Calmodulin Regulates Current Density and Frequency-Dependent Inhibition of Sodium Channel Nav1.8 in DRG Neurons

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Choi, Jin-Sung, Andy Hudmon, Stephen G. Waxman, and Sulayman D. Dib-Hajj. Calmodulin regulates current density and frequency-dependent inhibition of sodium channel Na\textsubscript{v}1.8 in DRG neurons. J Neurophysiol 96: 97–108, 2006. First published April 5, 2006; doi:10.1152/jn.00854.2005. Sodium channel Na\textsubscript{v}1.8 produces a slowly inactivating, tetrodotoxin-resistant current, characterized by recovery from inactivation with fast and slow components, and contributes a substantial fraction of the current underlying the depolarizing phase of the action potential of dorsal root ganglion (DRG) neurons. Na\textsubscript{v}1.8 C-terminus carries a conserved calmodulin-binding isoleucine–glutamine (IQ) motif. We show here that calmodulin coimmunoprecipitates with endogenous Na\textsubscript{v}1.8 channels from native DRG, suggesting that the two proteins can interact in vivo. Treatment of native DRG neurons with a calmodulin-binding peptide (CBP) reduced the current density of Na\textsubscript{v}1.8 by nearly 65%, without changing voltage dependency of activation or steady-state inactivation. To investigate the functional role of CaM binding to the IQ motif in the Na\textsubscript{v}1.8 C-terminus, the IQ dipeptide was substituted by DE; we show that this impairs the binding of CaM to the IQ motif. Mutant Na\textsubscript{v}1.8IQ/DE channels produce currents with roughly 50% amplitude, but with unchanged voltage dependency of activation and inactivation when expressed in DRG neurons from Na\textsubscript{v}1.8-null mice. We also show that blocking the interaction of CaM and Na\textsubscript{v}1.8 using CBP or the IQ/DE substitution causes a buildup of inactivated channels and, in the case of the IQ/DE mutation, stimulation even at a low frequency of 0.1 Hz significantly enhances the frequency-dependent inhibition of the Na\textsubscript{v}1.8 current. This study presents, for the first time, evidence that calmodulin associates with a sodium channel, Na\textsubscript{v}1.8, in native neurons, and demonstrates a regulation of Na\textsubscript{v}1.8 currents that can significantly affect electrogensis of DRG neurons in which Na\textsubscript{v}1.8 is normally expressed.

INTRODUCTION

Voltage-gated sodium channels (VGSCs) are major contributors to action potential electrogensis, and their modulation can regulate the function of excitable cells. Within the VGSC family, Na\textsubscript{v}1.8 channels are abundantly expressed in small neurons of dorsal root ganglion (DRG) (Akopian et al. 1996), most of which are nociceptive (Djouhri et al. 2003). Recent studies have provided strong evidence for an important role of Na\textsubscript{v}1.8 in inflammatory and neuropathic pain (Wood and Waxman 2005; Wood et al. 2004). Na\textsubscript{v}1.8 produces a slowly inactivating sodium current, characterized by depolarized voltage dependency of activation (Akopian et al. 1996, 1999; Sangameswaran et al. 1996), and rapid recovery from fast inactivation (Cummins and Waxman 1997; Elliott and Elliott 1993). Na\textsubscript{v}1.8 channels produce the majority of the inward current during the action potential upstroke in the DRG neurons in which they are present (Blair and Bean 2002; Renganathan et al. 2001) and contribute to the adaptation of firing induced by sustained stimulation, such as prolonged exposure to capsaicin (Blair and Bean 2003). Mechanisms that regulate current density and biophysical properties of Na\textsubscript{v}1.8 are therefore a major focus in the investigations of this channel’s role in nociception.

Calmodulin (CaM) binds directly to ion channel target proteins by the isoleucine–glutamine (IQ) motif (Rhoads and Friedberg 1997), in a Ca\textsuperscript{2+}-independent manner (Erickson et al. 2001; Kim et al. 2004; Mori et al. 2000; Xia et al. 1998), and has been shown to regulate current density and biophysical properties of sodium channels (Deschenes et al. 2002; Herzog et al. 2003b; Kim et al. 2004; Tan et al. 2002; Young and Caldwell 2005). The C-terminus of all VGSC contains a conserved IQ motif (Herzog et al. 2003b; Mori et al. 2000; Rhoads and Friedberg 1997). CaM has been shown to regulate current densities and gating properties of several sodium channels (Nav1.2, Nav1.4, Nav1.5, and Nav1.6) but, importantly, this channel modulation is isoform dependent (Deschenes et al. 2002; Herzog et al. 2003b; Kim et al. 2004; Tan et al. 2002; Young and Caldwell 2005). For example, the substitution of IQ with glutamic acid residues (EE) in Na\textsubscript{v}1.6, which blocks CaM binding, causes a hyperpolarizing shift of steady-state inactivation and a twofold slower fast inactivation, whereas these properties remain unchanged in Na\textsubscript{v}1.4IQ/EE (Herzog et al. 2003b). Additionally, the effect of CaM binding on Na\textsubscript{v}1.4 varies in HEK293 and CHO cell lines, suggesting a contribution of cell type–specific factors (Young and Caldwell 2005). Therefore it is important to investigate the effects of CaM on sodium channels in their native neurons.

Genetic assays previously showed that CaM can bind to the C-terminus of Na\textsubscript{v}1.8 in yeast (Malik-Hall et al. 2003). In this study, we investigated the ability of CaM to bind Na\textsubscript{v}1.8 by the IQ motif in the channel’s C-terminus and to the full-length channel in vivo, and to modulate the properties of this channel in native DRG neurons. We report here for the first time that CaM can associate with Na\textsubscript{v}1.8 channels in native neurons and show that CaM regulates the current density and the ability of this channel to respond to high-frequency depolarizations in DRG neurons.
METHO D S

Plasmids and antibodies

The plasmid pRK-Na,1.8 was a gift from Dr. John Wood (University College, London) and the CaM bacterial expression plasmid pSGC02, a generous gift from Drs. Geoffrey S. Pitt and Smita Ghosh (Columbia University, New York). The plasmid pEGFP, which encodes green fluorescent protein, was purchased from Clontech (Palo Alto, CA). The 1<sup>839</sup>Q<sup>840</sup>/DE substitution in the IQ motif of Na<sub>1.8</sub> was introduced by site-directed mutagenesis using the QuickChange XL system (Stratagene, La Jolla, CA). Na<sub>1.8</sub>-specific polyclonal antibody was purchased from Alomone Labs (Jerusalem, Israel). We verified the specificity of the Na<sub>1.8</sub> antibody on immunoblots of brain, spinal cord, and DRG tissues. A single Na<sub>1.8</sub> immunoreactive protein was observed in DRG samples, which was absent from brain tissue and was reduced in samples of axotomized DRG compared with control (data not shown). KS5/35 pan sodium channel antibody was purchased from Sigma (St. Louis, MO). Monoclonal calmodulin antibody was purchased from Upstate (Lake Placid, NY). Secondary antibody goat anti-mouse HRP conjugated immunoglobins (IgGs) was purchased from Dako (Hamburg, Germany). Rabbit polyclonal anti-6x-His was from Santa Cruz Biotechnology (Santa Cruz, CA).

