Gain-of-function mutation in Nav 1.7 in familial erythromelalgia induces bursting of sensory neurons

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Erythromelalgia is an autosomal dominant disorder characterized by burning pain in response to warm stimuli or moderate exercise. We describe a novel mutation in a family with erythromelalgia in SCN9A, the gene that encodes the Nav1.7 sodium channel. Nav1.7 produces threshold currents and is selectively expressed within sensory neurons including nociceptors. We demonstrate that this mutation, which produces a hyperpolarizing shift in activation and a depolarizing shift in steady-state inactivation, lowers thresholds for single action potentials and high frequency firing in dorsal root ganglion neurons. Erythromelalgia is the first inherited pain disorder in which it is possible to link a mutation with an abnormality in ion channel function and with altered firing of pain signalling neurons.

Keywords: channel; channelopathy; erythromelalgia; mutation; pain; sodium

Abbreviations: DRG = dorsal root ganglion

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Introduction

Sodium channels contribute to dorsal root ganglion (DRG) neuron hyperexcitability associated with acquired pain (Waxman et al., 1999; Black et al., 2002), but their role in hereditary pain syndromes is less well understood. Primary erythromelalgia (also called primary erythermalgia) is an autosomal dominant painful neuropathy with characteristics that include burning pain of the extremities in response to warm stimuli or moderate exercise (van Genderen et al., 1993). Recently, two mutations in SCN9A, the gene for the human Nav1.7 sodium channel, were reported in primary erythromelalgia (Yang et al., 2004). Nav1.7 channels are preferentially expressed in nociceptive DRG neurons and sympathetic ganglion neurons (Sangameswaran et al., 1997; Toledo-Aral et al., 1997; Djouhri et al., 2003), and produce 'threshold currents' close to resting potential, amplifying small depolarizations such as generator potentials (Cummins et al., 1998), while other sodium channel isoforms contribute most of the current underlying all-or-none action potentials in DRG neurons (Renganathan et al., 2001; Blair and Bean, 2002). The previously described mutations of Nav1.7cause a 13–15 mV hyperpolarizing shift in activation, slow deactivation and increase the response of the channels to small ramp depolarizations (Cummins et al., 2004). We now describe a third mutation in Nav1.7 which segregateswith the disease phenotype in a large pedigree of primary erythromelalgia, describe its effects on channel function and show, for the first time, that a mutation in a human sodium channel can lower the threshold for single action potentials and high frequency firing of DRG neurons.

Subjects and methods

Patients

A neurologist blinded to the genetic studies confirmed disease phenotype, based on formal clinical criteria (Drenth and Michiels, 1994), in 17 affected subjects, five unaffected subjects and three unaffected spouses after informed consent was obtained. In a study approved by the Yale Medical School Human Investigation Committee. A clinical description for part of this family (Finley et al., 1992) and linkage to chromosome 2q31–q32 have been reported (Drenth et al., 2001), but detailed genetic analysis had not been carried out previously.

Exon screening

Genomic DNA was purified from buccal swabs or venous blood from 25 family members (17 affected; eight unaffected). Human variation panel control DNA (25 males, 25 females; Caucasians) was obtained from the Coriell Institute (Camden, NJ). The genomic sequence of SCN9A (GenBank accession no. NC_000002) was used to design intron-specific primers to amplify coding and non-coding exons which produce Nav1.7 cDNA. Genomic sequences were compared with the reference Nav1.7 cDNA (Klugbauer et al., 1995) to identify sequence variation. Sequencing was performed at the Howard Hughes Medical Institute/Keck Biotechnology Center at Yale University. Sequence analysis used BLAST (National Library of Medicine) and Lasergene (DNAStar, Madison, WI).

Voltage-clamp analysis

The plasmid carrying the TTX-R version of human Nav1.7 cDNA (hNav1.7R) was described previously (Herzog et al., 2003). The F1449V mutation was introduced into hNav1.7R using QuickChange XL site-directed mutagenesis (Stratagene, La Jolla, CA). Wild-type or F1449V mutant h Nav1.7 R channels were co-transfected with the human b1 and b2 subunits (Lossin et al., 2002) into HEK293 cells, grown under standard culture conditions (5% CO2, 37□ C) in Dulbecco's modifiedEagle's medium supplemented with 10% fetal bovine serum, by calcium phosphate precipitation (Cummins et al., 2004).

Whole-cell patch-clamp recordings were conducted at room temperature (`21C), 40–72 h after transfection using an EPC-10 amplifier and Pulse 8.5 (HEKA, Germany) with 0.8–1.5 MV electrodes (access resistance 1.6 6 0.3 MV). Voltage errors were minimized using 80% series resistance compensation and linear leak subtraction; capacitance artefact was cancelled using computercontrolled circuitry. Recordings were started 3.5 min after establishing whole-cell configuration. The pipette solution contained: 140mM CsF, 1 mM EGTA, 10 mM NaCl and 10 mM HEPES (pH 7.3). The bathing solution was 140mMNaCl, 3mM KCl, 1 mMMgCl2, 1mM CaCl2 and 10mMHEPES (pH 7.3). Data were analysed using Pulsefit (HEKA) and Origin (Microcal, Northampton, MA) software.

