, 2 or 3</sub> subunits had no effects on INa<sub>p</sub> voltage sensitivity or amplitude. However, the study of Na<sub>1.6</sub> in a more physiological environment like in transiently transfected neurons revealed a persistent current similar to what we observed in HEK-293 cells [32]. This indicates that if a regulatory factor is lacking, it is probably specific to particular neurons. A second explanation to these differences in voltage and fluoride sensitivities of persistent current is that in native neurons, sodium channels usually consist of multiple molecular species and splice variants of alpha subunits [10]. It is possible that the underlying mechanisms for INa<sub>p</sub> could be different among sodium channel isoforms or splice variants. As a consequence, the behaviours of Na<sub>1.6</sub> channels in heterologous expression system may reflect a restricted population of sodium channels underlying INa<sub>p</sub> observed in native neurons.

The persistent current recorded in our whole-cell experiments represents 3% of the peak current. This magnitude is in agreement with that observed when Na<sub>1.6</sub> is expressed in neurons [32]. Surprisingly, in non-neuronal cells such as HEK-293 or tsA201 cells, this persistent current was previously characterised by amplitudes between 10% and 30% of the peak current [5, 13, 27, 33]. Since ankyrin-G is supposed to inhibit the Na<sub>1.6</sub> persistent current [33], an endogenous presence of ankyrin-G in HEK-293 could be implicated in this difference. However, although it was asserted that this protein is expressed in HEK-293 cells [27], we found no ankyrin-G RNA or protein in our cells (assessed by RT-PCR and Western blot; data not shown) as that was previously shown in tsA201 cells by Shirahata et al. [33]. The reason for this discrepancy is unknown. Since intracellular medium is important in persistent current regulation, this difference could be explained by the dilution of an important intracellular component that regulates the persistent current. Consequently, we took advantage of the cell-attached configuration to retain the intracellular medium integrity and study the persistent current.

### Na<sub>1.6</sub> Single channel behaviour

It is the first time to our knowledge that Na<sub>1.6</sub> single-channel properties have been characterised. The calculated single-channel conductance is 20.7±0.7 pS. This value is in the range of other VGSCs such as Na<sub>1.1</sub>, 1.4 and 1.5 [7, 9, 20, 39, 40] but is larger than the single-channel conductance of Na<sub>1.8</sub> which is 11 pS [43]. Na<sub>1.6</sub> presents an increase in the Po for potentials ranging from −30 to 0 mV. The maximum peak Po was 0.45±0.03 at 0 mV, which is similar to the Po reported for Na<sub>1.1</sub> [40]. In comparison, the Po was estimated at 0.69 in neocortical neurons [3]. In contrast to the increase in the Po with the potential, the open dwell times are not voltage-dependent for Na<sub>1.6</sub>.

At the single-channel level Na channels open once during the stimulation episode because they rapidly
inactivate [1]. Our data show that the single-channel behaviour of Na\(_a\),1.6 is different with reopenings during a long stimulation. The single-channel events of the late opening had similar amplitudes, suggesting that the channels responsible for the transient current are also responsible for the late Na current. The Po during the last 20 ms of the stimulation increases with depolarisation in a similar range to the number of late openings of one channel during one sweep. This indicates that an increased frequency of reopenings with the potential is responsible for the increased persistent Po observed. Interestingly, the persistent Po reaches a maximum of 0.0161 at 0 mV, whereas the peak Po is 0.45 at the same potential. This produces a ratio between the peak and persistent Po of 0.0161/0.45=3.57%, which is similar to the amount of the persistent current observed in whole-cell recording. This indicates that these reopenings underlay the native persistent Na current typical of Na v1.6 that in both of our conditions (whole cell and cell attached) represents about 3% of the peak current. These late openings are different from the modal gating of Na channels observed in skeletal Na channels by Patlak several years ago [31]. Indeed, in our work, the probability for one channel to enter into another gating mode during one stimulation was less than 2‰ which is insufficient to explain alone 3% of the persistent whole-cell current. This shows also that the persistent current is not an exacerbation of the modal gating, similar to what is observed in pathological states such as the long QT syndrome [11]. However, since the gating mode shift probability increases with potential, a participation of this mechanism to the total amount of persistent current could not be excluded.

Pathophysiological consequence of the persistent Na current

The persistent Na current has been described in many brain preparations and postulated to play an important pathophysiological role. Indeed, a sustained Na influx through the Na channel can induce calcium-related injuries by the reverse mode of the Na/Ca exchanger [35]. In demyelinated axons, there is a co-localisation of Na\(_a\),1.6 and the Na/Ca exchanger [15]. Since Na\(_a\),1.6 produces a persistent current, this co-localisation could trigger the reverse mode of the Na/Ca exchanger, leading to an increase in intracellular calcium and axonal injury [42]. It is also possible that in pathological states such as in multiple sclerosis where demyelisation takes place, a loss of factors or interacting proteins may exacerbate the persistent inward Na current. Consequently, from a therapeutic perspective it is crucial to understand the molecular mechanism underlying the persistent current. In this work, the persistent current measured with both the whole-cell configuration and cell-attached was similar. This shows that the molecular mechanism of the whole-cell persistent current is a consequence of the single Na\(_a\),1.6 channel behaviour. This current accounts for 3% of the transient peak current. If this amount is closed to the persistent Na\(_a\),1.6 observed by Rush et al. [32], it is at least three times smaller than the one observed by Burbidge et al., Shirahata et al. and Chen et al. [5, 13, 33]. However, Burbidge et al. [5] showed in the same cell line that this persistent current run-down in whole-cell experiments depending on the pipette medium. Indeed, these authors showed that the persistent current disappears with time when using a pipette solution containing fluoride which was confirmed by Shirahata et al. [33]. Moreover, the proportion of persistent current of human Na\(_a\),1.6 observed by Burbidge et al. was much greater in human cells like HEK-293 than that described for the same channel or the murine Na 1.6 expressed in Xenopus oocytes [5, 34]. This likely indicates that an endogenous factor depending on experimental conditions can strongly influence this persistent current in the whole-cell configuration. It would be interesting to investigate this avenue in future experiments.

References


18. Dubois JM, Bergman C (1975) Late sodium current in the node of Ranvier. Pflügers Arch 357:145–148


Differential expression of sodium channel β subunits in dorsal root ganglion sensory neurons

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*Running Title: Differential expression of β subunits in DRG neurons

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Background: Auxiliary β subunits regulate the voltage-gated sodium channels of DRG neurons. Results: β subunits are differentially expressed in subpopulations of DRG neurons and regulate Na\textsubscript{v}1.7 channels in an isoform-specific manner.

