A sodium channel mutation linked to epilepsy increases ramp and persistent current of Nav1.3 and induces hyperexcitability in hippocampal neurons

Mark Estacion 1, Andreas Gasser 1, Sulayman D. Dib-Hajj, Stephen G. Waxman *  

Department of Neurology, Yale University School of Medicine, New Haven, CT 06510, USA  
Center for Neuroscience and Regeneration Research, Yale University School of Medicine, New Haven, CT 06510, USA  
Rehabilitation Research Center, Veterans Affairs Connecticut Healthcare System, West Haven, CT 06510, USA

A R T I C L E   I N F O  
Article history:  
Received 15 February 2010  
Revised 12 April 2010  
Accepted 16 April 2010  
Available online 24 April 2010  

Keywords:  
Sodium channel  
Epilepsy  
Hippocampal neuron  
Voltage-clamp  
Current-clamp

A B S T R A C T  
Voltage-gated sodium channelopathies underlie many excitability disorders. Genes SCN1A, SCN2A and SCN9A, which encode pore-forming a-subunits Nav1.1, Nav1.2 and Nav1.7, are clustered on human chromosome 2, and mutations in these genes have been shown to underlie epilepsy, migraine, and somatic pain disorders. SCN3A, the gene which encodes Nav1.3, is part of this cluster, but until recently was not associated with any mutation. A charge-neutralizing mutation, K345Q, in the Nav1.3 DI/S5-6 linker has recently been identified in a patient with cryptogenic partial epilepsy. Pathogenicity of the Nav1.3/K345Q mutation has been inferred from the conservation of this residue in all sodium channels and its absence from control alleles, but functional analysis has been limited to the corresponding substitution in the cardiac muscle sodium channel Nav1.5. Since identical mutations may produce different effects within different sodium channel isoforms, we assessed the K345Q mutation within its native Nav1.3 channel and studied the effect of the mutant Nav1.3/K345Q channels on hippocampal neuron excitability. We show here that the K345Q mutation enhances the persistent and ramp currents of Nav1.3, reduces current threshold and produces spontaneous firing and paroxysmal depolarizing shift-like complexes in hippocampal neurons. Our data provide a pathophysiological basis for the pathogenicity of the first epilepsy-linked mutation within Nav1.3 channels and hippocampal neurons.

© 2010 Elsevier Inc. All rights reserved.

Introduction  
Monogenic linkage of sodium channelopathies and disorders of excitability is now well-documented in skeletal muscle (SCN4A/Nav1.4, Cannon 2006), sensory neurons (SCN9A/Nav1.7, Dib-Hajj et al., 2007), cardiac myocytes (SCN5A/Nav1.5, George 2005); and brain; (Meisler and Kearney, 2005). More than 300 sodium channel mutations, mostly in SCN1A which encodes sodium channel NaV1.1, together with a smaller number of mutations in SCN2A which encodes sodium channel NaV1.2 (Lossin 2009) have been associated with epilepsy (George 2004; Helbig et al., 2008; Noebels 2003; Sisodiya et al., 2007), the gene which encodes NaV1.3, is part of this cluster, but until recently was not associated with any mutation. A charge-neutralizing mutation, K345Q, in the Nav1.3 DI/S5-6 linker has recently been identified in a patient with cryptogenic partial epilepsy. Pathogenicity of the NaV1.3/K345Q mutation has been inferred from the conservation of this residue in all sodium channels and its absence from control alleles, but functional analysis has been limited to the corresponding substitution in the cardiac muscle sodium channel NaV1.5. Since identical mutations may produce different effects within different sodium channel isoforms, we assessed the K345Q mutation within its native NaV1.3 channel and studied the effect of the mutant NaV1.3/K345Q channels on hippocampal neuron excitability. We show here that the K345Q mutation enhances the persistent and ramp currents of NaV1.3, reduces current threshold and produces spontaneous firing and paroxysmal depolarizing shift-like complexes in hippocampal neurons. Our data provide a pathophysiological basis for the pathogenicity of the first epilepsy-linked mutation within NaV1.3 channels and hippocampal neurons.

© 2010 Elsevier Inc. All rights reserved.
requires functional analysis of the K354Q mutation within NaV1.3, and assessment of the contribution of the mutant NaV1.3/K354Q channels to excitability of CNS neurons. Here we report gain-of-function properties of NaV1.3/K354Q mutant channels and demonstrate that they produce epileptiform changes in transfected hippocampal neurons.

