Regulation of Na\textsubscript{1.6} and Na\textsubscript{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{1.6} and Na\textsubscript{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{1.6} and Na\textsubscript{1.8}) expressed in dorsal root ganglion (DRG) neurons. Single cell RT-PCR was used to show that Na\textsubscript{1.8}, Na\textsubscript{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{1.6} and Na\textsubscript{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{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{1.8} channels. The β\textsubscript{3}-subunit did not alter Na\textsubscript{1.8} gating but significantly reduced the current density by 31%. This contrasted with Na\textsubscript{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{1.6}. The β-subunits differentially regulated the expression and gating of Na\textsubscript{1.6} and Na\textsubscript{1.8}. 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{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{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{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; Vijayaragavan et al. 2001). The TTX-sensitive Na\textsubscript{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}-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}- and β\textsubscript{3}-subunits interact noncovalently with the α-subunit, whereas the β\textsubscript{2}- and β\textsubscript{4}-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{1.7} or Na\textsubscript{1.8} in Xenopus oocytes accelerates current decay kinetics, negatively shifts steady-state curves, and significantly enhances the expression of Na\textsubscript{1.8}. In addition, Na\textsubscript{1.8} + β\textsubscript{1} 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; Vijayaragavan 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 hyperexcitable 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 cerebral 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{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).
The β₃-subunit is expressed at high levels in small-diameter DRG neurons and in the II/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 β₃-subunit mRNA and protein levels are upregulated in various neuropathic pain models (Shah et al. 2001; Takahashi et al. 2003). Furthermore, β₂-subunit mutations are associated with early-onset lone atrial fibrillation, and β₂-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 β₂-subunit has been reported to induce negative shifts in the activation of several Na⁺ channel subtypes, including Na₁,1,1, Na₁,2,1, and Na₁,4, and Na₁,6, indicating that the β₂-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. 2008). 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 (Gricco 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- and β₂-subunits, whereas only ~10% express the β₃-subunit. We investigated how these β-subunits modulate the expression and gating properties of two different Na⁺ channel subtypes: Na₁,6 and Na₁,8. We demonstrate that the β₂-subunit induces a significant increase in the current density of Na₁,8 but has no effect on the current density of Na₁,6. In addition, the COOH-terminal domain of the β₁-subunit is involved in the modulation of the Na₁,8 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 harvested from all accessible levels. 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 NaHCO₃, 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 MgCl₂, 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-, and β₃-subunits. Primer sets were designed to span one or more exo-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/1 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. The 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 (QiEx II, Qiagen) and sequenced.

**Gene transfections and cell cultures.** Two human embryonic kidney (HEK)-293 cell lines stably expressing human Na₁,6 and rat Na₁,8 were used. Both HEK-293 cell lines were grown under 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). 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, and piERS/CD8). HEK-293 cell lines stably expressing Na₁,6, or Na₁,8 were transiently transfected with the same amount of individual β₁-β₂-subunits or empty vector pIRES/CD8 DNA. Transfected cells 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/β 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 (β₁β₂, β₂β₁, and β₁β₁) and COOH-terminal deletion variant (β₁ΔNa₁,8 and Na₁,8 REGULATION BY β-SUBUNITS 609) were generous gifts from Dr. Thomas Zimmer (Institute of Physiology II, Friedrich Schiller University, Jena, Germany). β₁β₂ contains the extracellular domain of the β₂-subunit and the transmembrane and intracellular domains of the β₁-subunit. β₂β₁ contains the extracellular and transmembrane domain of the β₂-subunit and the intracellular domain of the β₁-subunit. β₁ΔNa₁,8 contains the extracellular and transmembrane domains of the β₁-subunit and the intracellular domain of the β₂-subunit. β₁ΔNa₁,8 contains only the extracellular and transmembrane domains of the β₁-subunit. The 41 amino acid residues that form the COOH-terminal tail were deleted (see Fig. 6A). β₁β₂, β₂β₁, and β₁β₁ were constructed in the pIRES vector for expression in mammalian cells (piERS/CD8/β₁β₂, piERS/CD8/β₂β₁, and piERS/CD8/β₁β₁) and were individually transfected into the Na₁,8-expressing HEK-293 cell line. Transient transfections of the β₁/β₂-subunit chimeras and the COOH-terminal deletion variant were performed using the calcium phosphate method as previously described (Zhao et al. 2007). Transfected cells were identified for patch-clamp recordings by staining with anti-CD8 antibody (Dynabeads M450 CD8-a).
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 CsOH. 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 were 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 (Gₖ) was calculated from the peak current (Iₚ) using the following equation: 
\[ G_k = \frac{I_p}{V - E_k} \]
where \( V \) is the test potential and \( E_k \) 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 current and plotted against the conditioning pulse potential. Steady-state activation and inactivation curves were fit to a Boltzmann equation of the following form: 
\[ \frac{G}{G_{\text{max}}} = \frac{1}{1 + \exp(V_{1/2} - V)/k_v} \]
where \( G \) is conductance, \( G_{\text{max}} \) is maximal conductance, \( I \) is peak current, \( I_{\text{max}} \) is maximal current, \( V_{1/2} \) is the voltage at which the channels are half-maximally activated or inactivated, and \( k_v \) 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 noninactivated and available for activation. Using the \( V_{1/2} \) and \( k_v \) values of voltage-dependent activation and inactivation, the probability of Na⁺ channels being within the window was calculated using the following equation: 
\[ P = \frac{1}{1 + \exp(V_{1/2} - V)/k_v} \]