Conclusions: Differential β subunit expression and isoform-specific regulation has important implications for the sodium currents of DRG neurons.

Significance: β subunits are important determinants of sodium channel function and sensory neuron excitability.

SUMMARY

The small- (<25 μm) and large-diameter (>30 μm) sensory neurons of the dorsal root ganglion (DRG) express distinct combinations of tetrodotoxin-sensitive (TTX-S) and tetrodotoxin-resistant (TTX-R) sodium (Na\textsuperscript{+}) channels that underlie the unique electrical properties of these neurons. In vivo these Na\textsuperscript{+} channels are formed as complexes of pore-forming α and auxiliary β subunits. The goal of this study was to investigate the expression of β subunits in DRG sensory neurons. Quantitative single-cell RT-PCR revealed that β subunit mRNAs were significantly correlated in small (β\textsubscript{2}, β\textsubscript{3}) and large (β\textsubscript{1}, β\textsubscript{3}) DRG neurons indicating that these subunits are co-expressed in the same populations. Co-immunoprecipitation and immunocytochemistry indicate that Na\textsubscript{v}1.7 forms stable complexes with the β\textsubscript{1}, β\textsubscript{2} and β\textsubscript{3} subunits in vivo and that Na\textsubscript{v}1.7 and β\textsubscript{3} co-localize within the plasma membranes of small DRG neurons. Heterologous expression studies show that β\textsubscript{3} induces a hyperpolarizing shift in Na\textsubscript{v}1.7 activation while β\textsubscript{1} produces a depolarizing shift in inactivation and faster recovery. The data indicate that β\textsubscript{3} and β\textsubscript{1} subunits are preferentially expressed in small and large DRG neurons respectively and that these auxiliary subunits differentially regulate the gating properties of Na\textsubscript{v}1.7 channels.

The sensory neurons of the dorsal root ganglia (DRG) give rise to nerve fibers that convey information about thermal, mechanical, and chemical stimulation from peripheral tissues to the central nervous system. These neurons express a unique combination of tetrodotoxin-sensitive (TTX-S) and tetrodotoxin-resistant (TTX-R) sodium (Na\textsuperscript{+}) currents that produce the rapid rising phase of the action potentials. Much of what is currently known about Na\textsuperscript{+} channel expression in sensory neurons is derived from electrophysiological studies of cultured DRG neurons (1-3). The small-diameter neurons (<25 μm) isolated from the DRG represent the cell bodies of unmyelinated nociceptors and preferentially express TTX-R Na\textsuperscript{+} current while the large-diameter (>30 μm) neurons, typically associated with low-threshold mechanoreceptors,
predominately express TTX-S Na\(^+\) current. DRG sensory neurons express at least 6 distinct Na\(^+\) channel isoforms that display properties similar to the endogenous TTX-S (Na\(_{1.1}\), Na\(_{1.2}\), Na\(_{1.6}\), Na\(_{1.7}\)) and TTX-R (Na\(_{1.8}\), Na\(_{1.9}\)) Na\(^+\) currents observed in these neurons (4-7).

In vivo, voltage-gated sodium channels form complexes with auxiliary \(\beta\) subunits that regulate the trafficking, gating properties and kinetics of the endogenous Na\(^+\) channels (8-12). \(\beta\) subunits are relatively small proteins (33-36 kDa) composed of a single membrane-spanning \(\alpha\)-helix, a short intracellular C-terminus, and an large extracellular N-terminus incorporating an immunoglobulin-like fold similar to that found in adhesion molecules (8;13). Immunocytochemistry and \textit{in situ} hybridization indicate that all four isoforms of \(\beta\) subunits (\(\beta_1\),\(\beta_3\)) are expressed in sensory neurons (12;14;15).

This study employed a combination of single-cell RT-PCR, immunocytochemistry, immunoprecipitation and electrophysiology to further investigate \(\beta\) subunit expression in DRG sensory neurons. The data indicate that small and large DRG neurons express different complements of \(\beta\) subunits. The functional consequences of \(\beta\) subunit expression was evaluated by examining their regulation of Na\(_{1.7}\), a TTX-S Na\(^+\) channel widely expressed in sensory neurons and an important contributor to pain sensation (19,20).

The \(\beta_3\) and \(\beta_1\) subunits differentially regulated heterologously expressed Na\(_{1.7}\) channels. The preferential expression of \(\beta\) subunits in small (\(\beta_2\)), and large (\(\beta_1\), \(\beta_3\)) neurons coupled with the isoform-specific \(\beta\) subunit regulation of Na\(_{1.7}\) activation (\(\beta_3\)) and inactivation (\(\beta_1\)) predicts substantial differences in the TTX-S currents of DRG sensory neurons.

**EXPERIMENTAL PROCEDURES**

**Preparation of DRG neurons -** Neonatal (7 day old) Sprague Dawley rats (P7) were anaesthetized with isoflurane before decapitation and the dorsal root ganglia were harvested from all accessible levels. The ganglia were incubated for 30 min at 37\(^\circ\)C in 2 ml of HBSS/HEPES containing 1.5 mg/ml collagenase (Sigma-Aldrich) followed by 1 mg/ml trypsin (Sigma-Aldrich) for an additional 30 min. Trypsin was removed, and the ganglia were transferred to L-15 Leibovitz media supplemented with 1% fetal bovine serum (Gibco Life Technologies), 2 mM glutamine, 2% penicillin-streptomycin (Gibco Life Technologies) and 50 ng/ml of nerve growth factor (Sigma-Aldrich). The ganglia were disrupted using fire-polished Pasteur pipettes and dissociated neurons were plated onto poly-lysine coated glass cover slips and placed into 35 mm dishes containing supplemented Leibovitz media. Neurons were suitable for single-cell harvesting and electrophysiology for up to 8 hours after plating. Animal protocols were approved by the Animal Care and Use Committee of Thomas Jefferson University.