Transfection of DRG neurons and current-clamp recordings

DRGfrom wild-type C57/BL6 mice were treated (Rizzo et al., 1994) to obtain neurons for electroporation of sodium channel and green fluorescent protein (GFP) constructs (Amaxa Inc., Gaithersburg,MD; see on-line Supplementary material available at Brain Online). Current-clamp recordings were obtained 16–24 h post-transfection.

Whole-cell current-clamp recordings from small diameter DRG neurons (<30 mm) with robust GFP fluorescence were obtained at room temperature (21C) following transfection with either wild-type hNav1.7R–GFP or F1449V–GFP using an Axopatch 200B amplifier (Axon Instruments, Union City, CA). Micropipettes with resistances from 1 to 2.5 MV were filled with a solution of 140 mM KCl, 0.5 mM EGTA, 5 mM HEPES, 3 mM Mg-ATP and 3 mM Na-GTP, pH 7.3, adjusted to 315 mOsm/l with glucose. The external solution contained 140mMNaCl, 3mMKCl, 2mMMgCl2, 2 mM CaCl2, 10 mM HEPES, pH 7.3, adjusted to 320 mOsm/l with glucose. The pipette potential was adjusted to zero before seal formation; liquid junction potentials were not corrected. Capacity transients were cancelled before switching to current-clamp mode, and series resistance (3–6MV) was compensated by 70%. Traces were acquired from cells with stable resting potentials less than -40 mV using Clampex 8.1 software, filtered at 5 kHz and sampled at 20 kHz. When required, steady polarizing currents were applied to set a holding potential of -60 mV.

Results

Clinical description

The pedigree of this family contains 36 members; 16 subjects with the erythromelalgia phenotype (mean age 37 years; range 3–75) were clinically evaluated (10 women, six men). Mean age of onset of symptoms

was 3 years; four patients had onset in infancy, and all had onset of symptoms by their sixth birthday. All subjects experienced symptoms typical of erythromelalgia (Drenth and Michiels, 1994), with attacks of burning pain and erythema involving both surfaces of the hands, and feet up to or slightly proximal to the ankles. Ten subjects (63%) reported involvement of other areas, including face, ears, elbows and knees; one reported involvement of the vaginal area. Eleven (69%) reported attacks one or more times per day; three (19%) reported several attacks per month. All subjects but one (94%) reported that attacks are triggered by heat and improved by cold, although one reported that extreme cold can also trigger attacks.

Mutation in exon 23

Genomic DNA from the proband and control subjects was isolated from venous blood samples and used as template to amplify all known exons of SCN9A and compare the sequence with $Na_v1.7$ cDNA (Klugbauer et al., 1995). Proband and control templates produced similar amplicons which were purified and sequenced. Sequence analysis identified a T-to-G transversion in exon 23 (E23), corresponding to position 4393 of the reference sequence (see Supplementary material). This mutation substitutes phenylalanine (F) by valine (V) at position 1449 of the polypeptide, located at the N-terminus of loop 3 which joins domains III and IV. F1449 is invariant in all known mammalian sodium channels. Restriction digestion analysis (see Supplementary material) confirmed the presence of the F1449V mutation in 17 out of 17 affected individuals, and its absence in five out of five unaffected family members, three out of three unaffected spouses and 100 ethnically matched control chromosomes. Segregation of the T4393G mutation with disease was confirmed by DNA sequencing of E23 in all family members.

Voltage-clamp analysis

Wild-type $hNa_v1.7_R$ and the mutant channel F1449V were transiently expressed along with b1 and b2 subunits (Lossin et al., 2002) in HEK293 cells, where $Na_v1.7$ isplays biophysical properties (Cummins et al., 1998) similar to those in DRG neurons (Herzog et al., 2003). We examined the voltage dependence of activation using epolarizing test pulses from -100 mV. Mutant channels activated at potentials 5–10mVmore negative than wild-type channels. The midpoint of activation for F1449V (estimated by fitting with a Boltzmann function) was significantly shifted to -22.8 +/- 1.3 mV (n = 12) compared with wild-type currents (-15.2
+/- 1.3 mV, n = 11; $P < 0.05$), a smaller shift than for the previously described $Na_v1.7$ mutations (Cummins et al., 2004). The time course of activation, estimated using a Hodgkin–Huxley fit of currents elicited with a step depolarization to -20 mV, was not significantly different for wildtype (t = 482+/-25 ms) and F1449V channels (t = 431+/-17 ms). Deactivation kinetics, examined by eliciting tail currents at different potentials after briefly activating the channels (at -20 mV for 0.5 ms), were not altered at potentials ranging from -100 to -40 mV for the F1449V mutant channel, in contrast to the previously described $Na_v1.7$ mutations where deactivation was slower.