Analysis of electrophysiological data. Data were analyzed using a combination of pCLAMP software (version 9.0, Molecular Devices), Microsoft Excel, and SigmaPlot for Windows (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 Na⁺,1.8 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 Na⁺ 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 Na⁺,1.7, Na⁺,1.8, and Na⁺,1.9 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 \( \beta_1 \), \( \beta_2 \), or \( \beta_3 \)-subunit. Only a small percentage (17%) expressed the \( \beta_4 \)-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 Na1+ channel β-subunits on the expression of Na1.8, we transiently transfected individual β1–β4 subunits or empty vector into HEK-293 stable cells transiently 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 β4-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–β4 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- and β4-subunits. For the β1-subunit group, the Boltzmann analysis of Na1.8 conductance yielded a V1/2 of activation of −16.5 ± 1.4 mV (n = 18), which was 4.0 mV more negative than that of the empty vector control group (−12.5 ± 1.7 mV, n = 15, P < 0.01). 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 increased by 10.2 ± 3.2 mV compared with the control (−16.5 ± 1.4 mV, n = 18).

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 by 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.
produced a significant -16.7-mV shift (-29.2 ± 1.7 mV, n = 14, P < 0.01).

To investigate the effects of β1-β4 subunits on steady-state Na1.8 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 β1-β4-subunits were coexpressed with Na1.8, the β1- and β4-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 Na1.8 was coexpressed with β2 or β3-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 β1- and β4-subunits should induce alterations in window currents. Figure 3D shows that the Na1.8 channels were probably within this window, that is, were inactivated and were available for activation when coexpressed with β1- or β4-subunits. Compared with the empty vector control, the small but significant hyperpolarization caused by the β1-subunit shortened the window, indicating a lower probability of Na1.8 being activated. The peak probability shifted to a more depolarized potential, whereas the β4-subunit slightly enhanced the probability, with a peak probability of 2.8% at -41 mV and a larger fraction