Immunoprecipitation

Lumbar (L2–L6) DRGs were collected from two young adult male Sprague–Dawley rats (250–275 g) and homogenized in 0.5 ml buffer (200 mM NaCl, 20 mM Tris, pH 7.4) supplemented with 1% Triton X-100 and complete protease inhibitor cocktail containing 1 mM EDTA (Roche, Indianapolis, IN). The lysate was incubated on ice for 30 min and the Triton X-100 soluble proteins were collected in the supernatant after centrifugation for 20 min at 15,000 × g at 4°C. The insoluble material was resuspended and again subjected to homogenization and extraction as above. Supernatants were combined and precleared using 40 μl of a 50% slurry of Protein-A agarose (Upstate) for 1 h at room temperature. The DRG-cleared lysate was collected and incubated with 2 μg of Na<sub>1.8</sub>-specific polyclonal antibody (Alamone Labs) for 2–3 h before 40 μl of a 50% slurry of Protein-A agarose was added and the mixture continued to incubate for an additional 1.5 h at 4°C. The beads were collected, washed twice in (0.4 ml) each of lysis buffer (except 0.1% Triton X-100 was used), followed by two washes (0.4 ml) each in lysis buffer without Triton X-100. Protease inhibitors and 1 mM EDTA were included throughout the washes. The beads were immediately suspended in 40 μl of 2 × SDS sample buffer and denatured for 30 min at 37°C. All reagents used in protein electrophoresis were designed around the NuPAGE electrophoresis system (Invitrogen, Carlsbad, CA). The samples used for sodium channel analysis were subjected to SDS-PAGE on 4–12% gradient gels (1.5 h at 200 mM Tris, pH 7.4, 150 mM NaCl, 0.5% bovine serum albumin, and 0.2% Tween-20) before addition of 1:1,000 primary monoclonal antibodies [monoclonal anti-calmodulin (Upstate) or KS5/35 pan sodium channel antibody (Sigma)] in TBST. The membranes were incubated for 2 h in primary antibody and washed extensively for 45 min in TBST and incubated to secondary antibody (1:10,000 goat anti-mouse HRP conjugated immunoglobins; Dako). The membranes were washed extensively in TBST and the immunoreactive proteins were detected using chemiluminescence (NEN-PLUS, Perkin–Elmer Life Sciences, Boston, MA) and autoradiography.

Purification of Na<sub>1.8</sub> C-terminus 6X-His-tagged fusion proteins

A 6X-His fusion protein was constructed from the C-terminus of Na<sub>1.8</sub>, amino acids 1724 to 1956 (GenBank accession number U53833) and subcloned in pET-15b vector (Novagen, Madison, WI) using the Not I and Bam HI restriction sites. Substitution of the IQ dipeptide by DE in the consensus CaM binding sequence was accomplished using QuickChange XL site-directed mutagenesis (Stratagene) and confirmed by sequence analysis. Cotransfection with a CaM expression plasmid pSGC02 increased the production of wild-type channel C-terminus fusion protein as previously reported (Kim et al. 2004). BL21 cells (Stratagene) were transformed in the presence of carbenicillin (100 μg/ml) and chloramphenicol (100 μg/ml) to select for both expression plasmids. Large-scale growths were inoculated using an overnight starter culture (1:100) and grown to an OD<sub>600</sub> of 0.6 at 37°C in 2XYT media plus antibiotics. The cells were chilled on ice to <10°C and protein expression induced using 1 mM IPTG. After 4 h at room temperature, the bacteria were collected by centrifugation at 2,500 × g for 10 min, the pellets washed in ice-cold PBS, and frozen at −80°C. Bacterial pellets were resuspended in 20 ml of ice-cold lysis buffer (10 mM Tris, pH 8, 0.1 mM EDTA, 200 mM NaCl) supplemented with protease inhibitor cocktail (Roche) and lysed using a Microfluidics device (Newton, MA). The lysate was incubated on ice for 45 min in 1% Tween-20, briefly sonicated, and centrifuged at 10,000 × g for 20 min to remove insoluble material. The supernatant was diluted with 10 volumes of 50 mM phosphate buffer (pH 8.0 plus 300 mM NaCl and 20 mM imidazole) and applied to NTA-agarose for 2 h at 4°C (Invitrogen). Bound proteins were washed with 10 column volumes of phosphate buffer before elution (250 mM imidazole in phosphate buffer). Positive protein fractions were identified using Bradford and pooled and concentrated using Amicon Ultra 4 (10,000 molecular weight cutoff concentrators; Millipore, Bedford, MA). The purified fusion proteins were applied to a phenyl-Sepharose CL-4B in the presence of Ca<sup>2+</sup> to remove any CaM that copurified with the fusion proteins (Ohya et al. 1987). The proteins were concentrated and desalted (20 mM Tris, 200 mM NaCl, and 0.1 mM EDTA) using an Amicon Ultra 4 concentrator. Fusion proteins were quantified using Bradford (BSA as a standard) and equal protein levels were visually inspected using Coomassie Blue staining.

In vitro binding assays

CaM binding to the purified Na<sub>1.8</sub> C-tail wild-type and IQ/DE mutation was determined using CaM-Sepharose (Stratagene). CaM-Sepharose beads were blocked using 1% BSA and 0.2% 1-block (Tropix, Bedford, MA) and equilibrated in binding buffer (20 mM Tris, 200 mM NaCl, 0.1 mM EDTA, 5 mM EGTA, 0.1% Tween-20, and 0.1% BSA). Total CaM binding was measured using a batch-based pull-down assay. Briefly, similar levels of fusion proteins (4 μg) where added to 750 μl of binding buffer (20 mM Tris, 200 mM NaCl, 0.1 mM EDTA, 5 mM EGTA, 0.1% Tween-20, and 0.1% BSA) containing 20 μl of CaM-Sepharose beads (Stratagene). This binding reaction was incubated at room temperature on a rotating platform for 30 min, the beads collected by low-speed centrifugation, and washed twice in 1 ml of binding buffer minus BSA. The bound fusion proteins were eluted from the CaM-Sepharose beads with 2 × SDS sample buffer and heated to 90°C for 5 min. Electrophoresis was performed using the Novagen gel/transfer system (12% Tris-Bis gels) and MES as the running buffer (Invitrogen). The proteins were transferred to PVDF immobilized membrane (Invitrogen) and the membrane blocked overnight with 5% milk in TBST. After blocking, the membrane were rinsed and exposed to primary rabbit polyclonal anti-6x-His (Santa Cruz) at 1:1000 in TBST plus 0.1% BSA for 2 h at room temperature. Membranes were washed six times for 10 min each and an anti-rabbit goat secondary antibody-coupled to HRP (Dako) was used at 1:10,000.
for 1 h. Membranes were washed as above and the proteins visualized using chemiluminescence (Perkin–Elmer) and autoradiography. CaM binding was quantified using densitometric measurement of band intensity using 1D Image Analysis Software (Eastman Kodak, Rochester, NY). Multiple exposure times ensured linearity in the chemiluminescent intensity. One-way t-tests were performed to identify differences between the means. Comparison analyses were conducted using Origin 6.1 (Microcal Software, Northampton, MA).