**Single-cell RT-PCR -** Detailed methods for performing single-cell RT-PCR of dissociated DRG neurons were recently published (7). Small-(<25 \(\mu\)m) and large-diameter (>30 \(\mu\)m) DRG neurons are individually harvested by drawing them into a large bore pipette (30-50 \(\mu\)m diameter) containing sterile bath solution. The neurons are osmotically lysed by 10-fold dilution with sterile water and rapidly frozen. The mRNA present in the cell lysates was reverse-transcribed using random hexamer primers (Stratagene) in a standard 25 \(\mu\)l MMLV reverse transcription (RT) reaction (Fisher Scientific). Aliquots of the RT reaction (1-2 \(\mu\)l) were quantitatively analyzed using a SYBR green reaction cocktail on a MX3000P real-time PCR machine (Agilent Technologies). \(\beta\)-actin was quantitatively measured in each sample and used to normalize for differences in cellular mRNA expression. The absolute number of mRNA copies of each transcript was determined by comparing the threshold cycle (Ct) of the single-cell lysates with known cDNA standards assayed in parallel reactions. PCR primers are designed to span exon/intron borders to eliminate the detection of genomic DNA and concentrations (50-200 nM) optimized to achieve high amplification efficiency without the formation of primer dimers (Sigma-Proligo). The specificity of the real time detections were assessed using melting curve analysis and the identity of the amplified DNA determined by sequencing.

**Na\(_{1.7}\) stable cell line -** Rat Na\(_{1.7}\) cDNA was subcloned into the pcDNA3 expression vector (Invitrogen Corporation) and transfected into HEK293 cells using a standard calcium phosphate precipitation method (Invitrogen Corporation). After two weeks of selection for neomycin resistance (800 \(\mu\)g/ml) the remaining colonies were isolated and transferred to separate culture plates for expansion. Na\(_{1.7}\) expression was verified using RT-PCR and electrophysiology to
measure Na\textsuperscript{+} currents. The HEK293 cell line stably expressing Na\textsubscript{v}1.7 were maintained using standard culture conditions in DMEM media supplemented with FBS (10%), 2 mM L-glutamine, 100 U/ml penicillin, 10 mg/ml streptomycin and 400 \mu g/ml neomycin (Gibco Life Technologies).

**Electrophysiology** - Macroscopic Na\textsuperscript{+} currents of HEK293 cells stably expressing the Na\textsubscript{v}1.7 channel were recorded using the whole-cell patch-clamp technique. The pipette solution contained (in mM) 5 NaCl, 135 CsF, 10 EGTA, and 10 HEPES (pH 7.4). The bath solution contained (in mM) 150 NaCl, 2 KCl, 1.5 CaCl\textsubscript{2}, 1 MgCl\textsubscript{2} and 10 HEPES (pH 7.4). Patch electrodes were fashioned from 8161 borosilicate glass (Corning Incorporated) and coated with Sylgard (Dow-Corning Corporation) to minimize pipette capacitance. Recording pipettes had low access resistances (<1M\Omega) and the residual series resistance was 80% compensated. A correction for the liquid junction potential between the pipette and the bath solutions (-7 mV) was applied to the holding potential before the formation of gigaohm seals. After establishing the whole-cell configuration the cells were dialyzed for 10 minutes at room temperature (22\textdegree C) prior to recording Na\textsuperscript{+} currents. Voltage pulses were generated and currents recorded using pCLAMP and an Axopatch 200 amplifier (Molecular Devices). Whole cell currents were filtered at 5 kHz and digitized at 10 kHz with a Digidata 1440A (Molecular Devices).

Current-voltage relationships were obtained by plotting the current density (pA/pF) versus the test voltage. Normalized Na\textsuperscript{+} conductance (G\textsubscript{Na}) was calculated from the peak Na\textsuperscript{+} current (I\textsubscript{Na}) at each test potential (V): G\textsubscript{Na} = I\textsubscript{Na}/(V-E\textsubscript{Na}), where E\textsubscript{Na} is the measured Na\textsuperscript{+} ion reversal potential. The steady-state inactivation was determined by normalizing the peak Na\textsuperscript{+} current (I) measured after conditioning prepulses (-130 to -10, 500 ms) to the maximal Na\textsuperscript{+} current amplitude (I\textsubscript{max}) measured after prepulses to -140 mV and plotted against the conditioning voltage. The activation and steady-state inactivation were fitted to Boltzmann functions: G/G\textsubscript{max} = 1 / [1+exp \((V_{0.5} - V)/k\)], where V\textsubscript{0.5} is the midpoint and k, the slope factor. The predicted window currents were calculated from the product of the activation and steady-state inactivation curves as described previously (21). Recovery from inactivation was determined using depolarizing prepulses (-30 mV/20 ms) before returning to -100 mV for variable intervals (0-1200 ms). Standard test pulses (-30 mV/20 ms) were used to assess availability. The recovery time course was fitted to the sum of two exponentials yielding estimates of the fast (\tau\textsubscript{f}) and slow (\tau\textsubscript{s}) time constants.

Rat \beta\textsubscript{1}, \beta\textsubscript{2} and \beta\textsubscript{3} subunits were cloned in our laboratory as described previously (22). The \beta\textsubscript{4} subunit was a gift from Dr. Lori Isom (University of Michigan). The Na\textsuperscript{+} channel \beta\textsubscript{1-4} subunits (piRES/CD8/\beta\textsubscript{1-4}) and CD8 cDNA were subcloned into the piRES vector (Clontech Laboratories). HEK293 cells stably expressing the Na\textsubscript{v}1.7 channel were transient transfected with piRES/CD8/\beta\textsubscript{1-4} cDNA using a calcium phosphate precipitation method (23). Prior to recording the cells were briefly incubated in PBS containing CD8 antibody-coated beads to identify cells expressing the CD8 antigen (Dynal, Lake Success, NY).