Fig. 3. Whole cell Na1.8 currents in HEK-293 stable cells transiently transfected with individual β1-β4-subunits or empty vector. A: current-voltage relationship of Na1.8 transfected with individual β1 (n = 18), β2 (n = 21), β3 (n = 19), or β4 (n = 14)-subunits or empty vector (n = 15). Current densities were measured by normalizing current amplitudes to the membrane capacitance and were plotted versus voltage. The experimental protocol is the same as in Fig. 2A. B: histogram showing the average current densities of Na1.8 coexpressed with different β-subunits or empty vector. When cells were depolarized to 0 mV, coexpression with the β1-subunit produced a 2.3-fold increase in Na1.8 current density compared with the empty vector control (***P < 0.01). The β2- and β3-subunits caused no increase in Na1.8 current density (P > 0.05), whereas the β4-subunit induced a 30% decrease in Na1.8 current density (***P < 0.01). C: composite figure showing the steady-state activation and inactivation of Na1.8 coexpressed with individual β1-β4-subunits or empty vector. For clarity, we only show the symbols of the empty vector control group and the group that exhibited a significant difference, and we removed the symbols of the other groups. As such, only the symbols for the β1, β2, and control groups are shown, and the symbols for the β3 and β4 groups have been removed. Activation curves were generated using the same protocol as in Fig. 2A. Coexpression of β1- or β4-subunits with Na1.8 induced a hyperpolarizing shift of the activation curve from -15 to 10 mV for the β1 group (P < 0.01 or P < 0.05) and from -50 to 10 mV (P < 0.01) for the β4 group. Steady-state inactivation was determined using 20-ms test pulses to 15 mV after 500-ms prepulses to potentials ranging from -140 to 5 mV (see the inset under the inactivation curves for the protocol). Like voltage-dependent activation, coexpression of β1- or β4-subunits also caused a hyperpolarizing shift of the inactivation curves from -70 to -60 mV for the β1-subunit (P < 0.01 or P < 0.05) and from 0 to -30 mV for the β4-subunit (P < 0.01 or P < 0.05). Both the activation and inactivation curves were fitted to a single Boltzmann function (see MATERIALS AND METHODS). The midpoints (V1/2) and slope factors (k) of the activation and inactivation curves are shown in Table 1. D: effects of β1- and β4-subunits on the Na1.8 window current. The activation and inactivation curves of the Na+ channels overlap at a (relatively narrow) distinct voltage range (window), predicting a steady Na+ conductance over this range. The probability of Na1.8 channels being within this window was calculated using the equation given in MATERIALS AND METHODS. The β4-subunit decreased the window current, and the peak probability shifted to a more depolarized potential, whereas the β2-subunit slightly increased the window current of Na1.8.
(0.81%) of Nav1.8 channels that would be activated near the resting membrane potential.

Effect of $\beta_1$-$\beta_4$-subunits on the expression and gating properties of Nav1.6 in HEK-293 stable cells. To study the specificity of $\beta$-subunit regulation, we investigated the effect of $\beta_1$-$\beta_4$ subunits on the expression of Nav1.6 in the HEK-293 stable cell line. We transiently transfected individual $\beta_1$-$\beta_4$-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$\text{v}_1.6$

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<td>Nav1.8</td>
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<td>+$\beta_4$</td>
<td>$-29.2 \pm 1.7^\dagger$</td>
</tr>
<tr>
<td></td>
<td>+Empty vector</td>
<td>$-12.5 \pm 1.7$</td>
</tr>
<tr>
<td>Nav1.6</td>
<td>+$\beta_1$</td>
<td>$-34.8 \pm 1.7$</td>
</tr>
<tr>
<td></td>
<td>+$\beta_2$</td>
<td>$-35.3 \pm 2.6$</td>
</tr>
<tr>
<td></td>
<td>+$\beta_3$</td>
<td>$-37.3 \pm 1.1$</td>
</tr>
<tr>
<td></td>
<td>+$\beta_4$</td>
<td>$-44.3 \pm 0.5^\dagger$</td>
</tr>
<tr>
<td></td>
<td>+Empty vector</td>
<td>$-36.7 \pm 1.1$</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$\text{v}_1.8$ channels are half-maximally activated or inactivated; $k_v$, slope factor. $^*P < 0.05$ and $^\dagger P < 0.01$ compared with the control (+empty vector) group.