**DRG culture**

DRG cultures follow a protocol described previously (Rizzo et al. 1994). Briefly, adult C57BL/6 mice or Sprague–Dawley rats (1–2 mo old) were decapitated, and L4 and L5 DRG were quickly removed and desheathed in sterile complete saline solution (CSS), pH 7.2. The tissue was then enzymatically digested at 37°C for 25 min with collagenase A (1 mg/ml; Roche) and papain (30 U/ml; Worthington, Lakewood, NJ) in CSS at 37°C. The treated tissues were gently centrifuged (100 × g for 3 min), and the pellets were triturated in DRG media (1:1 DMEM/F12, 10% FCS, 100 U/ml penicillin, and 0.1 mg/ml streptomycin) containing 1.5 mg/ml BSA (Fraction V; Sigma) and 1.5 mg/ml trypsin inhibitor (Sigma). Cells were then plated on polyornithine–laminin-coated glass coverslips, flooded with DRG media after 1 h, and incubated at 37°C in a humidified 95% air–5% CO₂ incubator.

**Electroporation of Na,1.8 channels into Na,1.8-null DRG neurons**

DRG neurons were transfected with Na,1.8 or its mutant derivative IQ/DE using the Nucleofector system (Amazax, Gaithersburg, MD) as described previously (Dib-Hajj et al. 2005). Briefly, adult Na,1.8-null (Nav1.8⁻/⁻ in C57BL/6 genetic background) mice (Akopian et al. 1999) were decapitated and 10 pairs of DRG were quickly removed and desheathed in sterile calcium and magnesium-free Hank’s buffered saline solution (HBSS), pH 7.2. The tissue was treated at 37°C for 50 min with an enzyme mixture of dispase (5 mg/ml; Roche), collagenase A (2 mg/ml; Roche), and DNase I (0.1 mg/ml; Roche) in HBSS, desheathed in sterile complete saline solution (CSS), pH 7.2. The tissue was treated at 37°C for 50 min with an enzyme mixture of dispase (5 mg/ml; Roche), collagenase A (2 mg/ml; Roche), and DNase I (0.1 mg/ml; Roche) in HBSS, desheathed in sterile complete saline solution (CSS), pH 7.2. The tissue was treated at 37°C for 50 min with an enzyme mixture of dispase (5 mg/ml; Roche), collagenase A (2 mg/ml; Roche), and DNase I (0.1 mg/ml; Roche) in HBSS, desheathed in sterile complete saline solution (CSS), pH 7.2. The tissue was treated at 37°C for 50 min with an enzyme mixture of dispase (5 mg/ml; Roche), collagenase A (2 mg/ml; Roche), and DNase I (0.1 mg/ml; Roche) in HBSS, desheathed in sterile complete saline solution (CSS), pH 7.2. The tissue was treated at 37°C for 50 min with an enzyme mixture of dispase (5 mg/ml; Roche), collagenase A (2 mg/ml; Roche), and DNase I (0.1 mg/ml; Roche) in HBSS, desheathed in sterile complete saline solution (CSS), pH 7.2. The tissue was treated at 37°C for 50 min with an enzyme mixture of dispase (5 mg/ml; Roche), collagenase A (2 mg/ml; Roche), and DNase I (0.1 mg/ml; Roche) in HBSS.

**Electrophysiological recordings**

Whole cell patch-clamp recordings of sodium currents in acutely dissociated mouse (C57BL6) or rat (Sprague–Dawley) DRG neurons (2–12 h after plating) were performed to test the effect of the calmodulin-binding peptide (CBP, calmodulin-dependent protein kinase II 290–309 peptide) on biophysical properties of the slowly inactivating Na,1.8 TTX-R currents. DRG neurons displayed only short (<10 μm) axonal processes during the brief period of culture, facilitating the voltage clamp. Conventional whole cell patch-clamp recordings were made from small DRG neurons (≤25 μm diameter), using Axopatch 200B amplifiers (Axon Instruments, Foster City, CA). For currents >20 nA, we switched to the 50-MΩ feedback resistor (β of 0.1), which can pass ≈200 nA.

Micropipettes (0.6–0.9 MΩ) were pulled from patch-clamp capillary glass (PF10165-4; World Precision Instruments, Sarasota, FL) with a Flaming Brown P80 micropipette puller (Sutter Instruments, Novato, CA), and polished on a microforge. The average series resistance was 0.82 ± 0.05 MΩ (n = 79). Capacity transients were cancelled using computer-controlled circuitry, and series resistance was compensated (>85%) in all experiments. The pipette solution contained (in mM): 140 CsF, 1 EGTA, 10 NaCl, and 10 HEPES, pH 7.3 (adjusted to 310 mOsm/l with sucrose). To investigate the effect of intracellular free calcium ion on Na,1.8 currents in DRG neurons, the Ca²⁺-free pipette solution contained (in mM): 140 CsF, 5 BaCl₂, 10 NaCl, and 10 HEPES, pH 7.3, and to investigate the effect of high Ca²⁺ on the Na,1.8 currents, the 5 mM BaCl₂ in the pipette solution was replaced with 1 mM BaCl₂ and 1 mM CaCl₂ (free calcium concentration ≈10 μM). The full Na⁺ bath solution contained (in mM): 140 NaCl, 3 KCl, 1 MgCl₂, 1 CaCl₂, 20 TEA-Cl, 10 glucose, and 10 HEPES, pH 7.3 (adjusted to 320 mOsm/l with sucrose). Endogenous Ca²⁺ currents were blocked by 0.1 mM CdCl₂. Tetrodotoxin (TTX, 300 nM) was included in the bath solution to inhibit endogenous tetrodotoxin-sensitive (TTX-S) Na⁺ currents, which are completely blocked by this toxin concentration. The pipette potential was zeroed before seal formation and voltages were not corrected for liquid junction potential. Leakage current was digitally subtracted on-line using hyperpolarizing control pulses, applied after the test pulse (P/N subtraction). Whole cell currents were filtered at 5 kHz and acquired at 50 kHz using Clampex 1.1 software (Axon Instruments). All recordings were started 10 min after establishing whole cell configuration to allow currents to stabilize and minimize the contamination of residual persistent TTX-R Na,1.9 currents. For current density measurements, membrane currents were normalized to membrane capacitance and calculated as the integral of the transient current in response to a 5-mV hyperpolarizing pulse from the holding potential of −70 to −80 mV. The cell capacitance of the DRG neurons did not vary significantly (P > 0.05) under the experimental conditions of this study (data not shown). All experiments were performed at room temperature (21–25°C).

