SCN9A Mutations in Paroxysmal Extreme Pain Disorder: Allelic Variants Underlie Distinct Channel Defects and Phenotypes

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Summary

Paroxysmal extreme pain disorder (PEPD), previously known as familial rectal pain (FRP, or OMIM 167400), is an inherited condition characterized by paroxysms of rectal, ocular, or submandibular pain with flushing. A genome-wide linkage search followed by mutational analysis of the candidate gene *SCN9A*, which encodes hNa$_v$1.7, identified eight missense mutations in 11 families and 2 sporadic cases. Functional analysis in vitro of three of these mutant Na$_v$1.7 channels revealed a reduction in fast inactivation, leading to persistent sodium current. Other mutations in *SCN9A* associated with more negative activation thresholds are known to cause primary erythermalgia (PE). Carbamazepine, a drug that is effective in PEPD, but not PE, showed selective block of persistent current associated with PEPD mutants, but did not affect the negative activation threshold of a PE mutant. PEPD and PE are allelic variants with distinct underlying biophysical mechanisms and represent a separate class of peripheral neuronal sodium channelopathy.

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Introduction

Inherited disorders of ion channels are now known to account for a wide spectrum of human diseases characterized by paroxysmal dysfunction of excitable tissues. Understanding the molecular basis of these disorders has provided remarkable insights into normal physiological mechanisms (Ashcroft, 2000; Meisler and Kearney, 2005). The first such channelopathy to be described, hyperkalemic periodic paralysis, is caused by defects in the voltage-gated sodium channel of skeletal muscle Na$_v$1.4 (Ptacek et al., 1991; Rojas et al., 1991). Since this description, mutations in sodium channels have also been shown to cause epilepsy and inherited disorders of cardiac rhythm.

Here we describe a study of the molecular basis of Paroxysmal extreme pain disorder (PEPD), previously known as familial rectal pain (Fertleman and Ferrie, 2006), an autosomal dominant paroxysmal disorder of pain and autonomic dysfunction first identified by Hayden and Grossman (1959). Several further families have since been described (Mann and Cree, 1972; Schubert and Cracco, 1992), and there is evidence that this condition may be underdiagnosed (Elmslie et al., 1996). The distinctive features of PEPD are paroxysmal episodes of burning pain in the rectal, ocular, and mandibular areas accompanied by autonomic manifestations such as skin flushing. The anti-epilepsy drug carbamazepine is effective in many patients.

A genome-wide linkage search mapped the PEPD gene to a region of chromosome 2 encompassing *SCN9A*, which encodes the Na$_v$1.7 voltage-gated sodium channel. The expression pattern and function of this channel rendered *SCN9A* an excellent functional and positional candidate. Additionally, mutations in this channel were shown to underlie primary erythermalgia (PE) (Drenth et al., 2005; Yang et al., 2004), a disease associated with episodic burning pain, flushing of the extremities, inflammation, and swelling. Identification of a distinct repertoire of *SCN9A* mutations in PEPD, together with detailed functional analysis, suggest that these two disorders are allelic variants that are not only clinically but also mechanistically distinct, and that differences in the effect of the mutations on sodium channel activity account for the clear differences in the clinical phenotypes and in their differential responses to sodium channel-blocking drugs.

Results

Genetic Mapping of PEPD

We conducted a genome-wide linkage search in one large pedigree with PEPD, family 7 (Figure 1). We genotyped DNA from 13 affected and 13 unaffected family members using 455 microsatellite markers. The marker *D2S2330* on chromosome 2q gave a LOD score of 4.99 at $\theta = 0$. Haplotype analysis (Figure 1) identified critical recombinants at *D2S142* and *D2S335*, defining a 16 cM critical region containing a cluster of sodium channel genes. One of these, *SCN9A* (Klugbauer et al., 1995;