Steady-state fast inactivation of F1449V channels was shifted slightly in the depolarizing direction. The $V_{1/2}$ measured with 500 ms pre-pulses was -71.3 +/- 0.8 mV for wild-type (n = 16) and -67.0 +/- 1.4 mV for F1449V (n = 16; $P < 0.05$) channels. Voltage dependence of steady-state slow inactivation was shifted in the negative direction by the F1449V mutation. The time constants for open state inactivation were smaller for F1449V than for wild-type currents over the entire voltage range from -50 to +40 mV. At -0 mV, wild-type currents inactivated with a t = 1.4 +/- 0.1 ms (n = 7) and F1449V currents inactivated with a significantly smaller ($P < 0.05$) t = 1.0 +/- 2 ms (n = 8). Development of closed state inactivation was faster for F1449V channels, with significantly smaller ($P < 0.05$) time constants for inactivation at -80, -70 and -60 mV . Repriming (recovery from fast inactivation) was significantly faster ($P < 0.05$) for F1449V channels than for wild-type channels. The time constant for repriming of wild-type channels (t = 89 +/- 14 ms, n = 8) was 3-fold larger at -70 mV than for F1449V channels (t = 27 +/- 2 ms, n = 8). Ramp currents, elicited with slow (0.2 mV/ms) depolarizations from -100 to +20 mV, were not different for F1449V channels (0.4 +/- 0.1%; n = 7) and wild-type channels (0.4 +/- 0.1%; n = 12). In contrast, the I848T and L858H $hNa_v1.7$ erythromelalgia mutations elicited significantly larger ramp currents compared with wild-type channels (Cummins et al., 2004). Current-clamp analysis Reasoning that changes in voltage dependence could lower the firing threshold, we expressed wild-type or F1449V channels in small (<30

mm) DRG neurons which include nociceptors. Resting potential was similar (P > 0.05) in DRG neurons transfected with F1449V (-51.3 +/- 1.6 mV; n = 19) and with wild type (-49.0 +/- 1.3 mV; n = 16). To eliminate cell-to-cell variations, cells were held at -60 mV.
$Na_v1.7$ is important in early phases of electrogenesis in DRG neurons, producing graded depolarizations which may boost subthreshold inputs (Cummins et al., 1998) to bring DRG neurons to voltages at which $Na_v1.8$ [which has a more depolarized activation threshold (Akopian et al., 1996)] opens to produce all-or-none action potentials (Renganathan et al., 2001). Figure 6A, B and E shows the effect of the F1449Vmutation on the firing threshold. Figure 6A shows traces from a representative DRG neuron expressing wild-type $hNa_v1.7$. Subthreshold responses, which depolarized the cell slightly but not to -19 mV where an action potential is triggered, were elicited with 50–65 pA current injections. All-or-none action potentials required stimuli of -130 pA (current threshold for this neuron). In contrast, shows responses from a representative DRG neuron expressing F1449V, where action potentials were produced at a lower current threshold of 60 pA. Current threshold (current required to generate an all-or-none action potential) was significantly reduced (P < 0.05) following expression of F1449V (93.1 6 12.0 pA; n = 19) compared with wild-type (124.1 +/- 7.4 pA; n = 16). However, the voltage at which takeoff occurs for an all-or-none action potential was not significantly different (P > 0.5) in cells expressing wild-type (–21.4 +/- 0.9 mV; n = 16) or F1449V channels (–22.5 +/- 1.4 mV; n = 19). Similarly to native small DRG neurons, 50% of which fire repetitively in response to prolonged stimuli (Renganathan et al., 2001), the majority of neurons expressing wild-type $Na_v1.7$ (11 out of 16; 69%) or the F1449V channel (12 out of 19; 63%) can fire repetitively (Fig. 6C and D). Figure 6C shows the firing of a representative neuron expressing wildtype channels, which responded to a 950 ms stimulation of 150 pA with two action potentials. In contrast, a neuron expressing F1449V responds to an identical 150 pA depolarizing stimulus with high frequency firing (Fig. 6D). The firing frequency evoked with 100 pA current injections was increased from 1.24 +/- 0.58 Hz (n = 11) for wild-type to 5.34 +/- 1.21 Hz (n = 12; P < 0.01) for F1449V channels. Current injection of 150 pA evoked firing at 3.03 +/- 0.75 Hz (n = 9) following expression of wild-type and 6.48 +/- 1.41 Hz (n = 12; P < 0.05) following expression of F1449V channels. Thus, in addition to a lower current threshold for action potentials, the frequency of firing at graded stimulus intensities was higher for cells expressing F1449V.