**Experimental protocols and data analysis**

Wild-type DRG neurons were held at −70 mV to minimize the contamination of the recordings by the persistent TTX-R Na,1.9 currents (Akopian et al. 1999; Cummins et al. 1999). Activation curves were constructed with the membrane potential held at −70 mV and the application of a series of 40-ms test pulses to voltages that ranged from −70 to +60 mV in 10-mV increments. The peak value of Iₙa at each membrane potential (Vₘ) was plotted. The relationship of peak Iₙa versus Vₘ was fitted with the following equation

\[ Iₙa = G(Vₘ - Vₜₗ) \]

where Vₜₑᵥ is the reversal potential of Iₙa, and G is the voltage-dependent sodium current conductance. G was fitted using the following Boltzmann distribution equation

\[ G = Gₘₐₓ(1 + \exp([Vₘ - Vₜₗ]/k))] \]

where Gₘₐₓ is the maximum conductance, Vₜₗ is the membrane potential at half-maximal conductance, and k is the slope factor.

The voltage-dependent steady-state inactivation was estimated by measuring the peak current amplitude elicited by a 40-ms test pulse to −10 mV after a 500-ms prepulse to potential over the range of −80 to +10 mV with a 20-s interpulse period. The normalized curves (Iₙa/Iₙa) were fitted using the following Boltzmann distribution equation

\[ Iₙa/Iₙa = \frac{G_{ₘₐₓ}}{Gₘₐₓ + \exp([Vₜₗ - Vₘ]/k)}} \]
The fast-inactivation decay of Na\textsubscript{\textit{V}} current was fitted with an exponential function

$$I = A \exp(-t/\tau) + C$$

where \( I \) is the current, \( A \) is the percentage of the channels inactivating with time constant \( \tau \), and \( C \) is the steady-state asymptote.

An ANOVA was conducted, with a subsequent Student–Newman–Keuls test for identifying statistically significant differences between the mean values of the four experimental conditions (Ca\textsuperscript{2+}-free, high Ca\textsuperscript{2+}, Ca\textsuperscript{2+}-free + CBP, and high Ca\textsuperscript{2+} + CBP). Because of small sample sizes, we also conducted parallel, nonparametric analyses (a Kruskal–Wallis test with follow-up Mann–Whitney \( U \) tests), adjusting for the number of comparisons \( (n = 6) \) using the Bonferroni correction factor, and identical conclusions were found (results not shown). Elsewhere, Student’s \( t \)-tests were used, as indicated, with the criterion for statistical significance set at 0.05. Descriptive data are presented as means \( \pm \) SE. Data were analyzed using Clampfit 8.2 software (Axon Instruments) and Origin 6.1 (Microcal Software, Northampton, MA). The ANOVA and post hoc analyses were conducted using SPSS 10.1.3.

**RESULTS**

**Calmodulin coimmunoprecipitates with Na\textsubscript{\textit{V}}.1.8 channels from rat DRG**

Calmodulin (CaM) has been shown to bind to the distal part of the Na\textsubscript{\textit{V}}.1.8 C-terminus, which carries an IQ motif in a yeast two-hybrid assay (Malik-Hall et al. 2003; our unpublished data). To demonstrate that native Na\textsubscript{\textit{V}}.1.8 and CaM can associate in DRG neurons, we investigated the ability of anti-Na\textsubscript{\textit{V}}.1.8 antibodies to coimmunoprecipitate (co-IP) the channel and CaM from rat DRG lysate. Because the CaM-IQ motif interaction is Ca\textsuperscript{2+} independent (Erickson et al. 2001; Kim et al. 2004; Mori et al. 2000; Xia et al. 1998), we carried out the co-IP experiments using Ca\textsuperscript{2+}-free solutions containing 1 mM EDTA. DRG lysates were incubated with anti-Na\textsubscript{\textit{V}}.1.8 antibodies, the immunoprecipitated complex was split in half, and the constituent proteins were separated on two different gel systems to optimally resolve the high molecular weight channel proteins and the low molecular weight CaM protein. Pan sodium channel (Fig. 1A) and CaM (Fig. 1B) antibodies were used to detect the presence of sodium channels and CaM in the co-IP precipitate. Polyclonal anti-Na\textsubscript{\textit{V}}.1.8 antibodies specifically immunoprecipitated a protein that cross-reacted with the monoclonal pan sodium channel antibody (Fig. 1A, lane 1), whereas control IgG antibodies failed to do so (Fig. 1A, lane 2). Similarly, the polyclonal anti-Na\textsubscript{\textit{V}}.1.8 antibody specifically immunoprecipitated a protein that reacted with the CaM-specific monoclonal antibody (Fig. 1B, lane 1) and co-migrated with purified recombinant CaM (Fig. 1B, lane 3). Together with the results from in vitro binding assays (see following text) and yeast two-hybrid assays showing the interaction of CaM with the C-terminus polypeptide of Na\textsubscript{\textit{V}}.1.8 (Malik-Hall et al. 2003), our data demonstrate that CaM and native Na\textsubscript{\textit{V}}.1.8 channels can form a complex in DRG neurons in the absence of Ca\textsuperscript{2+}, in agreement with the view that this interaction is mediated by the apoCaM/IQ-motif binding.
determinants in the C-terminus of Na\textsubscript{1.2} channels when Ca\textsuperscript{2+} free or Ca\textsuperscript{2+} loaded (Kim et al. 2004).