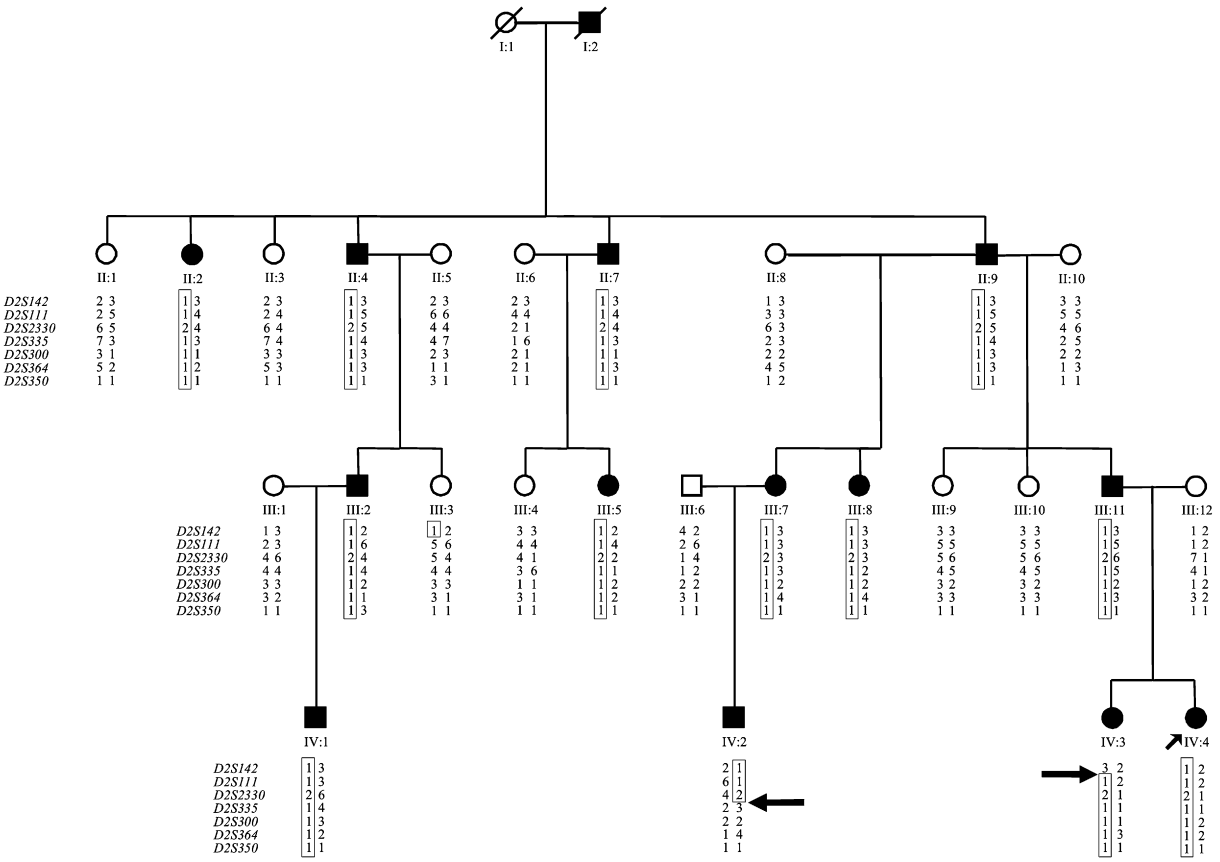

Figure 1. Familial Rectal Pain Maps to Chromosome 2q

Pedigree of family 7 showing haplotype of seven markers spanning a region of 26 cM on chromosome 2q24.1 to 32.1. DNA from all individuals was available apart from individuals I:1 and I:2. The disease chromosome is shown boxed and is inherited by all affected members of the pedigree. Two critical recombination events in individuals IV:2 and IV:3 delineate a critical region between *D2S142* and *D2S335* of approximately 16 cM. Pedigrees of other families with *SCN9A* mutations are available (see the Supplemental Data online).

Toledo-Aral et al., 1997), encoding the hNa$_v$1.7 voltage-gated sodium channel α-subunit, is expressed only in the peripheral nervous system, in both sympathetic neurons and the sensory neurons of the dorsal root ganglion (DRG) (Sangameswaran et al., 1997). Na$_v$1.7 mediates a tetrodotoxin (TTX) -sensitive, fast-inactivating current and has a role in acute and inflammatory pain (Nassar et al., 2004). We therefore investigated *SCN9A* as a positional and functional candidate.

Mutational Analysis of *SCN9A*

We designed intronic primers flanking the 26 exons of *SCN9A* to amplify genomic DNA from the probands of family 7 (individual IV:4, Figure 1), ten additional pedigrees (in family 15, only DNA from the proband's affected father was available), and two sporadic cases (families 8 and 9). We found a total of eight missense mutations in 8/13 families (Figure 2A) (See the Supplemental Data online for additional pedigrees and electropherograms). All patients are heterozygous for the respective mutations that cosegregated with the disorder in all affected members from whom DNA was available. In family 12, the proband is a compound heterozygote, having inherited R996C from his affected father, but also having a de novo mutation (V1298D). This individual is more severely affected than his father, indicating that R996C may represent a subtle mutation. Family 4, in which R996C is the only mutation, also appears to have a less severe phenotype not requiring medication.

None of these mutations were present in 192 ethnically matched control chromosomes (data not shown). All represent nonconservative amino acid changes likely to have an effect on channel function. Moreover, 7/8 mutations alter amino acids that are conserved across other human voltage-gated sodium channel α-subunits as well as those from other species (Figure 2B). Two mutations (I1461T, F1462V) occur within the highly conserved IFM amino acid motif in the linker region between domains III and IV (Figure 2Biii). Site-directed mutagenesis studies show that this motif forms the inactivation gate and plays a pivotal role in fast inactivation (West et al., 1992; Kellenberger et al., 1997). The T1464I mutation changes a completely conserved threonine residue immediately adjacent to the IFM domain (Figure 2Biii), and substitutions at this residue have also previously been shown to disrupt inactivation by substantially altering the stability of the inactivated state (Kellenberger et al., 1997). The residues V1298 and V1299 are also completely conserved (Figure 2Bii) and occur in the loop between domain III S4-S5, believed to interact with the domain III-IV inactivation motif (Smith and Goldin, 1997). The effect of mutations in the S4-S5 linker