Methods

Plasmid and transient transfection

For comparison of biophysical properties of WT NaV1.3 and the NaV1.3/K354Q mutant, we studied both by patch-clamp using a rat NaV1.3 construct. The construct, converted to a tetotoxin-resistant form (TTX-R, rNaV1.3s) by the Y834S substitution, permits the current produced by rNaV1.3 to be studied in isolation after expression in neuronal backgrounds when TTX is included in the bath solution, but does not alter the voltage-dependence or kinetics of the channel ([Cummins et al., 2001; Herzog et al., 2003]). The K354Q mutation was introduced using QuickChange XL II site-directed mutagenesis (Stratagene, La Jolla, CA). HEK293 cells, grown under standard culture conditions (5% CO2, 37 °C) in Dulbecco’s Modified Eagle’s Medium supplemented with 10% fetal bovine serum, were transiently-transfected with plasmids containing β1-fires-CD4 and β2-fires-GFP (Lossin et al., 2002) and either rNav1.3h (referred to as WT hereinafter) or rNaV1.3s/K354Q using Lipofectamine2000 (Invitrogen, Carlsbad, CA) with a stoichiometry of plasmids of 1:1:5 by mass. Since our HEK cells do not express endogenous Na-current, no TTX was necessary for the experiments.

Primary hippocampal neuron isolation and transfection

Animal protocols complied with NIH guidelines, and were approved by the VA Connecticut Healthcare System Animal Use Committee. Sprague–Dawley rat pups (Harlan, Indianapolis, IN) between postnatal days 10 and 14 were anesthetized with ketamine/xylazine (100/10 mg/kg, i.p.) and sacrificed by decapitation. Hippocampi were dissected out in ice-cold HABG [HA medium (Brain Bits, Springfi eld, IL) supplemented with 10% fetal bovine serum, were equilibrated with 80–90% humidity and 37 °C], sliced into 300 µm sections using a McIlwain tissue chopper (Warner Instruments, Hamden, CT) and digested (30 min at 37 °C) with papain (30 U/ml; Worthington, Lakewood, NJ) in calcium-free HA medium (Brain Bits) with 0.5 mM Glutamax. After digestion, tissue was resuspended in HABG and triturated with a 1 ml plastic pipette tip. After setting of remaining tissue, the supernatant was filtered through 40 µm mesh and centrifuged at 1000 × g (4 min, room temperature). For transfection, hippocampal neurons were resuspended in 100 µl of buffer (1.5 × 107 cells/ml). 10 µl aliquots were transfected by electroporation (MicroPorator, Harvard Apparatus, Holliston, MA, 2 pulses, each 15 ms, of 1500 V) using 0.5 µg of plasmid DNA per transfection (0.4 µg WT or K354Q, and 0.1 µg EGF). Following electroporation, cells were resuspended and incubated for 5 min in 45 µl calcium-free HABG medium at room temperature. 45 µl of complete medium [Neurobasal medium, supplemented with 2% B-27, 0.5 mM Glutamax, 10 µg/ml gentamycin, 5 ng/ml recombinant mouse FGF2, and 5 ng/ml recombinant mouse PDGFβb (Invitrogen)] was added. Cells were plated on 12 mm glass coverslips coated with poly-o-lysine and laminin (BD Biosciences, Bedford, MA), placed in a 24 well plate, and incubated at 37 °C for 1 h to allow attachment. Finally, 500 µl/well complete medium was added, and cells were incubated at 37 °C until used for recordings at 40–50h post-transfection. At this time culture neurites are sparse, permitting isolated pyramidal-shaped neurons to be studied by patch-clamp.

Electrophysiology: Voltage-clamp

Whole-cell voltage-clamp recordings were obtained using the following solutions. The extracellular solution contained (in mM):

- 140 NaCl, 3 KCl, 1 MgCl2, 1 CaCl2, and 10 HEPES, pH 7.3 with NaOH (adjusted to 320 mOsm with dextrose). The pipette solution contained (in mM): 135 Cs-Aspartate, 10 NaCl, 2 MgCl2, 0.1 CaCl2, 1.1 EGTA (pCa = 8), 10 HEPES, pH 7.2 with CsOH (adjusted to 310 mOsm with dextrose). Patch-pipettes had a resistance of 1–3 MΩ when filled with pipette solution, and once whole-cell recording mode was achieved the access resistance averaged 4–5 MΩ. The junction potential of 16 mV (calculated by JPCalc, CLAMP software) was compensated by setting holding potential during the seal test period to −16 mV. Once the seal had formed, these two solutions were no longer in contact and the applied potential was correct. Upon achieving whole-cell recording configuration, pipette and cell capacitance were manually minimized using Axopatch 200B (Molecular Devices, Union City, CA) compensation circuitry. To reduce voltage errors, 80–90% series resistance and prediction compensation were applied. Cells were excluded from analysis if the predicted voltage error exceeded 3 mV. Recorded currents were digitized using pCLAMP software (version 10) and a Digidata 1440A interface (Molecular Devices) at 50 kHz after passing through a low-pass Bessel fi lter setting of 10 kHz. Linear leak and residual capacitance artifacts were subtracted out using the P/N method (Clampex software). Sodium current recordings were initiated after a 5 min equilibration period once whole-cell configuration was achieved.