Reduction in current density of Na\textsubscript{1.8} does not arise from altered voltage-dependent activation or inactivation properties

To determine whether reduced Na\textsubscript{1.8} current density might have resulted from a shift in voltage dependency of the channel, the current–voltage relationship of Na\textsubscript{1.8} was investigated under the three experimental conditions (Fig. 3A). The \( V_{1/2} \) of activation of Na\textsubscript{1.8} channels did not differ (\( P > 0.05 \)) under the experimental conditions: Ca\textsuperscript{2+}-free solution: \(-16.51 \pm 0.46 \) mV, \( k = 6.24 \pm 0.32 \) (\( n = 14 \)); high Ca\textsuperscript{2+} solution: \(-16.48 \pm 0.40 \) mV, \( k = 5.36 \pm 0.30 \) (\( n = 18 \)); CBP plus high Ca\textsuperscript{2+}: \(-15.60 \pm 0.43 \) mV, \( k = 4.84 \pm 0.31 \) (\( n = 14 \)). Thus CaM modulation of the current density of Na\textsubscript{1.8} does not appear to result from changes in the voltage dependency of activation of the channels.

Steady-state inactivation (Fig. 3B) was also investigated under the three experimental conditions used in these studies. The \( V_{1/2} \) was measured with 500-ms prepulses to potentials over the range of \(-80 \) to \(+10 \) mV: Ca\textsuperscript{2+}-free solution: \(-43.94 \pm 0.85 \) mV, \( k = 6.46 \pm 0.44 \) (\( n = 14 \)); high Ca\textsuperscript{2+} solution: \(-43.05 \pm 0.44 \) mV, \( k = 7.36 \pm 0.12 \) (\( n = 10 \)); CBP plus high Ca\textsuperscript{2+}: \(-40.53 \pm 0.78 \) mV, \( k = 7.54 \pm 0.38 \) (\( n = 8 \)). The \( V_{1/2} \) did not significantly differ (\( P > 0.05 \)) under these experimental conditions. Thus our results suggest that CaM modulation of the current density of Na\textsubscript{1.8} is not a result of changes in the voltage dependency of steady-state inactivation of the channels.

Kinetic properties of activation and inactivation of Na\textsubscript{1.8} channel in the presence of CBP, with or without 10 \( \mu \)M free calcium, were also investigated. The time-to-peak (Fig. 3C) and decay (Fig. 3D) of Na\textsubscript{1.8} in 10 \( \mu \)M Ca\textsuperscript{2+} or in the presence of CBP in 10 \( \mu \)M Ca\textsuperscript{2+} were not different from those in Ca\textsuperscript{2+}-free solution at all tested voltages. These data demonstrate that CaM does not regulate the kinetics or voltage-dependent gating of Na\textsubscript{1.8}.

**The IQ-motif in the C-terminus of Na\textsubscript{1.8} is important for CaM regulation of current density in DRG neurons**

All voltage-gated sodium channels contain a CaM-binding IQ motif: IQXXXXXXR in their C-terminus (Herzog et al. 2003b; Mori et al. 2000; Rhoads and Friedberg 1997). CBP, which tightly binds CaM in the presence of Ca\textsuperscript{2+} and prevents CaM binding to its targets, caused a significant reduction in the current density of Na\textsubscript{1.8} (Fig. 2). To determine whether CaM regulation of Na\textsubscript{1.8} current density is dependent on the direct binding of CaM to the IQ motif in the C-terminus of the channel, we investigated the effect of substituting aspartic acid (D) and glutamic acid (E) for the I\textsuperscript{1859}Q\textsuperscript{1860} residues, respectively. Substitution of these conserved residues to charged amino acids blocks CaM binding to the IQ motifs of sodium channels (Herzog et al. 2003b; Young and Caldwell 2005) and calcium channels (Zuhlke et al. 1999). Immunoprecipitation of the native Na\textsubscript{1.8} channels from DRG neurons indicated that CaM is closely associated with the Na\textsubscript{1.8} sodium channel (see Fig. 1). Thus impairment of this interaction could lead to changes in the biophysical properties of the Na\textsubscript{1.8} channel in a manner similar to that of the competitive inhibition of this interaction by CBP.

To demonstrate direct binding of CaM to the C-terminus of Na\textsubscript{1.8}, we constructed a 6X-His tagged fusion protein spanning amino acids 1724–1956 of the C-terminus of Na\textsubscript{1.8} and a mutant fusion protein whereby the IQ residues at positions 1859/1860 were mutated to D and E, respectively. Wild-type (WT) and IQ/DE recombinant proteins were purified to homogeneity (Fig. 4A). Pull-down binding assays using CaM-Sepha-
rose beads were used to measure CaM binding to these purified proteins. The binding reaction contained 5 mM EGTA to ensure that any interaction would be the result of apoCaM binding (see functional data, Fig. 2). The signal of the bound WT and IQ/DE C-terminus protein was quantitated by densitometry. The average signal from four assays was 371.5 ± 47.0 arbitrary units (AU) for WT C-terminus and 44.4 ± 17.5 (AU) for the IQ/DE mutant C-terminus (Fig. 4, B and C). Mutation of the IQ residues to DE reduced CaM binding to the C-terminus of Na1.8 compared with WT by more than eightfold (PEM 0.001).

We studied the effect of the IQ/DE mutation on functional Na1.8 channels using patch-clamp methods after expression in Na1.8−/− DRG neurons. WT and mutant IQ/DE plasmids were electroporated into Na1.8−/− mouse DRG neurons (Akopian et al. 1999) to record this current in its native environment. DRG neurons of Na1.8−/− mice completely lack the slowly inactivating TTX-R sodium current produced by Na1.8 (Akopian et al. 1999) and thus provide a robust expression system for Na1.8 (Liu et al. 2005; this study) and other sodium channels that have been rendered TTX-R by site-directed mutagenesis, permitting the sodium current of interest to be recorded in isolation (Cummins et al. 2001; Herzog et al. 2003a,b; Rush et al. 2005). Figure 5A shows representative traces of WT and IQ/DE mutant Na1.8 currents expressed in transfected Na1.8−/− DRG neurons. Figure 5B shows that the current density of IQ/DE mutant (194.29 ± 36.08 pA/pF; n = 29) was significantly smaller (P < 0.05) than that of WT Na1.8 channels (376.67 ± 76.65 pA/pF, n = 33). The voltage dependency of channel activation of IQ/DE mutant channels (V1/2 = −24.21 ± 0.30 mV, k = 6.91 ± 0.20; n = 25) did not differ (P > 0.05) from that of WT Na1.8 channels (V1/2 = −22.29 ± 0.42 mV, k = 6.75 ± 0.30; n = 27) (Fig. 5C).