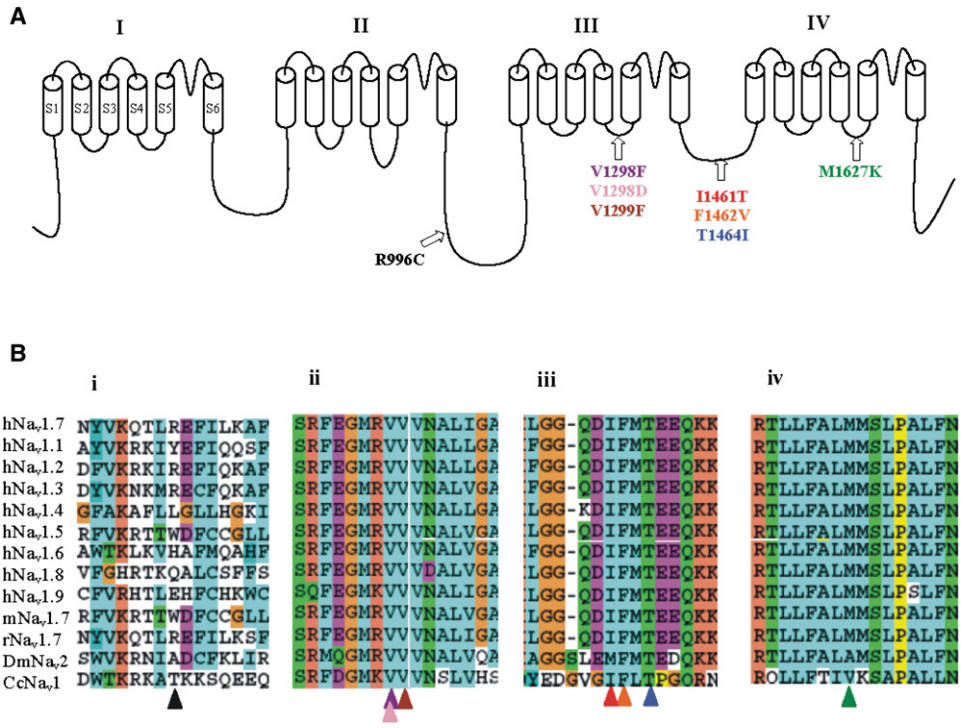

Figure 2. Mutations in the *SCN9A* Gene Underlie Familial Rectal Pain

(A) Diagram summarizing the nature and position of eight mutations within $Na_v1.7$. Text colors: black, R996C mutation (families 4 and 12); purple, V1298F (family 15); pink, V1298D (proband only, family 12); brown, V1299F (family 11); red, I1461T (family 1); orange, F1462V (sporadic case, family 9); blue, T1464I (family 7); green, M1627K (sporadic case, family 8).

(B) Sodium channel amino acid alignments. The human $Na_v1.7$ sodium channel ($hNa_v1.7$) amino acid sequence for the four distinct regions surrounding the eight PEPD missense mutations is compared with other human sodium channels ($hNa_v1.1$–1.9); mouse and rat $Na_v1.7$, and ancestral sodium channels from fruit fly and jellyfish. (Bi) Lack of conservation of the arginine residue at position 996. By contrast the other seven mutations alter highly conserved amino acids. (Bii) Complete conservation of valine residues at positions 1298 and 1299. (Biii) High degree of conservation of the IFM amino acid motif, which forms the inactivation gate and includes the residues I1461 and F1462, and of the adjacent threonine at position 1464. (Biv) Conservation of methionine at position 1627. The colored triangles indicating the positions of the mutated residues correspond to the colors used in (A) above.

in domain IV has previously been investigated in brain, heart, and skeletal muscle (McPhee et al., 1998). The M1627K mutation alters a conserved methionine residue (Figure 2Biv) that may form part of the inactivation gate receptor, as previous mutagenesis studies in heart ($Na_v1.5$) and skeletal muscle ($Na_v1.4$) channels suggest a role in fast inactivation (Filatov et al., 1998; Lerche et al., 1997; McPhee et al., 1998; Tang et al., 1996). In contrast, the arginine at position 996 is not conserved (Figure 2Bi), although the substitution by cysteine is not a conservative one and may have a subtle effect on channel function. This would be consistent with the less severe nature of the phenotype in family 4 and in the father in family 12.