Data analysis was performed using Clampfit (Molecular Devices) or Origin (Microcal Software, Northampton, MA). To generate activation curves, cells were held at −100 mV and stepped to −80 to +40 mV in 5 mV increments for 100 ms. Peak inward currents from activation protocols were converted to conductance values using the equation, G = I/(Vm−Em), where G is the conductance, I is the peak inward current, Vm is the membrane potential step used to elicit the response and Em is the sodium reversal potential (determined for each cell using the x-axis intercept of a linear fit of peak inward current responses). Conductance data were normalized by maximum conductance and fit with a Boltzmann equation of the form G = (Gmax − Gmin)/(1 + exp[(Vm−V1/2 − Vmin)/k]), where V1/2 is the activation midpoint and k is the slope factor. To generate steady-state fast-inactivation curves, cells were stepped to inactivating potentials of −180 to −20 mV for 500 ms followed by a 50 ms step to −10 mV. Peak inward currents obtained from steady-state fast inactivation protocol were normalized by maximum current amplitude and fit with a Boltzmann equation of the form I = Imin + (Imax − Imin)/(1 + exp[(Vm−V1/2)/k]), where Vm represents the inactivating pre-pulse membrane potential and V1/2 represents the midpoint of inactivation. Data are expressed as means ± standard error (SEM). Statistical significance was determined by Student’s t-test.

Electrophysiology: Current-clamp

Whole-cell current-clamp recordings were obtained using the Axopatch 200B amplifier, digitized using the Digidata 1440A interface and controlled using pCLAMP software. The bath solution for current-clamp recordings was the same as for voltage-clamp recordings specified above with the exception that CaCl2 and MgCl2 were increased from 1 mM to 2 mM. The pipette solution contained (in mM): 122 K-methanesulfonate, 0.9 EGTA, 9 HEPES, and 1.8 MgCl2, 4 Mg-ATP, 0.3 GTP (Tris salt) and 14 phosphocreatine (di-Tris salt) pH 7.4 with KOH (adjusted to 300 mOsm with dextrose). No junction potential correction was applied for current-clamp experiments. Recordings were obtained 40–50 h post-transfection from transfected hippocampal pyramidal neurons with small diameter (10–15 µm) triangular cell bodies that exhibited GFP fluorescence. While recordings from multiple cells could be obtained from the same coverslip, the coverslip was replaced if the time since transferring to the recording chamber exceeded an hour. Whole-cell configuration was obtained in voltage-clamp mode before proceeding to current-clamp recording mode. After initiating current-clamp, a small (−2 to
which directly gives the voltage midpoint (significantly higher than WT at −0.4%, peak inward current, the average persistent current measured at a −15 mV test potential for K354Q constructs and current-clamp recording conditions were used to study firing properties in transfected pyramidal neurons. The average capacitance of the recorded pyramidal neurons was 8.5 ± 0.5 pF (n = 20) for WT, and 7.5 ± 0.5 pF (n = 22) for NaV1.3/K354Q transfected cells. The input resistance for these neurons was high at 2.9 ± 0.7 GΩ for WT and 2.7 ± 0.4 GΩ for NaV1.3/K354Q transfected cells. The resting membrane potential started depolarized (−40 ± 3 mV for WT, −39 ± 3 mV for NaV1.3/K354Q transfected cells) but only needed small hyperpolarizing holding currents to re-establish a resting membrane potential near −80 mV. Only small current were required to reach threshold (38 ± 10 pA for WT, 16 ± 15 pA for NaV1.3/K354Q transfected cells; p = 0.025).

A notable difference between neurons transfected with NaV1.3/K354Q compared to neurons transfected with WT channels was a population of neurons that exhibited spontaneous firing. The NaV1.3/K354Q transfected neurons showed spontaneous firing of action potentials in 6 of 16 cells, while only 1 of 13 WT transfected neurons fired spontaneously. The majority of the NaV1.3/K354Q transfected neurons that exhibited spontaneous activity also showed fluctuations of resting membrane potential that sometimes elicited bursts of action potentials as illustrated in Fig. 3. Paroxysmal depolarizing shift-like complexes (arrows and panels D1, D2, and D3 in Fig. 3) were present in 4 of 16 neurons expressing the NaV1.3/K354Q mutation (including 3 neurons, such as the one shown in Fig. 4, which were clearly isolated and not contacted by axons), but were not present in any of the 13 WT expressing neurons studied by current-clamp.