The voltage dependency of steady-state inactivation of WT and IQ/DE mutant channels was also investigated. As shown in Fig. 5D, the V1/2 of steady-state inactivation curve of the IQ/DE mutant (−42.38 ± 0.46 mV, k = 6.46 ± 0.44; high Ca2+ (●), n = 10), V1/2 = −43.05 ± 0.44 mV, k = 7.36 ± 0.12; 10 μM CBP in high Ca2+ (●, n = 8), V1/2 = −40.53 ± 0.78 mV, k = 7.54 ± 0.38. Kinetics of activation (C) and inactivation (D) of Na1.8 are not affected by CBP or Ca2+ concentration. Time to peak (C) and the time constant of inactivation decay (τinactivation) as a function of test potentials were not different under conditions of Ca-free (●, n = 8), high Ca2+ (●, n = 6), or in the presence of CBP in high Ca2+ buffer (●, n = 6).

### IQ/DE mutant channels exhibit slower recovery from inactivation

To investigate the effect of direct binding of CaM to the IQ motif in the C-terminus of Na1.8 on recovery from inactivation, we compared recovery from inactivation of WT Na1.8 and the IQ/DE mutant channels using a double-pulse protocol (Fig. 6). Data were fitted with a double-exponential function as shown in Fig. 6. Figure 6 (bottom) shows recovery from inactivation of wild-type and IQ/DE mutant channels where channels displayed two components of recovery from inactivation: one rapid and the other much slower. Recovery from inactivation of wild-type channels was well fitted by a double-exponential function with fast and slow components of recovery time constants of 5.94 ± 0.88 and 1.930 ± 220 ms, respectively. Similarly, recovery from inactivation of IQ/DE mutant channel was also well fitted by a double-exponential function with fast and slow components of recovery time constants of 6.28 ± 1.06 and 3.650 ± 300 ms, respectively.
The slower recovery component of the IQ/DE mutant (3,650 ± 300 ms) was significantly different (P < 0.05, t-test) from that of the wild-type channel (1,930 ± 220 ms), whereas the faster components were not significantly different (P > 0.05). Surprisingly, IQ/DE mutant channels do not fully recover from inactivation even after 10 s of interpulse period of recovery (Fig. 6), suggesting that the change of the slope of steady-state inactivation curve could be caused by accumulation of inactivated channels.

IQ/DE mutant channels demonstrate strong frequency-dependent current reduction

Slow recovery from inactivation of the IQ/DE mutant channels suggested that these channels may undergo frequency-dependent inhibition. Figure 7 shows the frequency-dependent inhibition of WT and IQ/DE mutant channels. Twenty repetitive 100-ms depolarizing pulses to −10 mV, from a holding potential of −70 mV, were applied at 0.1- and 1-Hz frequencies (Fig. 6A). The peak current amplitude of WT Na\textsubscript{v}1.8 channel at the 20th pulse did not change, compared with that at the first pulse, at a depolarization frequency of 0.1 Hz (0.98 ± 0.03, n = 6), whereas the peak current amplitude at the 20th pulse at 1 Hz decreased to 0.69 ± 0.04 (n = 11) of that of the first pulse. As expected, the peak amplitude of IQ/DE mutant channels at 20th pulse, compared with the first pulse, was decreased even at 0.1 Hz (0.80 ± 0.03, n = 5), with more inhibition at 1 Hz (0.46 ± 0.05, n = 14). Higher frequencies produced an even greater reduction in the peak current amplitude; compared with the peak current amplitude at the first pulse, the residual peak current amplitude of the IQ/DE mutant channels at the 20th depolarizing pulse at 2 Hz was 0.37 ± 0.05 (n = 8) and at 5 Hz was 0.18 ± 0.08 (n = 5). Current amplitudes of wild-type channels were also reduced at higher frequencies to 0.56 ± 0.08 (n = 8) at 2 Hz and 0.48 ± 0.14 (n = 4) at 5 Hz (Fig. 6B). The frequency-dependent inhibition of the wild-type channels was significantly smaller than that of the IQ/DE mutant channels at all tested frequencies (P < 0.05).

The current density reported in Fig. 2 was measured after a series of depolarizations to construct the I–V curve of Na\textsubscript{v}1.8 in the presence of CaM antagonists and for the IQ/DE mutant channels. Because the recording protocol could have led to the accumulation of inactivated channels leading to a reduced current density, we further investigated the mechanism underlying the reduction of Na\textsubscript{v}1.8 current density by CaM antagonists, by measuring the peak current amplitude of the first pulse to −10 mV, 10 min after establishing whole cell configuration. The peak current density of Na\textsubscript{v}1.8 in the presence of CBP was significantly decreased (P < 0.05, t-test) to 160.31 ± 35.72 pA/pF (n = 15) compared with control conditions (398.48 ± 104.08 pA/pF, n = 12). Although accumulation of inactivated channels may contribute to the reduction in current density, this finding shows that it does not fully account for the entire reduction.

CBP increases the frequency-dependent inhibition of endogenous Na\textsubscript{v}1.8 currents in native rat DRG neurons

Two populations of slowly inactivating TTX-R currents, TTX-R1 and TTX-R2, were described in rat DRG neurons with different degrees of frequency-dependent inhibition (Rush et al. 1998). We hypothesized that the association, or lack thereof, of Na\textsubscript{v}1.8 channels and CaM might underlie the two frequency-dependent states. To test this possibility, we investigated the recovery from inactivation and frequency-dependent inhibition of Na\textsubscript{v}1.8 currents at 1 Hz in rat small-diameter DRG neurons in the absence and the presence of CaM antagonist CBP. Figure 8A shows recovery from inactivation of Na\textsubscript{v}1.8 channels in native DRG neurons. Data were fitted with a double-exponential function as shown in Fig. 6. Recovery from inactivation of Na\textsubscript{v}1.8 channels in the absence of CBP was well fitted by a double-exponential function with fast and slow components of recovery time constants of 5.2 ± 0.6 and 3,396 ± 259 ms (n = 11), respectively. Recovery from inactivation of Na\textsubscript{v}1.8 channels in the presence of CBP was also well fitted by a double-exponential function with fast and slow components of recovery time constants of 5.7 ± 0.7 and 4,442 ± 221 ms (n = 10), respectively. Similar to the effect of
IQ/DE mutation in recombinant Na\textsubscript{v}1.8 channels in transfected DRG neurons, the slow component of recovery from inactivation of native Na\textsubscript{v}1.8 in the presence of CBP was significantly slower than that in the absence of CBP (\(P < 0.05\)). Interestingly, the slow component of recovery from inactivation of Na\textsubscript{v}1.8 channels in native neurons is slower than that in neurons transfected with WT Na\textsubscript{v}1.8 channel, suggesting that the frequency-dependent inhibition of Na\textsubscript{v}1.8 channels in acutely isolated native neurons should be stronger than that in transfected neurons.