Functional Analysis of Three PEPD Mutations

The highly conserved nature of seven of the eight amino acids mutated in these patients supports a causative role in the disorder, and their location makes it likely that channel inactivation would be affected. Therefore, we investigated the electrophysiological properties of three of these mutations (I1461T, T1464I, M1627K) using cDNA constructs of $hNa_v1.7$ transfected into HEK293 cells (Figure 3). Wild-type $hNa_v1.7$ expressed in HEK293 cells exhibits normal inactivation (Figures 3A–3C). All three mutant channels exhibited altered inactivation (Figures 3D–3F) with associated persistent currents, which were maintained for more than several hundred milliseconds in the two inactivation gate mutants (Figures 3D and 3E). There is a depolarizing (rightward) shift in steady-state inactivation (h_∞) curves for all three mutants (Figures 3G–3I) and inactivation is incomplete for I1461T and T1464I. For M1627K, the greater shift and reduction in voltage-dependence would widen the range of potentials over which "window current" (activation-inactivation gating overlap) could operate.

We further investigated the properties of T1464I and the in vitro effects of carbamazepine, as symptoms in patients with this mutation are effectively controlled using this drug. There was a depolarizing shift in the voltage-dependence of activation in this mutant (6.8 mV compared with wild-type, Figure 3C). The average inactivation time constant (τ_h) was less voltage-dependent and the kinetics of this partial inactivation were rapid (see the Supplemental Data available with this article online). The reduced sensitivity of inactivation rate to membrane potential is seen in other sodium channels with mutations in the domain III-IV linker (Cannon, 2000).

In response to long depolarizing steps, the T1464I persistent current was maintained (Figure 4A). In the presence of carbamazepine, this current was reduced in a concentration-dependent manner (Figures 4A–4C,

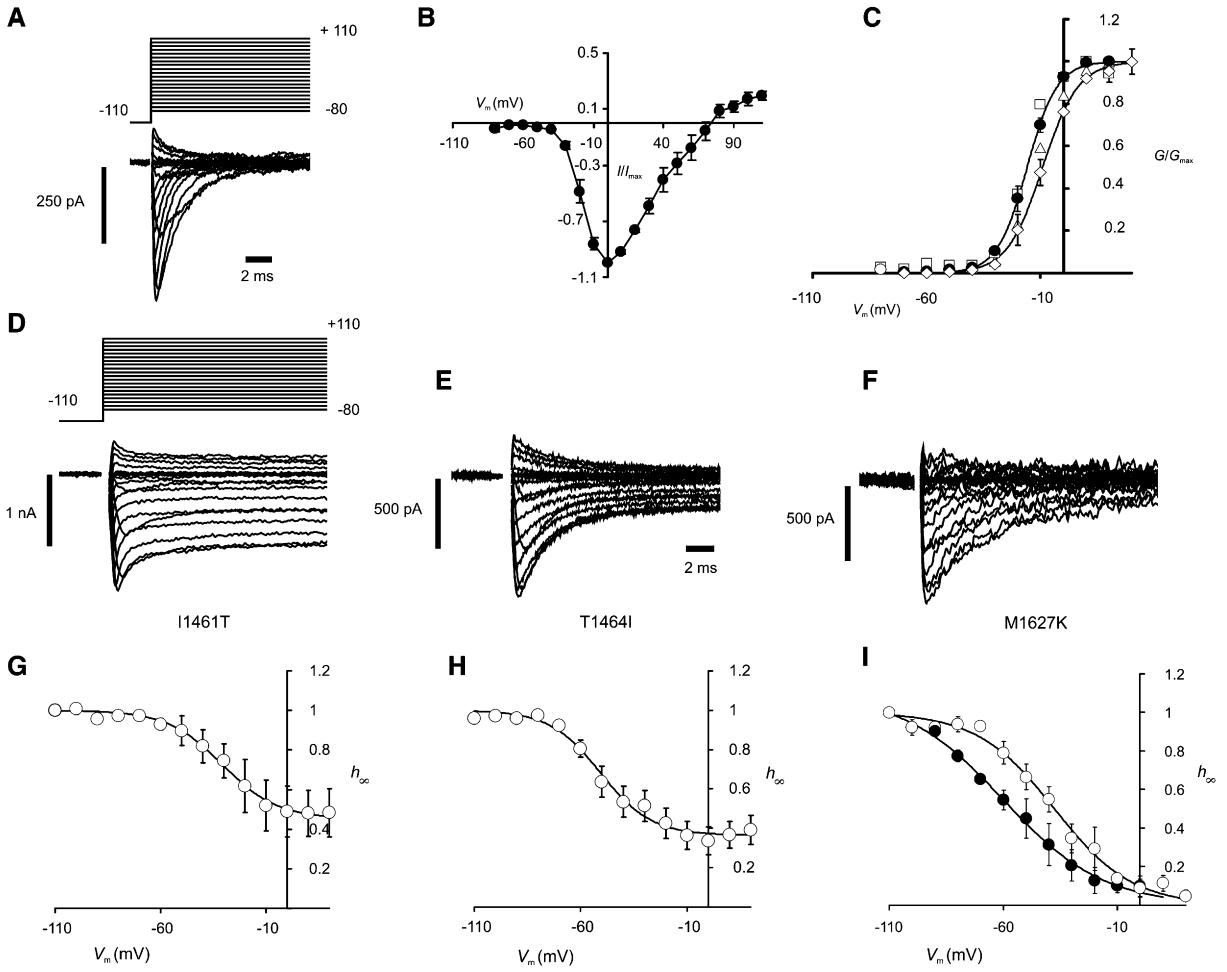

Figure 3. Mutants of hNa$_V$1.7 Associated with Familial Rectal Pain Exhibit Defects in Inactivation

(A) Example control tetrodotoxin (TTX) -sensitive currents recorded in transfected HEK293 cells in response to depolarizing voltage-clamp steps.