**\beta subunit chimeras** - The \beta\textsubscript{1}/\beta\textsubscript{2} chimeras (\beta\textsubscript{211}, \beta\textsubscript{221}, \beta\textsubscript{112}, \beta\textsubscript{11A}) were a gift from Dr. Thomas Zimmer (Friedrich Schiller University, Jena, Germany). The three subscripted numbers refer to the extracellular N-terminal, membrane-spanning and intracellular C-terminal domains. In this nomenclature the wild-type \beta, and \beta\textsubscript{1}, \beta\textsubscript{2} subunits are designated \beta\textsubscript{111} and \beta\textsubscript{222} respectively. \beta\textsubscript{211} contains the extracellular domain of \beta\textsubscript{2} and the membrane-spanning and intracellular domains of \beta\textsubscript{1}. \beta\textsubscript{221} incorporates the extracellular and membrane-spanning domains of \beta\textsubscript{2} and the intracellular domain of \beta\textsubscript{1}. \beta\textsubscript{112} contains the extracellular and membrane-spanning domains of \beta\textsubscript{1} and a deletion of the 41 amino acids from the intracellular C-terminal domain (Fig. 6. A). \beta\textsubscript{211}, \beta\textsubscript{221}, \beta\textsubscript{112}, and \beta\textsubscript{11A} were transfected to the piRES vector for expression in mammalian cells (piERS/CD8/\beta\textsubscript{211}, piERS/CD8/\beta\textsubscript{221}, piERS/CD8/\beta\textsubscript{112}, piERS/CD8/\beta\textsubscript{11A}) and transiently transfected into our Na\textsubscript{v}1.7 stable cell line.

**Immunoprecipitation and Western analysis** - Rat dorsal root ganglia (DRG) were harvested and immediately placed in ice cold Hank's Balanced Salt Solution (HBSS). The ganglia were washed with ice-cold HBSS and pelleted by low-speed centrifugation at 4\textdegree C. HBSS was replaced with ice cold lysis buffer (50mM Tris, 1.0mM EDTA, 1.0mM EGTA, 150mM NaCl, 1.0% Triton X 100) supplemented with protease inhibitors (Sigma-
The samples were homogenized on ice and centrifuged (15,000 rpm/20 mins) at 4°C. The supernatant was recovered and assayed for protein concentration using the Bradford method (Bio-Rad Corporation). Lysates (1 mg) were incubated overnight at 4°C in 1 ml of lysis buffer containing either 10 μg control mouse IgG or 10 μg mouse monoclonal N68/6 anti-Na,v1.7 antibody (NeuroMab). The N68/6 anti-Na,v1.7 antibodies do not cross-react with other Na⁺ channels isoforms or channel proteins extracted from adult rat brain. Protein G agarose resin (Thermo Scientific) was added (100 μl) and the lysates incubated 6 hours at 4°C before washing with ice-cold lysis buffer. Proteins were eluted from the protein G agarose by addition of 50 μl of 0.2M glycine buffer (pH 2.5). The pH was neutralized by adding 10 μl of Tris buffer (1M, pH 9.0), mixed with 3x sample buffer and separated on 12% SDS-PAGE gels. Proteins were transferred to Protran nitrocellulose membranes (Whatman International), blocked with 5% BSA, washed with Tris buffered saline with 0.1% Tween 20 (TBST) and incubated overnight with rabbit polyclonal SCN1B (Cell Applications), rabbit polyclonal SCN2B (Sigma-Aldrich) or rabbit polyclonal SCN3B (Abcam) antibodies in TBST containing 5% BSA. These commercial antibodies (SCN1B, SCN2B, SCN3B) are highly specific and do not display cross-reactivity with other members of the β subunit family. The membranes were incubated with HRP conjugated goat anti-rabbit secondary antibody (Thermo Scientific) for one hour at room temperature and labeled proteins were detected using chemiluminescence (Thermo Scientific). We routinely failed to observe Na,v1.7 or β subunit precipitation from cell lysates preincubated with control IgG further supporting the specificity of the Na,v1.7 pull downs. The low level expression of the β4 subunits in DRG neurons (Figure 1) combined with the poor quality of available β4 antibodies prevented detailed analysis of this protein.

Immunocytochemistry - Dissociated DRG neurons were plated onto poly-lysine coated glass cover slips and fixed in PBS containing 4% paraformaldehyde for 10 minutes. Cells were permeabilized with 0.1% Triton-X100 in PBS for 5 minutes before several washes with PBS. Non-specific antibody binding was reduced by incubating the cells with 5% BSA and 5% goat serum in TBST for 60 minutes. Permeabilized cells were incubated with mouse monoclonal anti-

RESULTS

The expression of β subunits was investigated in acutely dissociated DRG sensory neurons isolated from 7 day old neonatal rats. Neurons were individually harvested and the mRNA present in the cell lysates quantitatively measured (mRNA copies/neuron) using real-time PCR. Figure 1 compares the expression of the β subunit transcripts in small- (<25 μm) and large-diameter (>30 μm) DRG neurons. The data indicate that small neurons preferentially express the β2 and β3 isoforms (2000-4000 copies/neuron). Although β1 was also detected in these neurons the mRNA copy number was 5-fold lower (<400 copies/neuron). This contrasts with large-diameter neurons that highly expressed β1 and β3 mRNA (~4500 copies/neuron) while β3 was present at lower levels (<2000 copies/neuron). The β4 subunit was expressed at comparatively low levels in both the small (<500 copies/neuron) and large (<2000 copies/neuron) neurons. The data indicate that small (β2, β3) and large (β1, β2) DRG neurons express different complements of auxiliary β subunits.

To investigate the relationship between Na,v1.7 and β subunits the mRNA encoding for these subunits were quantitatively measured in small and large DRG neurons. Figure 2 plots the number of Na,v1.7 mRNA copies versus the β subunit mRNA measured from the same neurons. The data were statistically evaluated using Pearson produce-moment correlation analysis to determine the strength of mRNA co-expression in these neurons. The Na,v1.7-β2 and Na,v1.7-β3 mRNAs were found to be significantly correlated with...
Pearson coefficients (r) of 0.777 and 0.775 respectively (p<0.001). Despite the low expression of α1 subunit mRNA (344 copies/neurons) these subunits were significantly correlated with Na+,1.7 (r=0.537, p<0.01) although the physiological relevance of this association is not clear. The Na+,1.7 and β4 mRNAs were not associated in these neurons (r=0.193). These data indicate that β1 and β3 subunit transcripts are abundantly expressed in small DRG neurons and are significantly correlated with Na+,1.7 mRNA.

Figure 2 also shows the correlation of Na+,1.7 and β1 subunit mRNA in large neurons. Na+,1.7 expression was significantly correlated with the β1 (r=0.732) and β2 (r=0.680) subunits (p<0.001). This contrasted with β3 (r=0.357, p=0.112) and β4 (r=0.342, p=0.152), which were not correlated with Na+,1.7. The data indicate that the Na+,1.7, β1 and β2 subunit mRNAs are co-expressed in the same population of large-diameter neurons.