Na\textsubscript{v}1.8 channels in native neurons show similar frequency-dependent inhibition in Ca\textsuperscript{2+}-free and high Ca\textsuperscript{2+} pipette solutions (Fig. 8B) with average residual currents at the 20th pulse of 48.9 \pm 3.9\% (\(n = 15\)) in Ca\textsuperscript{2+}-free and 48.6 \pm 4.1\% (\(n = 11\)) in high Ca\textsuperscript{2+} solutions, compared with the current amplitude at the first pulse. However, the presence of the CBP peptide in high Ca\textsuperscript{2+} buffer in the pipette solution significantly reduced (\(P < 0.02\)) the average of the frequency-dependent inhibition of the peak current to 33.1 \pm 2.8\% (\(n = 8\)) of the peak current at the first pulse.

Consistent with the differences in the recovery from inactivation of Na\textsubscript{v}1.8 channels in native and transfected DRG neurons, the frequency-dependent inhibition of Na\textsubscript{v}1.8 currents in native neurons (49\%, Fig. 8B) is much stronger than that of recombinant WT Na\textsubscript{v}1.8 in transfected neurons (69\%, Fig. 7B). One major difference in these assays is time in culture: 2–12 h for the acutely isolated native DRG neurons and 24–52 h for neurons transfected with Na\textsubscript{v}1.8 channels. To investigate possible effects of time in culture on the frequency-dependent inhibition of Na\textsubscript{v}1.8 channels in native neurons, we compared this inhibition in acutely isolated native neurons and after 24 h in culture. Acutely tested DRG neurons show a frequency-dependent reduction of the Na\textsubscript{v}1.8 current at 1-Hz stimulation (0.51 \pm 0.32; \(n = 29\)), whereas neurons after 24 h in culture show less frequency-dependent inhibition of the current (0.69 \pm 0.02, \(n = 31\)) (Fig. 8C). Frequency-dependent inhibition of Na\textsubscript{v}1.8 in native neurons after 24 h in culture (0.69 \pm 0.02, \(n = 31\)) is not significantly different from that in neurons transfected with recombinant WT Na\textsubscript{v}1.8 channels (0.69 \pm 0.04; Fig. 7B).

**DISCUSSION**

This study shows that sodium channel Na\textsubscript{v}1.8, which contributes most of the current for the action potential (Blair and Bean 2002; Renganathan et al. 2001) in nociceptive DRG neurons where it is normally expressed (Akopian et al. 1996; Black et al. 1996; Djouhri et al. 2003), associates with, and is modulated by, calmodulin (CaM). The voltage dependency and kinetics of activation and inactivation of Na\textsubscript{v}1.8 were unchanged in the IQ/DE mutant, which impairs CaM binding, or after the application of a CaM antagonist [calmodulin-binding peptide (CBP)]. CBP in the recording pipette causes a reduction in Na\textsubscript{v}1.8 current density and in the ability of this channel to follow high-frequency depolarizations. Both of the CaM-mediated effects on Na\textsubscript{v}1.8 are Ca\textsuperscript{2+}-independent, consistent with direct binding of apoCaM to the IQ motif in the C-terminus of the channel.

CaM interacts with the IQ motif in the C-terminus of several sodium channels and was previously shown to influence the properties of Na\textsubscript{v}1.4, Na\textsubscript{v}1.5, and Na\textsubscript{v}1.6 currents in an isoform-specific manner (Deschenes et al. 2002; Herzog et al. 2003b; Tan et al. 2002). Although we were not able to show direct binding of GST-Na\textsubscript{v}1.8 C-terminus and CaM in our earlier studies (Herzog et al. 2003b), recent studies on the interaction of Na\textsubscript{v}1.5 C-terminus and CaM (Kim et al. 2004)
suggested that misfolding of the bacterially expressed channel C-terminus polypeptides might account for the lack of binding of GST-Nav1.8 and CaM in our previous assays. Adopting the coexpression protocol developed by Kim et al. (2004) as well as a 6X-His tag, a much smaller affinity tag than GST, we observed CaM binding to the C-terminus of Nav1.8 in direct binding assays in this study, in agreement with a previous report of their interaction in a yeast two-hybrid assay (Malik-Hall et al. 2003). This interaction appears to involve apoCaM to the IQ motif in the C-terminus of the channel because it is not Ca2+/H11001-dependent, and is significantly reduced by more than eightfold by the substitution of the IQ residues with acidic residues DE. Importantly, we now show in this study that CaM coimmunoprecipitates with endogenous full-length Nav1.8 channels from native DRG neurons and modulates the Nav1.8 current within these cells. These data provide strong evidence that CaM is a functional partner of sodium channels in native neurons.

We investigated regulation of Na\textsubscript{v}1.8 by CaM in native DRG neurons because cell background has been shown to influence biophysical properties of other sodium channels, such as Na\textsubscript{v}1.3 and Na\textsubscript{v}1.6 currents (Cummins et al. 2001; Herzog et al. 2003a). Consistent with a role of cell typespecific regulation of sodium currents, the effects of CaM on Na\textsubscript{v}1.4 channels was recently shown to significantly differ in HEK293 and CHO cell lines (Young and Caldwell 2005). The use of DRG neurons as an expression system is also important because it permits direct comparison of the effect of CaM on Na\textsubscript{v}1.8 (this study) and Na\textsubscript{v}1.4 and Na\textsubscript{v}1.6 (Herzog et al. 2003b), as discussed in the following text. Thus the effects of CaM on Na\textsubscript{v}1.8 channels that we describe here are biologically relevant and are predicted to significantly impact the electrogenic properties of DRG neurons.

CaM binding to the C-terminus of sodium channels has been shown to regulate the current densities of several channels. Mutations of IQ motifs of Na\textsubscript{v}1.4, Na\textsubscript{v}1.6 (Herzog et al. 2003b), and Na\textsubscript{v}1.8 (this study) reduce sodium current density to different levels. Although most of the IQ motif in Na\textsubscript{v}1.4, Na\textsubscript{v}1.6, and Na\textsubscript{v}1.8 channels is conserved, it is not identical (Herzog et al. 2003b); thus the differential sensitivity of various channels to the IQ mutations could be caused by altered affinity of CaM binding to the mutant motifs. The N and C lobes of CaM contribute to the binding to the channel; the C

FIG. 6. Slow component of recovery from inactivation is slower in the IQ/DE channels. Recovery from inactivation of WT and IQ/DE channels was studied with a standard double-pulse protocol; the first prepulse of a 40-ms depolarizing potential of −10 mV from a holding potential of −70 mV was followed by the second identical pulse after increasing time intervals between 2 and 10,000 ms at −70 mV. Every cycle of the double-pulse protocol was 20 s. Peak currents elicited by the second test pulse were normalized against the peak currents obtained by the first prepulse and plotted as a function of various interpulse intervals. Inset: rescaled data to show early times. Curves were obtained by fitting the plotted data to a double-exponential function, which yielded the fast component of recovery time constants of 5.94 ± 0.88 and 6.28 ± 1.06 ms, and the slow component of time constants of 1,930 ± 220 and 3,650 ± 300 ms for WT (●, n = 7) and IQ/DE (○, n = 8), respectively. Compared with WT channels, the IQ/DE mutant channels show a similar faster component (P > 0.05), whereas the slower component is significantly slower (P < 0.05).