(B) Control peak current-voltage relation for hNa$_V$1.7 (n = 4), expressed as a ratio of the maximal peak current.

(C) Average conductance expressed as a ratio of peak average conductance (G/G_{max}) for control (black circles, n = 4), I1461T (open triangle, n = 5), M1627K (open square, n = 6) and T1464I (open diamond, n = 5). Smooth curves are Boltzmann relations drawn with best-fit parameters describing activation voltage-dependence for control and T1464I, with mid-points of −15.9 and −9.1 mV, respectively. Standard errors are plotted for control and T1464I, calculated from normalized currents.

(D–F) Recordings of currents from mutants I1461T, T1464I, and M1627K, respectively, that reveal partial inactivation. All currents could be completely blocked by superfusion of 250 nM TTX.

(G and H) Steady-state inactivation (h_∞) curves for I1461T and T1464I, respectively. Mutant I1461T and T1464I exhibit rightward-shifted and incomplete inactivation, measured with 100 ms prepulses (plotted as open circles, I1461T mid-point = −30.8 mV, maximal inactivation = 54.7%, n = 3; T1464I mid-point = −50.7 mV, maximal inactivation = 63.5%, n = 6; best-fit values for Boltzmann relations).

(I) Control h_∞, (plotted as black circles, n = 3), measured using 60 ms duration prepulses. Smooth curve through inactivation data is a Boltzmann relation, with a mid-point of −60.1 mV and a slope-factor of −21.9 mV. The h_∞ curve for M1627K is shown (open circles) (mid-point of −37.9 mV, slope = −17.0 mV, n = 5, 60 ms prepulse), and is significantly shifted to positive values in comparison with control data (between groups $p < 0.001$, two-way ANOVA, Sigma-Stat).

Where possible, data are given as mean ± SEM.

and the Supplemental Data) while the transient current evoked in the same protocol was spared. The dissociation constant (K_d) obtained from individual data sets is 32.7 ± 8.2 μM (n = 4), assuming that complete block is possible, a value within the range of therapeutic plasma concentrations observed in patients (Breton et al., 2005). Although these observations indicate that this mutant channel is sensitive to carbamazepine in vitro (as is the I1461T mutant, see the Supplemental Data), the therapeutic effect in vivo may also depend on its effects on the other sodium channels, including Na$_V$1.8, expressed in the same sensory neurons. The negative activation potential of a PE mutant, I848T (Yang et al., 2004) (Figure 4D), was unaffected by exposure to 100 μM carbamazepine, demonstrating the molecular basis for the difference in drug effectiveness in PEPD and PE.

Discussion

We have identified eight *SCN9A* mutations in families with PEPD. All are missense, indicating a gain-of-function or dominant-negative mechanism for the autosomal dominant inheritance displayed in familial cases. PEPD is the second inherited pain disorder to be shown to be