Fig. 4. Representative Na$\text{v}_1.6$ current traces in HEK-293 cells transiently transfected with individual $\beta_1$ (A)-, $\beta_2$ (B)-, $\beta_3$ (C)-, or $\beta_4$ (D)-subunits or empty vector (E) and normalized by membrane capacitance. The inset in A shows the protocol. Na$\text{v}_1.6$ currents were evoked with depolarizing voltage steps from $-100$ to $90$ mV in $5$-mV increments for $50$ ms at a holding potential of $-140$ mV.
Fig. 5. Whole cell Na\textsubscript{1.6} currents in HEK-293 stable cells transiently transfected with individual \(\beta_1\)-\(\beta_4\)-subunits or empty vector. A: current-voltage relations of Na\textsubscript{1.6} currents were obtained using the same protocol as in Fig. 4A. For clarity, we removed the overlapping symbols of the \(\beta_1\), \(\beta_2\), and \(\beta_3\)-subunit transfection groups and only kept their corresponding lines (\(\beta_1\): \(n = 9\), \(\beta_2\): \(n = 8\), \(\beta_3\): \(n = 8\), \(\beta_4\): \(n = 11\), and empty vector: \(n = 9\)). The \(\beta_1\)-\(\beta_4\)-subunits did not modulate the expression of Na\textsubscript{1.6} in HEK-293 cells. B: voltage dependence of activation and inactivation of Na\textsubscript{1.6} coexpressed with different \(\beta\)-subunits or empty vector. For clarity, we show only the symbols for the \(\beta_4\) 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 \(\beta_3\)-subunit caused a negative shift in the activation curve from \(-65\) to \(-20\) mV (\(P < 0.01\) or \(P < 0.05\)). However, coexpression of \(\beta_1\)-\(\beta_4\)-subunits did not affect the voltage-dependent inactivation of Na\textsubscript{1.6} in HEK-293 cells. The values of \(V_{1/2}\) and \(k_\alpha\) are shown in Table 1. C: effect of the \(\beta_4\)-subunit on the Na\textsubscript{1.6} window current. The y-axis shows the probability of Na\textsubscript{1.6} 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 Na\textsubscript{1.6} opening within the window and shifted the peak probability in a more hyperpolarizing direction. D: representative resurgent Na\textsuperscript{+} current in HEK-293 cells coexpressing Na\textsubscript{1.6} and the \(\beta_4\)-subunit. The voltage protocol used to elicit the currents is shown under the current trace. HEK-293 cells coexpressing Na\textsubscript{1.6} and the \(\beta_4\)-subunit were depolarized from a holding potential of \(-120\) mM by a prepulse to \(30\) mV for \(40\) ms to activate and inactivate Na\textsuperscript{+} 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 Na\textsubscript{1.6} and the \(\beta_4\)-subunit.

currents was approximately \(-60\) mV, and the maximum inward currents ranged from \(-25\) to \(-15\) mV. The reversal potential was \(-85\) mV in the five groups, which was in good agreement with the calculated value (86.2 mV). Coexpression of \(\beta_1\)-\(\beta_4\)-subunits with Na\textsubscript{1.6} in HEK-293 cells failed to increase the current densities of Na\textsubscript{1.6} compared with the empty vector control (Na\textsubscript{1.6} + \(\beta_1\): \(-127.0 \pm 11.8\) pA/pF, \(n = 9\); Na\textsubscript{1.6} + \(\beta_2\): \(-127.2 \pm 8.7\) pA/pF, \(n = 8\); Na\textsubscript{1.6} + \(\beta_3\): \(-125.8 \pm 13.1\) pA/pF, \(n = 8\); Na\textsubscript{1.6} + \(\beta_4\): \(-124.6 \pm 13.4\) pA/pF, \(n = 11\); and control: \(-120.5 \pm 14.9\) pA/pF, \(n = 9\)).

Figure 5B is a composite figure showing the steady-state activation and inactivation curves of Na\textsubscript{1.6}. Coexpression of \(\beta_1\)-, \(\beta_2\)-, or \(\beta_3\)-subunits with Na\textsubscript{1.6} did not cause a shift in the activation and inactivation curves compared with the empty vector control group. However, the \(\beta_4\)-subunit induced a 7.6-mV hyperpolarized shift in the \(V_{1/2}\) value of Na\textsubscript{1.6} activation but did not appreciably alter the voltage dependence of inactivation. As such, only the \(\beta_4\)-subunit appeared to regulate Na\textsubscript{1.6} voltage dependence in HEK-293 cells. The leftward shift of steady-state activation of Na\textsubscript{1.6} caused by the \(\beta_4\)-subunit produced an enhanced window current, as shown in Fig. 5C. Compared with the empty vector control, coexpression of the \(\beta_4\)-subunit increased the opening probability of Na\textsubscript{1.6} within the window (\(\beta_4\): 0.26% and control: 0.15%). The peak probability of the \(\beta_4\)-subunit group was shifted in a more hyperpolarizing direction (\(\beta_4\): \(-60\) mV and control: \(-50\) mV), with an increased fraction of Na\textsubscript{1.6} channels that would be activated near the resting membrane potential (\(\beta_4\): 0.08% and control: 0.045%).