FIG. 7. IQ/DE mutation increases frequency-dependent inhibition of Na\textsubscript{v}1.8. A: WT (filled symbols) and IQ/DE (open symbols) Na\textsubscript{v}1.8 channels were subjected to 20 repetitive 100-ms depolarizing pulses of −10 mV, from a holding potential of −70 mV, that were applied at 0.1 Hz (●, n = 6; ○, n = 5) and 1 Hz (●, n = 11; ○, n = 14). Peak amplitudes of current at every pulse were normalized by the peak amplitudes of current obtained at the first number of pulse and then plotted vs. the pulse numbers. B: normalized current of the 20th depolarization pulse at 0.1–5 Hz, respectively, for WT and IQ/DE mutant channels (n = 5–14; *P < 0.05; **P < 0.01).
and the presence of CA2+ yielded the fast component of recovery time constants of 5.2 ms, and the slow component of time constants of 3.396 were obtained by fitting the plotted data to a double-exponential function that

DRG neurons was studied with a standard double-pulse protocol as in Fig. 5. Curves in the C-terminus, other cytosolic parts of the channel, or other channel protein partners (Young and Caldwell 2005). Thus the local environment of the IQ motif in the C-terminus and perhaps isoform-specific sequences in other cytosolic regions

of the channel may influence regulation of sodium channels by CaM.

Na1.8 (this study) and Na1.5 (Young and Caldwell 2005) are regulated by CaM in a Ca2+-independent manner. In contrast, Herzog et al. (2003b) showed that CaM regulation of inactivation kinetics of the TTX-S channel Na1.6 were Ca2+ sensitive. More recently, Young and Caldwell (2005) reported that CaM modulation of inactivation of another TTX-S channel, Na1.4, was also Ca2+ sensitive. However, neither the current density nor gating properties of Na1.8 were different under Ca2+-free or 10 μM Ca2+ conditions. Also, frequency-dependent inhibition of Na1.8 was regulated by CaM but in a Ca2+-independent manner. The lack of Ca2+ modulation of CaM regulation of Na1.5 and Na1.8 suggests that TTX-R channels, which are genetically linked, might have evolved a Ca2+-independent form of CaM regulation, unlike that which evolved for the TTX-S channels.

The reduction of the current density of Na1.8 was not accompanied by significant changes in activation and inactivation properties of the channel. Removal of channels from the cell surface by endocytic pathways might account for the reduction of the current density. Regulation of channel density of Na1.2 at the cell surface by clathrin-mediated endocytosis has been linked to a conserved motif in the C-terminus of sodium channels including Na1.8 (Garrido et al. 2001). Recently, Na1.8 has been shown to interact by the membrane-proximal portion of its C-terminus with a novel protein, CAP-1A, which can act as an adaptor molecule linking Na1.8 and clathrin (Liu et al. 2005). Overexpression of CAP-1A and Na1.8 in Na1.8-/- mouse DRG neurons caused a reduction of the current density by clathrin-mediated endocytosis (Liu et al. 2005). Thus CaM binding to the IQ motif in the membrane-distal portion of the C-terminus might interfere with the binding of components of the endocytic machinery to the channel, resulting in the stabilization of channels at the cell surface, thus increasing the current density. Although removal of channels from the cell surface by endocytosis can underlie a reduction in current density, it does not account for the slowed recovery from inactivation of Na1.8IQ/DE mutant channel.

Alternatively, the lack of an effect of CaM antagonists or the IQ/DE mutations on the voltage dependency of activation and inactivation of Na1.8 suggests that CaM may regulate single-channel properties, leading to a reduction in current density and thus negatively influencing frequency-dependent inhibition of the channel. In agreement with this view, it has recently been reported that DRG neurons contain two populations of Na1.8-like channels with single-channel conductances of 9.2 and 14.9 pS, and with different frequency-dependent inhibition at 1-Hz stimulation frequency (Cardenas et al. 2004). The CBP-mediated and IQ/DE-mediated reduction in the current density and the CBP-mediated frequency-dependent inhibition of Na1.8 are consistent with a CaM binding effect on single-channel properties. Additional experiments are needed to determine the contribution of these alternative mechanisms to the current density reduction of Na1.8, which is caused by blocking CaM binding to the channel.

In addition to the reduction in Na1.8 current density by >50%, the application of CBP to native DRG, or the introduction of the IQ/DE mutation, hindered the slow component of recovery from inactivation of this channel, an effect that may underlie the greater frequency-dependent inhibition of Na1.8 IQ/DE mutant channel.

FIG. 8. CBP increases frequency-dependent inhibition of Na1.8 in native DRG neurons. A: recovery from inactivation of Na1.8 channel in native neurons was studied with a standard double-pulse protocol as in Fig. 5. Curves were obtained by fitting the plotted data to a double-exponential function that yielded the fast component of recovery time constants of 5.2 ± 0.6 and 5.7 ± 0.7 ms, and the slow component of time constants of 3.396 ± 259 and 4,442 ± 221 ms in the absence of CBP (●, n = 11) and the presence of CBP with high calcium [Ca2+] (○, n = 10), respectively. B: normalized Na1.8 current in rat small DRG neurons of the 20th depolarization pulse at 1 Hz in the absence ([Ca2+]n = 15) and the presence (n = 11) of Ca2+, and with CBP in the presence of 10 μM Ca2+ (n = 9). C: frequency-dependent inhibition of Na1.8 in native DRG neurons was measured under different culture conditions; acute isolated (2–10 h, n = 29) and cultured (24–52 h, n = 31). Data are presented as means ± SE (*P < 0.05; **P < 0.001).
Na,1.8 that we observed. The net outcome of this change was a dramatic frequency-dependent inhibition of the current even at a frequency as low as 0.1 Hz. Interestingly, two populations of Na,1.8 TTX-R currents with different frequency-dependent inhibition profiles have been described in DRG neurons (Cardenas et al. 2004; Rush et al. 1998). These previous reports and our current data are in agreement with those of Blair and Bean (2003), who reported a 35–50% variability range in use-dependent inhibition of Na,1.8 in native rat DRG neurons. What is remarkable is that the application of CBP to native DRG neurons reduced the variability of frequency-dependent inactivation by 10.220.32.247 on October 9, 2016 http://jn.physiology.org/ Downloaded from

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