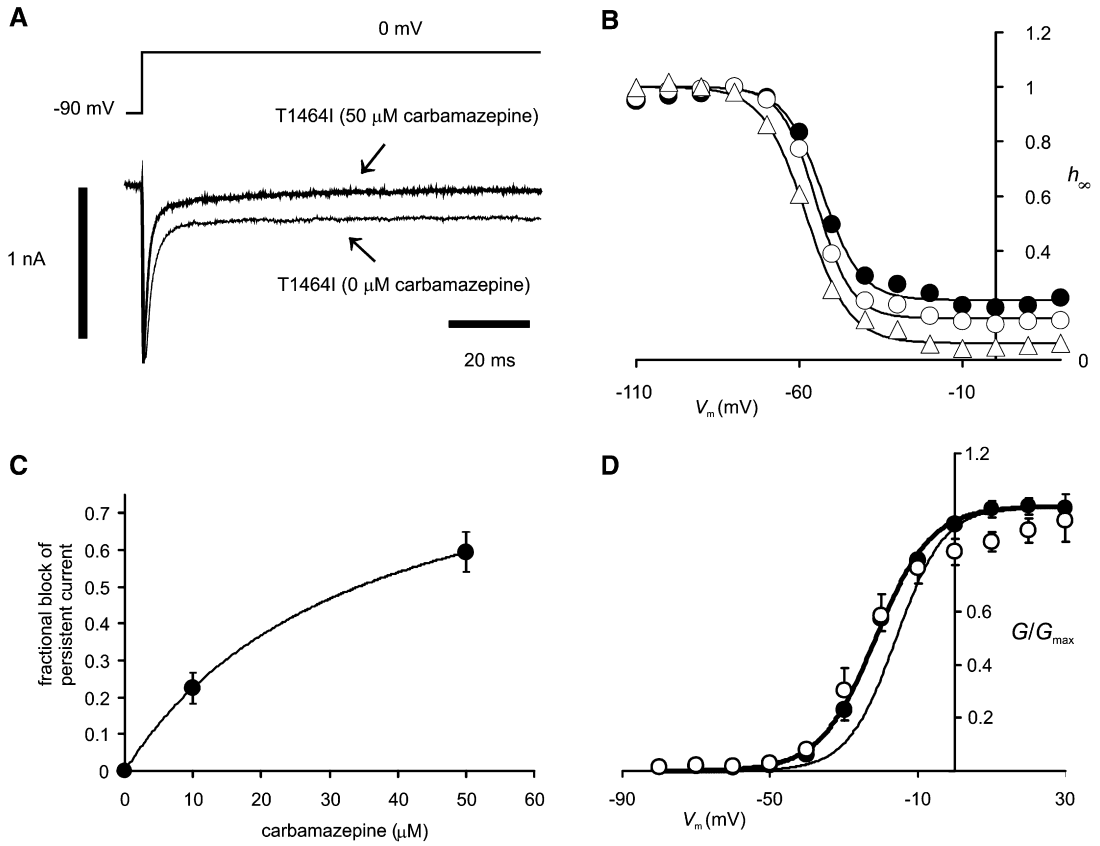

Figure 4. Carbamazepine Blocks Persistent Currents Generated by PEPD Mutant T1464I, But Does Not Affect the Activation Threshold of I848T Erythermalgia Mutant

(A) Persistent current generated following partial inactivation of T1464I is blocked by carbamazepine, whereas the transient current is spared in the voltage-clamp protocol.

(B) The effect of a range of drug concentrations on the h_∞ curve (100 ms duration prepulses) for the same cell as in (A) with the minimum value of *h* approaching zero as the drug concentration is increased. Carbamazepine appears to increase the maximal amount of inactivation in the mutant in a concentration-dependent manner (control, black circles; 10 and 50 μM, open circles and open triangles, respectively). Similar results were found in four other cells.

(C) The apparent K_d for block of persistent current was estimated from the asymptote of the h_∞ curve, without the drug, and in the presence of 10 and 50 μM carbamazepine (n = 4). Smooth curve is a rectangular hyperbola drawn according to best-fit parameters to the mean data, with an apparent K_d of 34.3 μM maximal block of 100% (K_d estimated from individual data sets, 32.7 ± 8.2 μM).

(D) Erythermalgia mutant I848T has a more negative activation threshold than normal. I848T (black circles, n = 7) half-maximal activation potential is −21.4 mV (best-fit Boltzmann curve, heavy line), shifted over 5 mV negative when compared with control curve (fine line, same as that shown in Figure 3C). Exposure to 100 μM carbamazepine (open circles, n = 4) does not shift the mutant activation threshold to more positive values.

attributable to mutations in *SCN9A*. Nine mutations have recently been shown to be associated with PE (Dib-Hajj et al., 2005; Drenth et al., 2005; Michiels et al., 2005; Yang et al., 2004). In contrast to phenotypes associated with mutations in *SCN1A* (Meisler and Kearney, 2005), sporadic cases with haplo-insufficiency have not been observed in these *SCN9A*-related disorders, and variable phenotypic expression is limited within families, suggesting that modifier genes are less important in channelopathies of peripheral nerves than in those of central neurons. Our results suggest that around two-thirds of families with PEPD have mutations in *SCN9A*, suggesting the existence of locus heterogeneity, as in other sodium channelopathies (George, 2005).

Altered function of hNa$_v$1.7 in nociceptive and sympathetic neurons is likely to underlie the pain and flushing common to both PEPD and PE. However, there are marked clinical differences between the phenotypes, as well as differential responses to medication. PEPD is essentially a visceral pain condition, often unprovoked, with marked cranial nerve involvement, whereas PE is a condition primarily affecting the extremities in which the triggers, such as exercise and temperature change, are well documented. To what extent can these differences, including the responses to sodium channel blockers, be accounted for by the functional effects caused by the mutations studied? Three PE mutations have been studied. Two, I848T and L858H (Yang et al., 2004), cause a significant hyperpolarizing shift in activation, slow deactivation, and increased response to small ramp depolarizations (Cummins et al., 2004). A third mutation, F1449V, produces a smaller hyperpolarizing shift in activation and a depolarizing shift in steady-state inactivation, leading to a lowering of the threshold for single action potentials and high-frequency firing in DRG neurons (Dib-Hajj et al., 2005). These effects are likely to reduce the activation threshold for sensory and nociceptive neurons, causing the chronic hyperalgesia,

inflammation, and central sensitization associated with activation through nonnoxious stimulation, e.g. walking.