When Na\textsuperscript{+} channels open transiently during recovery from inactivation, they generate a “resurgent current,” which has been reported to be carried mainly by Na\textsubscript{1.6} \(\alpha\)-subunits. The cytoplasmic tail of the \(\beta_4\)-subunit may be the endogenous open channel blocker responsible for the production of resurgent currents (Grieco et al. 2005; Raman and Bean 1997). However, we did not detect a resurgent current in Na\textsubscript{1.6}-expressing HEK-293 cells coexpressing the \(\beta_4\)-subunit (Fig. 5D) or empty vector (data not shown). Similarly, no resurgent current was observed in Na\textsubscript{1.8}-expressing HEK-293 cells cotransfected with the \(\beta_4\)-subunit (data not shown).
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

![Diagram](image-url)
the β₁-subunit intracellular domain was sufficient to modulate the current density of Nav1.8, chimeras β⁺₂⁺ and β₁⁺β₂ were expressed with Nav1.8. β⁺₂ increased the current density of Nav1.8 (−152.7 ± 16.0 pA/pF, n = 9, P < 0.01). Current densities of the β⁺₂ and β⁺β₂ 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 Nav1.8 was observed in the β⁺β₁⁺ and β⁺β₁⁺ groups, which contain the NH₂-terminus and transmembrane domain of the β₁-subunit but lack the COOH-terminus of the β₁-subunit (β⁺β₁⁺: −81.5 ± 9 pA/pF, n = 14, and β⁺β₁⁺: −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 Nav1.8 observed in the wild-type β₁, β⁺₂, and β⁺β₂ groups is due to the COOH-terminal region of the β₁-subunit.

To determine whether the increase in the peak current densities of Nav1.8 caused by COOH-terminal of the β₁-subunit was accompanied by changes in the biophysical properties of Nav1.8, 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, β⁺₂, and β⁺β₂ groups produced significant negative shifts in the V½ 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 V½ 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, β⁺₂, and β⁺β₂ groups.

DISCUSSION

The main goal of the present study was to investigate the regulation of Nav1.6 and Nav1.8 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 Nav1.6 and Nav1.8 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 Nav1.8 by auxiliary β-subunits. The single cell RT-PCR analysis of acutely dissociated DRG neurons indicated that Nav1.8 channels are widely expressed in small-diameter neurons (Fig. 1B), which is consistent with recent work showing that the TTX-resistant Na⁺ channel is highly expressed in unmyelinated C-fibers (Ho and O’Leary 2010). These neurons also express β₁-, β₂-, and β₃-subunits, raising the possibility that these auxiliary subunits may associate with and regulate endogenous Nav1.8 channels expressed in these neurons. To further investigate this potential regulation, Nav1.8 and β-subunits were coexpressed in HEK-293 cells, and the biophysical properties of the channel complexes were assessed using an electrophysiology approach. In the absence of auxiliary subunits, heterologously expressed Nav1.8 channels produced robust Na⁺ currents that activated at relatively depolarized voltages (−50 mV) and displayed properties similar to the dominant TTX-resistant Na⁺ current of DRG neurons (Zhao et al. 2007). Coexpression of the β₁-subunit increased the current density twofold and produced hyperpolarizing shifts in activation (−4 mV) and steady-state inactivation (−4.6 mV). These shifts produced a twofold reduction in the window current, resulting in a decrease in the fraction of Nav1.8 channels predicted to be open at voltages near the resting membrane potential (approximately −65 mV) of sensory neurons (Zhao et al. 2007). Our results indicated that Nav1.8 and the β₁-subunit are coexpressed in the same population of DRG sensory neurons and that the association with the β₁-subunit increases the cell surface expression and alters the gating of the channels. Coexpression of the β₁-subunit did not alter Nav1.8 gating but produced a 31% decrease in the peak current density (Fig. 3B). Whereas β₂ subunits were widely expressed in small-diameter DRG neurons (47%), this auxiliary subunit did not significantly alter the expression or gating of heterologously expressed Nav1.8 channels. The β₁β₂-subunit produced dramatic shifts in activation (−16.7 mV) and inactivation (−9.3 mV), resulting in an increase in the window current. These changes predicted that Nav1.8 + β₁β₂ channels would activate at more hyperpolarized membrane potentials and would increase the likelihood of a persistent Na⁺ current at more hyperpolarizing voltages. β₁-subunit regulation may increase the excitability of DRG neurons expressing this combination of subunits. However, unlike the β₁-subunit, the β₁β₂-subunit was expressed in a relatively small percentage (17%) of small-diameter sensory neurons. This suggested that, although β₁-subunits are potent regulators of Nav1.8 channels, they are expressed in a small subpopulation of unmyelinated C-fibers.