Discussion

This study demonstrates, in a large family with primary erythromelalgia, a single substitution of phenylalanine by valine (F1449V) at codon 1449 in the sodium channel $Na_v1.7$. This single amino acid substitution alters the biophysical properties of $hNa_v1.7$ and reduces the threshold for action potential firing and bursting of DRG neurons. F1449 is located within L3, the cytoplasmic loop which joins domains III and IV, 11 amino acid residues N-terminal to the fast inactivation IFM (isoleucine– phenylalanine–methionine) motif (West et al., 1992). F1449V substitution produces an 8 mV hyperpolarizing shift in voltage dependence of activation, smaller than the 13–15 mV shifts in the previously described I848T and L858H $hNa_v1.7$ erythromelalgia mutations (Cummins et al., 2004). F1449V substitution also produces an 4 mV depolarizing shift in fast inactivation, which is expected to increase the fraction of channels available for activation close to resting potential. Increased overlap between activation and steady-state inactivation for F1449V channels also increases the predicted window current. These changes each would be expected to lower the threshold of nociceptive DRG neurons which express mutant channels. Current-clamp recordings demonstrated, in fact, a lower threshold for single action potentials and high frequency firing in response to graded stimuli in DRG neurons expressing the mutant channel. The F1449V mutation also changed some $hNa_v1.7$ properties in ways that might decrease excitability, by enhancing slow inactivation and the rate of closed state inactivation. Because slow closed state inactivation promotes larger ramp currents, F1449V substitution would be expected to attenuate ramp currents. Indeed, in contrast to the I848T and L858H $hNa_v1.7$ erythromelalgia mutations which increased ramp currents (Cummins et al., 2004), F1449V did not alter ramp currents compared with wild-type channels. Domain III/S6 and the III–IV linker sequences are highly conserved among the known sodium channels. This region is crucial to fast inactivation (Patton et al., 1992), and has been implicated in other inherited disorders of excitability. Several mutations in the skeletal muscle sodium channel ($Na_v1.4$) that underlie myotonic disorders are located in this region. The $Na_v1.4$ V1293I mutation, at a position corresponding to V1444 in $hNa_v1.7$, five amino acids N-terminal to F1449, causes a mild variant of paramyotonia congenita. The functional consequences of the V1293I mutation in $hNa_v1.4$ (Green et al., 1998) are similar to those of F1449V

substitution in hNav1.7. Both mutations shift activation in a hyperpolarizing direction and inactivation in a depolarizing direction, and accelerate recovery from inactivation. In a model of muscle excitability, V1293I substitution lowered the threshold for action potential generation and caused myotonia; the 6 mV shift in activation was the major factor leading to this enhanced excitability (Green et al., 1998). However, myotonia produced by the Nav1.4-V1293I mutation is often precipitated by cold, whereas cooling reduces pain in most erythromelalgia patients with the F1449V mutation.

Current-clamp recordings demonstrated a lower current threshold for single action potentials and higher frequency firing in response to graded stimuli, in DRG neurons expressing mutant F1449V channels. Thus, even though F1449V displays a shift in activation that is relatively modest, deactivation that is not prolonged and ramp currents that are not enhanced in comparison with previously described mutations in Nav1.7 (Cummins et al., 2004), F1449V substitution imparts a gain-of-function change on DRG neurons, consistent with the autosomal dominant inheritance of familial erythromelalgia (Drenth et al., 2001; Yang et al., 2004) and our finding that affected family members are heterozygous for the mutation. Nevertheless, the inflection in voltage recordings, at which the upstroke of the all-or-none action potentials arose (approximately -20mV), was essentially the same in neurons expressing wild-type and F1449V channels. This probably reflects the role of Nav1.7 as a 'threshold channel' which activates at more hyperpolarized potentials so as to boost small, slow depolarizing inputs below action potential threshold (Cummins et al., 1998), and the fact that Nav1.8, with a threshold closer to 20 mV (Akopian et al., 1996), generates most of the current underlying the action potential upstroke in DRG neurons (Renganathan et al., 2001; Blair and Bean, 2002). The present results demonstrate that a sodium channel mutation can reduce the firing threshold and produce abnormal repetitive firing in sensory neurons in an inherited painful syndrome, primary erythromelalgia. Two anecdotal reports describe partial relief frompain in patients with erythromelalgia treated with lidocaine and mexilitine (Kuhnert et al., 1999; Davis and Sandroni, 2002), and a study on four patients reported reduced pain lasting for at least 2 years with oral mexilitine (Legroux-Crespel et al., 2003). Identification of this mutation and its role in the pathophysiology of erythromelalgia suggest that rational treatment with sodium channel blockers may be efficacious in this disorder.

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