In marked contrast to the functional effects of PE mutations, the three PEPD mutants studied here display marked deficits in sodium channel fast inactivation. Similar effects on fast inactivation have been described for muscle sodium channels in myotonia (Cannon, 2000; Wu et al., 2005). In PEPD such inactivation deficits may only become apparent after near-normal activation and would promote prolonged action potentials and repetitive firing in response to provoking stimuli, such as stretching and experiencing cold. In either case, it is likely that the role of $Na_v1.7$ as a "threshold channel" is a factor, with $Na_v1.8$ probably contributing to the regenerative current at the nerve ending (e.g., Strassman and Raymond, 1999; Renganathan et al., 2001; Carr et al., 2002).

Such allelic heterogeneity is not uncommon in the channelopathies. There are several well-documented examples of channels in which different mutational mechanisms lead to clinically distinct phenotypes. These include generalized epilepsy with febrile seizures plus (GEFS+) and severe myoclonic epilepsy of infancy in *SCN1A*; the skeletal muscle disorders, hyperkalaemic periodic paralysis (HYPP) and myotonia, caused by mutations in *SCN4A*; and the cardiac disorders long-QT and Brugada syndromes, caused by mutations in *SCN5A* (reviewed in detail in George, 2005). It is of interest to note that GEFS+, long-QT, and HYPP, in a situation analogous to that of PEPD, are largely the result of gain-of-function mutations leading to persistent sodium current.

That there are clear mechanistic differences between PEPD and PE is supported by their differential responses to drug therapy. Oral mexiletine and topical lidocaine, both local anesthetic/type 1b anti-arrhythmia drugs, have been reported to give relief in PE (Legroux-Crespel et al., 2003), whereas many PEPD patients respond well to the anti-epilepsy drug carbamazepine. Mexiletine is also effective in skeletal muscle myotonias arising from mutations in $hNa_v1.4$ (Mohammadi et al., 2005). It is noteworthy that certain myotonia mutations (e.g., substitutions of R1448) respond well to the drug, an effect ascribed to an increased probability of the channel being in closed-state inactivation and/or enhanced recovery from fast inactivation (Mohammadi et al., 2005). A similar effect on channel kinetics is seen in the PE mutant F1449V, so the response to mexiletine may be mutation-specific. Carbamazepine also blocks sodium channels in a use-dependent manner and has long been established as a treatment for neuropathic pain (Backonja, 2002), but has little or no effect in PE (Waxman and Dib-Hajj, 2005). This is consistent with pain in PEPD being precipitated by persistent Na current (because carbamazepine preferentially suppresses persistent Na current, as demonstrated for two of the mutants studied—see the Supplemental Data) in comparison with the pain in PE being caused by lowered activation thresholds.

In summary, these observations establish the existence of allelic heterogeneity in a class of sodium channelopathy affecting peripheral neurons. PEPD and PE are both inherited autosomal dominant inflammatory pain conditions, but have entirely distinct phenotypes. Our data demonstrate that these distinct phenotypes are the result of distinct repertoires of mutations leading to either lowered thresholds of activation (PE) or defective inactivation (PEPD) These data further emphasize the critical role of $Na_v1.7$ in human inflammatory pain and explain the differential drug sensitivity of PEPD and PE.

Experimental Procedures

Subjects

This study was approved by the joint UCL/UCLH committees on the ethics of human research. Informed consent was obtained from all participants or their parents. Twelve families and two sporadic cases (family 8 and 9) with PEPD were ascertained using the diagnostic criteria outlined below.

Diagnostic Criteria

PEPD is a rare, autosomal dominant paroxysmal disorder of pain and autonomic dysfunction that presents with four types of painful episode: (1) birth crisis, when babies are born red and stiff; (2) rectal crisis, which is triggered by defecation in infants and young children and by a variety of emotional factors in older children and adults; (3) ocular crisis, which may be provoked, but is more usually spontaneous, and (4) mandibular crisis, which is often triggered by eating and yawning. The distinctive features of this condition are paroxysmal episodes of burning pain in the rectal, ocular, and mandibular areas, accompanied by the autonomic manifestations such as skin flushing, reflex asystolic syncopal events, lacrimation, and rhinorrhoea. In between episodes, physical examination is normal, and investigations such as imaging or tissue biopsy are normal. Diagnosis therefore relies on the history and either direct observation or video of the episodes. The former is, of course, more helpful in adults and the latter in infants and children.

Family 1 was originally referred with an erroneous diagnosis of hyperekplexia. Other families were ascertained from previous publications (Mann and Cree, 1972; Schubert and Cracco, 1992) or by direct contact from those managing affected children. Family 7 is a recently ascertained large pedigree from Berkshire, UK and was selected for linkage analysis. Detailed clinical documentation of this family will be published elsewhere. In all cases, DNA was extracted from venous blood or buccal swabs from patients and unaffected family members using standard methods.