Na+,1.7-β interactions were further investigated using co-immunoprecipitation and Western blotting (Figure 3). Figure 3A shows a western blot of DRG homogenates isolated from P7 animals probed with Na+,1.7 antibody. The Na+,1.7 antibody labeled a single high-molecular weight protein (≈270 kD) that is characteristic of Na+,1.7 channels. Immunoprecipitated Na+,1.7 complexes were separated on acrylamide gels, transferred to nitrocellulose membranes and probed with β-specific antibodies. Figures 4B-4D show that the β subunit antibodies labeled low molecular weight proteins (32-34 kDa) that are slightly smaller than what was previously reported for the β1 (36 kDa) and β2 (33 kDa) of adult rats (24). β subunits are highly glycosylated proteins containing 30-36% carbohydrate by weight (24;25). Differences in the carbohydrate content of these subunits account for variations in the molecular weight of the β1 subunit expressed in skeletal muscle (25). We speculate that the lower molecular weights observed in neonatal P7 animals (≈1-2 kDa) may represent partially glycosylated β subunits (26). The immunoprecipitation data show that the β1, β2 and β3 subunits form stable complexes with Na+,1.7 channels isolated from the DRG and are therefore candidates for regulating these channels in vivo. Unfortunately, it is impossible to associate the Na+,1.7-β interactions detected using immunoblotting techniques with specific subpopulations of small and large DRG neurons.

Potential Na+,1.7-β subunit interactions were further investigated using immunocytochemistry. Figure 4 shows the confocal imaging of small neurons labeled with Na+,1.7 and β-specific antibodies. The cytoplasm of these neurons displayed diffuse labeling for the Na+,1.7, β1 and β2 subunits. Merged images revealed some overlap of Na+,1.7 with β1 and β2 subunits, predominately within the intracellular compartment. By contrast, the majority of the β3 immunofluorescence was localized along the cell periphery consistent with the labeling of membrane bound proteins. The merged images display considerable overlap of Na+,1.7 and β3 around the cell periphery consistent with the co-localization of these proteins near the plasma membrane.

Initial attempts to investigate the β subunit regulation of endogenous Na+,1.7 channels in dissociated DRG neurons were complicated by the variable expression of Na+,1.7 and β subunits and the presence of multiple overlapping components of TTX-S Na+ current in these neurons. We therefore conducted heterologous expression studies to further investigate the β subunit regulation of Na+,1.7 channels. HEK293 cells stably expressing Na+,1.7 were transiently transfected with β subunits. Figure 5 shows examples of whole-cell Na+ currents recorded from cells expressing Na+,1.7 alone or with co-expressed β1 or β3 subunits. In the absence of β subunits the Na+,1.7 channels produced rapidly gating Na+ current. Co-expressing β subunits (β1, β4) had no effect on the current kinetics or peak Na+ current amplitudes.

To investigate potential changes in voltage-dependent gating the Na+ conductance was calculated from the peak currents and plotted versus the test voltage (Figure 6A). Co-expressing the β3 subunit produced a significant hyperpolarizing shift (-9 mV) in Na+,1.7 activation. Steady-state inactivation was determined using 500 ms prepulses to voltages between -130 and -5 mV. β1 induced a depolarizing shift (+5 mV) in the midpoint of Na+,1.7 inactivation (Figure 6A). By contrast, co-expressing the β2 or β4 subunits did not alter the activation or the steady-state inactivation of the channels.

Recovery from inactivation was determined by applying depolarizing prepulses (-30 ms/20 mV) before returning to -100 mV for varying intervals (0-1200 ms). The recovery time course of Na+,1.7 channels was biexponential with fast (τf) and slow
Differential expression of β subunits in DRG neurons

(τᵣ) time constants of 26 ms and 153 ms respectively (Figure 6B). Co-expressing β₁ significantly reduced both τᵣ (14 ms) and τᵢ (67 ms) consistent with more rapid recovery from inactivation (Figure 6B). The remaining β subunits (β₂,β₃) had no effect on recovery from inactivation (Table 1).

The overlap of activation and steady-state inactivation of Na⁺ channels defines a range of voltages (i.e. window) where Na⁺ channels can be partially activated but are not fully inactivated. Na⁺ channels within this hyperpolarized range of voltages may become persistently activated resulting in inward Na⁺ currents that could potentially depolarize the resting membrane potential and increase neuronal excitability. β-induced increases in the overlap of Na⁺ channel activation and inactivation tend to expand this window and consequently the fraction of persistently activated channels. The β₁ subunit produced a +5 mV depolarizing shift in steady-state inactivation while β₁ produced a -9 mV shift in Na₁.7 activation (Table 1) that could potentially increase the window currents. Figure 6C shows the predicted window currents of Na₁.7 channels co-expressed with either the β₁ or β₃ subunits. Despite acting by different mechanisms the β₁ and β₃ subunits produce similar 2-3 fold increases in the Na₁.7 window current.

To gain a better understanding of the mechanism of β subunit regulation chimeras were generated by exchanging the structural domains of the β₁ subunit that shifted steady-state inactivation and accelerated recovery from inactivation with the homologous domains of the β₂ subunit that had no effect on Na₁.7 gating (Table 1). The extracellular N-terminal, intracellular C-terminal and membrane-spanning domains of β₁ were systematically replaced with those of β₂ and transiently expressed in HEK293 cells stably expressing Na₁.7 channels. Chimeras that retained the extracellular N-terminal domain of β₁ (β₁₁₂, β₁₁₃) fully recapitulated the hyperpolarizing shift in steady-state inactivation and faster recovery observed with the wild-type β₁ subunit (Table 1). Conversely, substitutions that replaced the N-terminus of β₁ (β₂₁₁, β₂₂₁) completely abolished Na₁.7 regulation. C-terminal deletions of the β₁ subunit (β₁₁₃) retained full activity indicating that intracellular domain is not essential. The data indicate that the extracellular N-terminal domain of β₁ is critical for the functional regulation of Na₁.7 channels.

DISCUSSION

The goal of these studies was to investigate the expression of auxiliary β subunits in DRG neurons and to characterize the β subunit regulation of Na₁.7, a TTX-S Na⁺ channel widely expressed in sensory neurons. Single-cell analysis found that β subunit mRNAs were differentially expressed in small (β₂, β₃) and large (β₁, β₂) DRG neurons (Figure 1). Comparisons of Na₁.7, β₂ and β₃ mRNA measured from individual small neurons found that the expression of these subunits were significantly correlated indicating that these transcripts are co-expressed in the same neurons (Figure 2). By contrast, the Na₁.7 mRNA of large neurons was found to be significantly correlated with the β₁ and β₂ subunits. These data indicate that the Na₁.7 channels present in small and large DRG neurons are co-expressed with different complements of auxiliary β subunits.