Discussion

Although more than 300 epilepsy-linked sodium channel mutations have been reported, the great majority have been mutations of NaV1.1 and NaV1.2. Only one mutation, K354Q, has recently been reported in NaV1.3 in a patient with cryptogenic epilepsy (Holland et al., 2008). This mutation was not examined physiologically within NaV1.3 but, because of challenges associated with instability of NaV1.3 during cloning and maturation, was functionally profiled within cardiac NaV1.5 sodium channels (Holland et al., 2008). The functional effects of single amino acid substitutions, however, can differ substantially depending on the channel isoform in which they occur (Lampert et al., 2006a; Lossin et al., 2002). In the present study, we expressed the K354Q mutation in its native NaV1.3 background, and have demonstrated by voltage-clamp that this mutation enhances the NaV1.3 persistent current nearly two-fold and ramp current more than two-fold. We demonstrate by current-clamp that transfection of hippocampal pyramidal neurons with the mutant NaV1.3 channel reduces current threshold, and produces spontaneous firing and paroxysmal depolarizing shift-like complexes.

The present results build upon a previous demonstration of persistent sodium current in unjured mammalian hippocampal neurons (French et al., 1990), which are known to express NaV1.1, NaV1.2, NaV1.3, and NaV1.6 isoforms of sodium channels (Whitaker et al., 2001). Previous studies have demonstrated that NaV1.3 can produce a non-inactivating, persistent current (Lampert et al., 2006a; Sun et al., 2007). A number of studies (e.g. Alzheimer et al., 1993) indicate that persistent current participates in the control of intrinsic neuronal excitability. There is, for example, also evidence indicating that persistent sodium currents can drive spontaneous neuronal firing in a variety of cell types (Baker et al., 2003; Pennartz et al., 1997; Taddei...
Fig. 1. Voltage-clamp of WT and K354Q mutant channels. (A) Superimposed selected traces recorded from a HEK293 cell expressing WT channels in response to the activation stimulation protocol (100 ms duration pulses, see inset). To better illustrate the persistent current remaining at the end of the 100 ms pulses, portions of three of the traces are plotted on an expanded scale. Note that the unit of the vertical scale bar for the inset is in units of percent of maximal peak current. (B) Superimposed selected traces from the same cell as in (A) in response to the fast-inactivation pulse protocol (see inset). (C) Selected traces recorded from a HEK293 cell expressing K354Q channels are illustrated in the same format as in panel (A). (D) Superimposed selected traces from the same cell as in (C) in response to the fast-inactivation pulse protocol. (E) The peak currents for each cell are analyzed to obtain the conductance–voltage (G–V) relation as described in the Methods. The G–V curves for both WT (filled squares) and K354Q (open squares) cells are normalized and then averaged to obtain the plotted data. (F) The responses to the fast-inactivation protocol are analyzed to obtain the voltage-dependence of fast-inactivation as described in the Methods. Normalized and averaged data are shown for both WT (filled squares) and K354Q (open squares) channels. Error bars are standard error of the mean (SEM).
and Bean 2002) including hippocampal neurons (Azouz et al., 1996) and, in fact, large persistent currents tend to be present in subicular neurons isolated from patients with temporal lobe epilepsy (Vreugdenhil et al., 2004). A number of mutations of NaV1.1 associated with epilepsy have been demonstrated to enhance persistent current (Escayg et al., 2000; Lossin et al., 2002, 2003; Rhodes et al., 2004). Interestingly, persistent sodium current tends to be reduced by the anti-convulsants phenytoin, carbamazepine, and topiramate (Lampl et al., 1998; Sun et al., 2007). Enhanced persistent current, as produced by the NaV1.3/K354Q mutation, would be expected to lead to neuronal hyperexcitability. The evidence presented here thus establishes the potential of the K354Q mutation, in NaV1.3, as pathogenic for epilepsy.