Genotyping and Linkage Analysis

Primers flanking a total of 455 polymorphic loci primarily identified from the Généthon map spanning the human genome were used in the genome search in family 7. Primers were obtained from MRC HGMP Resource Centre, Research Genetics, MWG Biotech, and Invitrogen. These microsatellites were genotyped using a fluorescent-based semi-automated method on automated DNA sequencing machines (ABI 373A, ABI 3100).

Mutation Screening

Primers were designed for the mutation screen of *SCN9A* from the genomic sequence obtained from the Human Genome Browser (http://genome.ucsc.edu/). Intronic primers were designed to flank each exon (or groups of exons if separated by small introns). Exons from each of the eight probands and appropriate controls were amplified and sequenced on an automated DNA sequencer (ABI 373A or ABI 3100) and the sequences aligned and compared using Sequence Navigator v1.0.1.

Cloning and Mutagenesis

A full-length version of the human $Na_v1.7$ cDNA was cloned into a modified version of the expression vector pcDNA3 (Klugbauer et al., 1995). For functional analysis, three mutated constructs were made (I1461T, T1464I, M1627K). These single base substitutions were introduced into this wild-type clone by PCR-based site-directed mutagenesis using the QuikChange II XL kit (Stratagene, La Jolla, CA). Correct introduction of these substitutions was confirmed by complete sequencing of the inserts.

Transfection

HEK293 cells were grown in sparse culture in 35 mm diameter Petri dishes. Transfections were carried out in serum-free medium by incubating with pcDNA3 incorporating $Na_v1.7$ cDNA and mutant DNA 0.8–1.3 μg 3 μl lipofectamine 2000, 0.2 μg pBS500 for 2 hr at 37°C. The medium was then replaced with normal culture medium incorporating 5% serum. Recordings were made from 1–2 days later.

Solutions and Electrophysiology

Conventional voltage-clamp recordings in the whole-cell patch-clamp configuration were made from HEK293 cells 1–2 days following transfection, using an Axopatch 200B amplifier (Axon Instruments, Union City, California, USA) driven from a PC generating pulse protocols (Pclamp 9, Axon Instruments). The extracellular solution contained the following: 140 mM NaCl, 10 mM Tetraethylammonium Cl, 10 mM HEPES, 2.1 mM $CaCl_2$, 2.12 mM $MgCl_2$, 0.5 mM 4-Aminopyridine, 7.5 mM KCl, and 0.1 mM CdCl. The intracellular solution contained the following: 130 mM CsCl, 13 mM CsF, 3 mM EGTA (Na), 10 mM Tetraethylammonium Cl, 10 mM HEPES, 1.21 mM $CaCl_2$, 3 mM ATP(Mg), and 500 μM GTP (Li). The external and internal solutions were buffered to pH 7.2–7.3 with the addition of NaOH and CsOH, respectively. Electrodes were made from thin-walled glass capillaries (Harvard Apparatus, Edenbridge, Kent, UK), with initial resistances of 1–2 MΩ when filled with recording solution. During recording, series-resistance compensation was always used and set to 70% or higher. Families of sodium currents were recorded in response to a series of incrementing depolarizing voltage-clamp steps from a holding potential of −90 mV. Steady-state inactivation protocols comprised an incrementally depolarizing prepulse, 60 or 100 ms in duration, followed immediately by a depolarizing step to +10 mV. Records were usually filtered at 5 KHz (4-pole Bessel) and sampled at 20 or 10 KHz. Current traces were generated by averaging three or more records. Leak subtraction was achieved using a *P/N* protocol, where five reverse polarity clamp-steps were used to produce the leakage record. TTX was applied to ascertain that the currents were indeed TTX-s sodium currents. The HEK293 cells failed to generate TTX-s currents unless they were transfected with an $hNa_v1.7$ clone. Because HEK293 cells can generate nonlinear leakage currents, records of membrane currents were made both before and after the application of TTX, and the TTX-sensitive component was isolated by off-line digital subtraction. TTX (250 nM) and carbamazepine (up to 200 μM) were applied by local superfusion. Carbamazepine was made up as a stock solution of 50 mM or 200 mM in DMSO. Recordings were made at room temperature.

Data Analysis

Peak currents were converted to conductances, assuming that the sodium equilibrium potential (E_{Na}) was +70 mV, allowing activation curves to be plotted. Boltzmann relations were drawn according to best-fit parameters (Sigma-Stat) for activation curves and h_∞ curves. The maximal block in the presence of carbamazepine was derived from the asymptote of the h_∞ curve or (as in the Supplemental Data) by measuring the residual current amplitude at 0 mV. Concentration block data for carbamazepine was described as a rectangular-hyperbola (Langmuir isotherm), assuming that 100% block was achievable, from which the apparent K_d for block could be derived.

Supplemental Data

The Supplemental Data for this article includes six figures and can be found online at http://www.neuron.org/cgi/content/full/52/5/767/DC1/.

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