Interactions between Na₁.7 and β subunits were further explored using co-immunoprecipitation of Na₁.7 channels. Na₁.7 co-precipitated with the β₁, β₂ and β₃ subunits indicating that these subunits form stable complexes in vivo (Figure 3). Despite supporting a direct physical interaction between Na₁.7 and the β₁-β₃ subunits the neurons in which these interactions occurred (i.e. small vs. large) is impossible to ascertain using immunoprecipitation techniques. However, immunofluorescent imaging showed that Na₁.7 and β₃ co-localized near the periphery of the small DRG neurons (Figure 4). Although β₂ subunits are also highly expressed in small neurons they failed to display obvious co-localization with Na₁.7 channels. The combination of Na₁.7-β₃ mRNA correlation (Figure 2), co-immunoprecipitation (Figure 3) and co-localization near at the plasma membrane (Figure 4) support the idea that β₃ subunits partner with Na₁.7 channels. While these data do not preclude Na₁.7 interaction with other β subunits it suggests an important contribution of Na₁.7-β₃ channels to the TTX-S Na⁺ currents of small DRG neurons.

Previous studies of β subunit regulation of heterologously expressed Na₁.7 channels have produced conflicting data. Initial studies of Na₁.7 channels expressed in Xenopus oocytes indicated that the β₁ and β₂ subunits failed to alter the
expression or gating properties of NaV1.7 suggesting that these channels may be not regulated by these auxiliary subunits (27;28). Subsequent work, also in oocytes, found that co-expressing β1 accelerated inactivation and recovery kinetics and produced a hyperpolarizing shift in NaV1.7 activation (29). The regulation of NaV1.7 channels by the β3 and β4 subunits has not been investigated.

In this study HEK293 cells stably expressing NaV1.7 channels were employed to further investigate the functional consequences of NaV1.7-β interactions. Co-expressing β subunits (β1-β4) did not alter the peak Na+ current densities or NaV1.7 current kinetics. However, β1 produced a depolarizing shift in steady-state inactivation and faster recovery from inactivation (Table 1). At voltages near the resting membrane potentials of DRG neurons (∼60 mV), depolarizing shifts in inactivation would tend to increase the fraction of NaV1.7 channels available to open in response to depolarization. Similar increases in availability along with the associated increase in Na+ current density are well known to reduce the threshold for initiating action potentials (30-32). The rate of Na+ channel recovery from inactivation is an important determinant of the absolute and relative refractory periods of action potentials. The faster recovery of NaV1.7-β1 channels predicts rapid repriming at hyperpolarized voltages that may reduce the duration of the refractory periods thereby enabling increased firing frequency in large-diameter neurons highly expressing the NaV1.7-β1 combination.

The β3 subunit produced a -9 mV shift in NaV1.7 activation causing the channels to open at more hyperpolarized voltages (Table 1). Such shifts in activation and the accompanying increase in Na+ current at more hyperpolarized voltages are predicted to increase neuronal excitability and could potentially reduce the threshold for firing action potentials in small-diameter neurons. This mechanism is consistent with studies showing that Na+ channels with low activation thresholds are critical determinants of action potential initiation at the axon initial segment (33;34).

NaV1.7-β subunit interactions that induce hyperpolarizing shifts in activation (β3) or depolarizing shifts in inactivation (β1) tend to increase the overlap of activation and inactivation gating (Figure 6C). At voltages within this overlap region Na+ channels are partially activated but not fully inactivated increasing the potential of persistent window currents (35). At -50 mV, the peak window current probability predicts that a small percentage (0.1%) of NaV1.7 channels will be persistently activated. Co-expressing the β1 or β3 subunits increased the probability of persistent activation by 2-3 fold. Persistent activation of Na+ channels is partially activated but not fully inactivated increasing the potential of NaV1.7 channels by the β3 and β4 subunits has not been investigated.

Previous studies have employed chimeras, deletion analysis and mutations to define the structural domains of β subunits that are critical for Na+ channel regulation (21;39-42). The findings indicate that the extracellular N-terminal domain of β1 is essential for the functional regulation of neuronal and skeletal muscle Na+ channels. This contrasts with the β1 regulation of cardiac Na+ channels, where the membrane-spanning domain was found to be critical for the increased expression and accelerated recovery of Na+ channels (43). These data imply that different structural domains and therefore different molecular interactions are responsible for β1 regulation of neuronal and cardiac Na+ channels.

β1 mRNA is highly expressed in large DRG neurons (Figure 1) where it is significantly correlated with NaV1.7 indicating that these subunits are co-expressed in the same population of large-diameter neurons (Figure 2). β subunit chimeras were employed to identify the structural domains of β1 required to produce the observed depolarizing shift in steady-state inactivation and accelerated recovery of NaV1.7 channels (Table 1). Chimeras incorporating the extracellular N-terminal domain of β1 (β112) retained the shift in inactivation and faster recovery while replacing the extracellular domain (β211) completely eliminated these effects. These data indicate that the N-terminal domain of β1 subunit is required for NaV1.7 regulation. β1 subunits with a truncated C-terminus (β113) retained full functional regulation indicating that the intracellular domain is non-essential. Interactions between the N-terminus of β1 and extracellular loops of NaV1.7 may be important for the functional regulation of these.
Differential expression of \( \beta \) subunits in DRG neurons

Recent work employed a similar approach to investigate the \( \beta_1 \) regulation of Na,1.8, a TTX-R channel that produces the majority of the inward Na\(^+\) current in small-diameter DRG neurons (21). Substitution of the extracellular N-terminal domain of \( \beta_1 \) had no effect on the expression or gating properties of Na,1.8 channels. Rather the intracellular C-terminal domain of \( \beta_1 \) was found to be the critical determinant of Na,1.8 regulation. These data indicate that the N- and C-terminals of the \( \beta_1 \) subunit differentially regulate the gating properties of Nav1.7 and Na,1.8 channels.