Regulation of Nav1.6 by auxiliary β-subunits. Nav1.6 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 Nav1.6 channels generate a rapidly inactivating TTX-sensitive current that activates at a relatively hyperpolarized (−60 mV) voltage (Fig. 5A). The β₁-subunit and Nav1.6 have reciprocal functions, such that β₁-subunit-mediated neurite outgrowth requires Na⁺ current carried by Nav1.6, and the β₁-subunit is required for normal expression/high-frequency action potential firing of Nav1.6 at the axon initial segment (Brackenbury et al. 2010). Our results showed that coexpression of β₂-subunits (β₁β₂β₃ does not alter the peak current density or voltage-dependent gating of heterologously expressed Nav1.6 channels (Fig. 5). The sole exception was the β₁-subunit, which produced a hyperpolarizing shift (−7.6 mV) in Nav1.6 activation. This may be significant because it resulted in a twofold increase in the window current and shifted Nav1.6 activation into a range of voltages considered to be near the resting membrane potential of sensory neurons. These changes may increase the excitability of Nav1.6 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 Nav1.8 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 Na1,2 channel indicated that the NH2-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 NH2-terminal domain of the \(\beta_1\)-subunit has been previously postulated for the skeletal muscle Na1,4 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 Na1,2 and Na1,4 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 Na1,2 regulation (Meadows et al. 2001). Current evidence suggests that both NH2- 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 Na1,5 channel, where the membrane-spanning domain coupled with secondary interactions with either the NH2- or COOH-terminal of the \(\beta_1\)-subunit were reported to be required for Na1,5 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 Na1,8 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 Na1,8 channels. The observed changes in expression and gating were not altered by replacing the extracellular NH2-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 \(\beta_3\)-subunit and neuronal Na1,1 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 Na1,8 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 \textit{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 Na1,2 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 Na1,2 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 Na1,2 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 \textit{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 Na1,8 channels by auxiliary \(\beta\)-subunits. Early work examining Na1,8 channels expressed in \textit{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 Na1,8 channels (Vijayaragavan et al. 2004). A similar \(\beta_1\)-subunit-induced shift in activation was reported for the heterologously expressed human Na1,8 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 Na1,8 channels expressed in mammalian cells (Zhao et al. 2007).

There are conflicting results concerning the regulation of Na1,8 channels by the \(\beta_3\)-subunit. Coexpression of the \(\beta_3\)-subunit in oocytes results in an increase in Na1,8 current and a hyperpolarizing shift in activation (Shah et al. 2000). This contrasts with previous work showing that coexpression of the \(\beta_3\)-subunit in oocytes produces a depolarizing shift in Na1,8 inactivation but no change in current density (Vijayaragavan et al. 2004). \(\beta_3\)-Subunits have been reported to directly bind to

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**Table 2. Effect of \(\beta_1/\beta_2\)-subunit chimeras and \(\beta_1\) COOH-terminal deletion mutant on the activation and inactivation of Na1,8 channels in HEK-293 cells**

<table>
<thead>
<tr>
<th></th>
<th>(V_{1/2}) mV</th>
<th>(n)</th>
<th>(k_v) mV</th>
<th>(n)</th>
<th>(V_{1/2}) mV</th>
<th>(n)</th>
<th>(k_v) mV</th>
<th>(n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na1,8 (+) (\beta_1)</td>
<td>(-17.7 \pm 1.8^*)</td>
<td>14</td>
<td>(-8.1 \pm 0.6^\dagger)</td>
<td>14</td>
<td>(-47.7 \pm 1.5^*)</td>
<td>13</td>
<td>7.8 (\pm 0.5^\dagger)</td>
<td>13</td>
</tr>
<tr>
<td>Na1,8 (+) (\beta_2)</td>
<td>(-14.4 \pm 1.2)</td>
<td>13</td>
<td>(-9.4 \pm 0.7)</td>
<td>13</td>
<td>(-45.9 \pm 0.9)</td>
<td>11</td>
<td>6.5 (\pm 0.5)</td>
<td>11</td>
</tr>
<tr>
<td>Na1,8 (+) (\beta_{111})</td>
<td>(-17.3 \pm 1.5^*)</td>
<td>14</td>
<td>(-7.9 \pm 0.3^\dagger)</td>
<td>14</td>
<td>(-48.1 \pm 2.2^*)</td>
<td>14</td>
<td>7.1 (\pm 0.3^\dagger)</td>
<td>14</td>
</tr>
<tr>
<td>Na1,8 (+) (\beta_{211})</td>
<td>(-19.1 \pm 1.1^*)</td>
<td>9</td>
<td>(-8.2 \pm 0.5^\dagger)</td>
<td>9</td>
<td>(-49.0 \pm 1.2^*)</td>
<td>12</td>
<td>7.0 (\pm 0.4^*)</td>
<td>12</td>
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<tr>
<td>Na1,8 (+) (\beta_{12})</td>
<td>(-14.5 \pm 2.0)</td>
<td>14</td>
<td>(-9.0 \pm 0.5^*)</td>
<td>14</td>
<td>(-44.7 \pm 1.4)</td>
<td>12</td>
<td>6.6 (\pm 0.6)</td>
<td>12</td>
</tr>
<tr>
<td>Na1,8 (+) (\beta_{112})</td>
<td>(-15.7 \pm 2.1)</td>
<td>16</td>
<td>(-8.9 \pm 0.5^*)</td>
<td>16</td>
<td>(-47.3 \pm 1.3)</td>
<td>15</td>
<td>7.3 (\pm 0.6^\dagger)</td>
<td>15</td>
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</table>