While persistent sodium currents can amplify EPSPs (Llinas and Sugimori 1980; Stuart and Sakmann 1995; Lipowsky et al., 1996), the hippocampal neurons that we studied had been dissociated and cultured for less than 50 h prior to recording, suggesting that they had not developed robust synaptic connections. Consistent with the appearance of paroxysmal depolarizing shift-like complexes in neurons expressing the NaV1.3/K354Q mutation in the absence of synaptic events, similar complexes characterized by multiple action potentials superimposed on prolonged depolarizations can be recorded from peripheral sensory axons following axotomy (Kocsis and Waxman, 1983). The appearance of these complexes, which can

**Fig. 2.** K354Q channels exhibit an enhanced ramp response. The currents evoked during a smoothly increasing voltage ramp from $-100$ mV to $+20$ mV over 600 ms are shown from a WT (gray line) and K354Q (black line) expressing cell. The traces have been normalized to the maximal peak current recorded from that cell. The amplitude and corresponding voltage of the inward peak of the slow ramps were averaged and plotted as the gray circle (WT) and black circle (K354Q). Error bars are standard error of the mean (SEM).

**Fig. 3.** K354Q-expressing hippocampal neurons exhibit spontaneous activity. Continuous segments of current-clamp recording from hippocampal pyramidal cells illustrate patterns of spontaneous action potential firing seen in a subset of cells expressing the K354Q mutant channels. Cells were held at a resting potential of $-80$ mV. Traces A–D are two minute recordings from four different cells. Small regions of trace (D) are expanded and labeled D$_1$, D$_2$, and D$_3$ to illustrate action potentials riding on top of transient paroxysmal depolarizing shift-like depolarizations of the membrane potential, which are indicated by arrows in traces B and C.
be observed along axonal trunks many millimeters distant from, and in electrical isolation from, regions of synaptic output, has been correlated with the up-regulation of Na<sub>1.3</sub> along sensory axons following transection (Waxman et al., 1994; Black et al., 1999). In both cases, enhanced levels of activity of Na<sub>1.3</sub> appear to produce neuronal hyperexcitability.

The S5–S6 linkers of voltage-gated sodium channels [i.e. the part of the channel where the K354Q mutation is located] contribute to the channel pore, thus mutations in these extracellular linkers may induce allosteric effects that alter gating properties. Indeed, missense mutations in the DI/S5–S6 linker of sodium channel Na<sub>1.1</sub> have been linked to epilepsy (Lossin, 2009), including the Na<sub>1.1</sub>/R356G mutation associated with severe infantile epilepsy (Marini et al., 2007) which, like Na<sub>1.3</sub>/K354Q, neutralizes a positive charge within this linker. However, Na<sub>1.1</sub> channels carrying these mutations have not been functionally tested to determine the effects on the gating properties of the mutant channels. It is possible that mutations of similar chemical nature (charge neutralization in this case) of closely positioned conserved residues within the DI/S5–S6 linker may result in different types of epilepsy, depending on the sodium channel isoform.

Transcription of Na<sub>1.3</sub> channels is up-regulated within dorsal root ganglion (DRG) neurons following nerve injury (Black et al., 1999; Kim et al., 2001; Waxman et al., 1994), a finding that has prompted a search for subtype-specific Na<sub>1.3</sub> blockers as potential pharmacotherapeutics for neuropathic pain. Supporting the hypothesis that a paucity of this channel in adult CNS tissues might make Na<sub>1.3</sub> a tractable target for pain pharmacotherapeutics, expression of Na<sub>1.3</sub> is high in the embryonic rat brain, but is down-regulated as development proceeds (Beckh et al., 1989; Black et al., 1996; Felts et al., 1997; Waxman et al., 1994). However, the available immuno-cytocchemical evidence suggests that Na<sub>1.3</sub> is present in the adult human brain (Whitaker et al., 2001), although the function of these channels in the CNS has remained unexplored. The present results indicate that gain-of-function mutations of Na<sub>1.3</sub> can decrease threshold and induce epileptiform activity in hippocampal neurons, and provide strong support for the idea that Na<sub>1.3</sub> mutations can produce epilepsy in postnatal humans. These results more generally suggest that Na<sub>1.3</sub> channels may be functional in the postnatal human brain, and indicate the need for careful assessment of CNS function in studies on Na<sub>1.3</sub> blockade in human subjects.

Acknowledgments

We thank Lynda Tyrrell and Bart Toftness for excellent technical assistance. This work was supported by the Medical Research Service and Rehabilitation Research Service, Dept. of Veterans Affairs and by grants from the National Multiple Sclerosis Society and the Erythromelalgia Association. The Center for Neuroscience and Regeneration Research is a Collaboration of the Paralyzed Veterans of America and the United Spinal Association with Yale University.

References


M. Estacion et al. / Experimental Neurology 224 (2010) 362–368
368  M. Estacion et al. / Experimental Neurology 224 (2010) 362–368