Much of what is currently known about \( \beta \) subunit expression in the DRG is derived from immunocytochemistry and in situ hybridization (4;12;15-18;44). These studies indicate that all four isoforms of \( \beta \) subunits (\( \beta_1-\beta_4 \)) are present in the DRG and that these subunits are differentially expressed in subpopulations of sensory neurons (12;15). \( \beta_3 \) subunits are prominently expressed in small- and medium-sized neurons while \( \beta_1 \) and \( \beta_4 \) are preferentially expressed in large neurons (4;16;17). \( \beta_2 \) appears to be widely expressed in the DRG and does not show a clear preference for neuronal size (15;45). These findings are in good agreement with our single-cell analysis of gene expression and are consistent with the conclusion that \( \beta \) subunits are differentially expressed in subpopulations of DRG neurons. Unfortunately, histological approaches do not provide quantitative assessments of \( \beta \) subunits expression levels or insight into the functional regulation Na\(^+\) channels by \( \beta \) subunits. Our data indicate that the differential expression of \( \beta \) subunits in DRG neurons combined with isofrom-specific \( \beta \) subunit regulation of Na,1.7 activation (\( \beta_3 \)) and inactivation (\( \beta_1 \)) predicts substantial differences in the predominant TTX-S Na\(^+\) currents of small and large sensory neurons.

Previous work investigated the role of the \( \beta_1 \) and \( \beta_2 \) subunits in sensory neurons using SCN1B and SCN2B null mice (45;46). Whole-cell recordings from DRG neurons isolated from the \( \beta_1 \) knockouts revealed small changes in the amplitudes and gating properties of TTX-S and TTX-R Na\(^+\) currents (46). The relatively subtle effects of the SCN1B knockout on DRG Na\(^+\) currents coupled with the low level expression of \( \beta_1 \) subunits in small-diameter sensory neurons suggests that these subunits may not be important regulators of the Na\(^+\) channels expressed in nociceptors. Neurons from the SCN2B null mice displayed reductions in TTX-S Na\(^+\) current amplitude, Na\(^+\) channel mRNA and protein (46). Although the underlying mechanism is unclear the SCN2B knockout appears to reduce TTX-S Na\(^+\) currents by decreasing Na\(^+\) channel mRNA and protein expression. Based on the comparison of Na\(^+\) currents recorded from control and SCN2B null mice the \( \beta_2 \) subunits were proposed to increase Na\(^+\) channel expression (Na,1.1, Na,1.6, Na,1.7), produce hyperpolarizing shifts in activation and accelerate the kinetics of the endogenous TTX-S Na\(^+\) currents (46). These effects were not recapitulated in our heterologous expression studies of Na,1.7-\( \beta_2 \) channels where no changes in Na\(^+\) current density, voltage-dependence or current kinetics were observed. Rather our findings are consistent with previous work showing that the \( \beta_2 \) subunit has no effect on the expression or gating properties of the Na,1.3, Na,1.6 or Na,1.8 channels (47;48). The reasons for the apparent discrepancy between \( \text{in vivo} \) knockdown and heterologous expression studies are not known but may reflect contributions by endogenous regulatory pathways that are specific to the DRG or the compensatory upregulation of other \( \beta \) subunits in the sensory neurons of SCN2B null mice.

References

Differential expression of β subunits in DRG neurons


Differential expression of $\beta$ subunits in DRG neurons


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Figure Legends

Figure 1. Single-cell analysis of β subunit mRNA. Small- (<25 μm) and large-diameter (>30 μm) DRG neurons were individually harvested, the mRNA present in the cell lysates reverse transcribed and quantitatively measured using real-time PCR. The data are expressed as the number of mRNA copies present in each neuron. The data are the means and SEM of 74 and 21 small and large neurons respectively.

Figure 2. Correlation of Na\textsubscript{v}1.7 and β subunit mRNA expression in small and large DRG neurons. The mRNA (copies/neuron) of Na\textsubscript{v}1.7 and β subunits were measured from the same populations of small-diameter (<25 μm) and large-diameter (>30 μm) neurons. A-D. Plots of Na\textsubscript{v}1.7 mRNA versus β\textsubscript{1} (Panel A), β\textsubscript{2} (Panel B), β\textsubscript{3} (Panel C) and β\textsubscript{4} (Panel D). The straight lines are simple linear regressions. The data represent the means and SEM of mRNA measurements from 29 small and 21 large DRG neurons.

Figure 3. Co-immunoprecipitation of Na\textsubscript{v}1.7 and β subunits. A. DRG homogenates were separated on SDS-Page gels, transferred to nitrocellulose membranes and probed with Na\textsubscript{v}1.7-specific antibodies. B-D. Na\textsubscript{v}1.7 channel complexes were immunoprecipitated from DRG lysates, separated on SDS-page gels and probed with antibodies specific for β\textsubscript{1} (Panel B), β\textsubscript{2} (Panel C) or β\textsubscript{3} (Panel D). Tick marks indicate the position of molecular weight markers in kilodaltons (kDa).

Figure 4. Imaging of Na\textsubscript{v}1.7 and β subunits in small DRG neurons. Small-diameter (<25 μm) DRG neurons were immunolabeled with Na\textsubscript{v}1.7- and β-specific (β\textsubscript{1}-β\textsubscript{3}) antibodies and reacted with fluorochrome-conjugated secondary antibodies before confocal imaging. The left panels show the Na\textsubscript{v}1.7 immunostaining, the middle panels are the β subunit staining and the right panels are the merged images.

Figure 5. β subunit regulation of heterologously expressed Na\textsubscript{v}1.7 channels. Whole-cell Na\textsuperscript{+} currents of HEK293 cells stably expressing the Na\textsubscript{v}1.7 channels. Currents were elicited by depolarizing voltage pulses between -90 and +50 mV from a holding potential of -120 mV. A-C. Representative Na currents of Na\textsubscript{v}1.7 channels expressed alone (panel A) or co-expressed with β\textsubscript{1} (panel B) or β\textsubscript{3} subunits (panel C). D. Plot of the peak current density (pA/pF) of Na\textsubscript{v}1.7 channels alone or with co-expressed β subunits (β\textsubscript{1}-β\textsubscript{4}). Data are the means and SEM of 13 (Na\textsubscript{v}1.7), 26 (β\textsubscript{1}), 9 (β\textsubscript{2}), 18 (β\textsubscript{3}) and 8 (β\textsubscript{4}) determinations.