Values are means \(\pm\) SE; \(n\), number of cells. \(*P < 0.05\) and \(\dagger P < 0.01\) compared with the Na1,8 + empty vector group (values are shown in Table 1).
Na,1.8 channels via the COOH-terminus of the β3-subunit and to help translocate Na,1.8 from the endoplasmic reticulum to the plasma membrane (Zhang et al. 2008). However, in the present study, coexpression of the β3-subunit in mammalian cells produced a significant 31% decrease in Na,1.8 current density (Fig. 3B). This result is in agreement with a previous study showing that β3-subunits do not improve the functional expression of Na,1.8 in COS-7 cells (Swanwick et al. 2010). Collectively, these findings suggest that coexpression of the β3-subunit either has no effect or reduces Na,1.8 current without altering voltage dependence or gating kinetics. The role of the β3-subunit in neuropathic pain is closely associated with Na,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 β3-subunit depolarizes the voltage-dependent activation and inactivation of Na,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 β2- and β3-subunits share 35% sequence similarity, and both subunits covalently associate with Na+ channels via disulfide bonds (Yu et al. 2003). In the present study, the β2-subunit did not alter the expression, kinetics, or voltage dependence of Na,1.8 channels (Table 1). This is in good agreement with previous work showing that the β2-subunit does not regulate Na,1.8 channels expressed in Xenopus oocytes (Vijayaragavan et al. 2004). In contrast, the β3-subunit produced hyperpolarizing shifts in the activation of Na,1.8 and Na,1.6 channels. Similar changes in activation have been reported for Na,1.1, Na,1.2, Na,1.4, and Na,1.6 channels coexpressed with β3-subunits (Chen et al. 2008; Yu et al. 2003; Aman et al. 2009). The hyperpolarizing shift in activation produced by the β3-subunit expanded the predicted Na,1.8 window current (Fig. 3D) and increased the likelihood of Na,1.8 activation and persistent TTX-resistant Na+ currents at hyperpolarized voltages. While the COOH-terminal domain of the β3-subunit has been proposed to act as the endogenous open channel blocker of Na+ channels (Grieco et al. 2005), we could not detect a reduction in current density or resurgent currents when Na,1.6 and Na,1.8 channels were coexpressed with the β3-subunit.

Conclusions. The present study examined the functional regulation of neuronal Na,1.6 and sensory neuron-specific Na,1.8 channels by auxiliary β-subunits. Single cell RT-PCR revealed that the Na,1.8 channel and several β-subunits (β1, β2, and β3) were coexpressed in the same population of small-diameter neurons. The high level expression of TTX-resistant Na+ currents and the preferential expression of Na,1.7, Na,1.8, and Na,1.9 transcripts in these neurons were consistent with what has been reported for unmethylated C-fibers. Association with the β3-subunit increased Na+ current density and produced shifts in gating, leading to Na,1.8 activation at voltages near the resting membrane potential of sensory neurons. The predicted increase in TTX-resistant Na+ currents could have important implications for the electrical excitability of sensory neurons. Previous work has shown that β3-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,1.8 channels and β1-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,1.8 also express transcripts coding for the β1-subunit, which provides support for this possibility. Further studies are required to determine whether these β3-subunits are associated with Na,1.8 channels and whether they regulate the expression and gating of Na,1.8 channels in these neurons. (Table 2).

GRANTS

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DISCLOSURES

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

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