Figure 6. β subunits shift activation and inactivation of Na\textsubscript{v}1.7 channels. A. The normalized conductance was determined from the peak Na\textsuperscript{+} currents and plotted versus the test potential. Also plotted in the steady-state inactivation obtained using 500 ms prepulses to voltages between -130 and -5 mV. The smooth curves are fits of the activation and inactivation data to Boltzmann functions with the parameters listed in Table 1. Data are the means and SEM of 14 (Na\textsubscript{v}1.7), 26 (β\textsubscript{1}), 9 (β\textsubscript{2}), 21 (β\textsubscript{3}) and 8 (β\textsubscript{4}) determinations. B. Na channels were inactivated by a brief depolarization (-30 mV/20 ms) and the recovery time course (0-1200 ms) measured at −100 mV. The smooth curves are biexponential curve fits with fast and slow time constants listed in Table 1. Data are the means and SEM of 15 (Na\textsubscript{v}1.7), 22 (β\textsubscript{1}), 10 (β\textsubscript{2}), 17 (β\textsubscript{4}) and 8 (β\textsubscript{4}) determinations. C. Window current probabilities predicted from the activation and steady-state inactivation of the Na\textsubscript{v}1.7 channels expressed alone or with either the β\textsubscript{1} or β\textsubscript{3} subunits.
Table 1. β subunit regulation of Nav1.7 gating

<table>
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<th>Inactivation $V_{0.5}$ (mV)</th>
<th>$k_v$ (mV)</th>
<th>Recovery $\tau_f$ (ms)</th>
<th>$\tau_s$ (ms)</th>
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<td>-43 ± 1</td>
<td>5.3 ± 0.3</td>
<td>-87 ± 1</td>
<td>7.2 ± 0.2</td>
<td>22 ± 2</td>
<td>128 ± 12</td>
<td>70</td>
</tr>
<tr>
<td>β₃</td>
<td>-51 ± 1*</td>
<td>5.0 ± 0.3</td>
<td>-88 ± 1</td>
<td>7.1 ± 0.2</td>
<td>24 ± 2</td>
<td>142 ± 11</td>
<td>70</td>
</tr>
<tr>
<td>β₄</td>
<td>-41 ± 1</td>
<td>6.9 ± 0.2</td>
<td>-91 ± 2</td>
<td>7.6 ± 1.0</td>
<td>25 ± 2</td>
<td>136 ± 11</td>
<td>73</td>
</tr>
</tbody>
</table>

β₁-β₂ Chimeras

<table>
<thead>
<tr>
<th></th>
<th>Activation $V_{0.5}$ (mV)</th>
<th>$k_v$ (mV)</th>
<th>Inactivation $V_{0.5}$ (mV)</th>
<th>$k_v$ (mV)</th>
<th>Recovery $\tau_f$ (ms)</th>
<th>$\tau_s$ (ms)</th>
<th>$A_r$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β₁₁₂</td>
<td>-44 ± 1</td>
<td>5.9 ± 0.2</td>
<td>-83 ± 1*</td>
<td>6.3 ± 0.1</td>
<td>16 ± 1*</td>
<td>58 ± 6*</td>
<td>73</td>
</tr>
<tr>
<td>β₁₁Δ</td>
<td>-44 ± 1</td>
<td>4.9 ± 0.2</td>
<td>-82 ± 1*</td>
<td>6.3 ± 0.2</td>
<td>16 ± 2*</td>
<td>60 ± 6*</td>
<td>71</td>
</tr>
<tr>
<td>β₂₁₁</td>
<td>-43 ± 1</td>
<td>5.4 ± 0.3</td>
<td>-87 ± 1</td>
<td>7.2 ± 0.2</td>
<td>22 ± 1</td>
<td>126 ± 11</td>
<td>69</td>
</tr>
<tr>
<td>β₂₂₁</td>
<td>-44 ± 1</td>
<td>4.6 ± 0.2</td>
<td>-88 ± 1</td>
<td>6.9 ± 0.2</td>
<td>22 ± 2</td>
<td>133 ± 10</td>
<td>67</td>
</tr>
</tbody>
</table>

The parameters were obtained from curve fits of Nav1.7 activation, inactivation and recovery from inactivation (Figure 6). The data were tested for significant differences using ANOVA (p<0.001) followed by post-hoc Dunnett’s test at a significance level of p<0.05. For Dunnett’s test the effects of beta subunits were compared to values measured for Nav1.7 channels expressed alone. Data are the means and SEM of between 8 and 30 experiments.
Figure 1

Differential expression of β subunits in DRG neurons

- β1
- β2
- β3
- β4

mRNA Expression (copies/neuron)

<25 μm
>30 μm
Differential expression of β subunits in DRG neurons

Figure 2

A

\[ \beta_1 \text{ mRNA (copies/neuron)} \]

\[ \text{Nav1.7 mRNA (copies/neuron)} \]

<25 μm

>30 μm

B

\[ \beta_2 \text{ mRNA (copies/neuron)} \]

\[ \text{Nav1.7 mRNA (copies/neuron)} \]

C

\[ \beta_3 \text{ mRNA (copies/neuron)} \]

\[ \text{Nav1.7 mRNA (copies/neuron)} \]

D

\[ \beta_4 \text{ mRNA (copies/neuron)} \]

\[ \text{Nav1.7 mRNA (copies/neuron)} \]
Figure 3

Differential expression of β subunits in DRG neurons
Differential expression of β subunits in DRG neurons

Figure 4
Figure 5

Differential expression of β subunits in DRG neurons

A

B

C

D

Nav1.7

Nav1.7-β1

Nav1.7-β3

2 nA

2 ms

2 nA

2 ms

-100 -80 -40 -20 20 40 60

-1000

-800

-400

-200

200

mV

pA/pF

-1000

-800

-400

-200

200

-400

-200

-100

-80

-40

-20

20

40

60
Figure 6

A

Voltage (mV)

Normalized Current

-120 -100 -80 -60 -40 -20 0

-1.0 -0.8 -0.6 -0.4 -0.2 0.0

Nav1.7

$\beta_1$

$\beta_2$

$\beta_3$

$\beta_4$

B

Recovery Interval (ms)

Normalized Current

0.1 1 10 100 1000

0.0 0.2 0.4 0.6 0.8 1.0

C

Voltage (mV)

Probability

-100 -80 -60 -40 -20 0 20

0.0000 0.0005 0.0010 0.0015 0.0020 0.0025 0.0030

Nav1.7

$\beta_1$

$\beta_3$