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

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ABSTRACT

Voltage-gated sodium channelopathies underlie many excitability disorders. Genes *SCN1A*, *SCN2A* and *SCN9A*, which encode pore-forming α-subunits $Na_V1.1$, $Na_V1.2$ and $Na_V1.7$, 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 $Na_V1.3$, is part of this cluster, but until recently was not associated with any mutation. A charge-neutralizing mutation, K345Q, in the $Na_V1.3$ DI/S5-6 linker has recently been identified in a patient with cryptogenic partial epilepsy. Pathogenicity of the $Na_V1.3$/K354Q 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 $Na_V1.5$. Since identical mutations may produce different effects within different sodium channel isoforms, we assessed the K354Q mutation within its native $Na_V1.3$ channel and studied the effect of the mutant $Na_V1.3$/K354Q channels on hippocampal neuron excitability. We show here that the K354Q mutation enhances the persistent and ramp currents of $Na_V1.3$, 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 $Na_V1.3$ channels and hippocampal neurons.

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 $Na_V1.1$, together with a smaller number of mutations in *SCN2A* which encodes sodium channel Na_V 1.2 (Lossin 2009) have been associated with epilepsy (George 2004; Helbig et al., 2008; Noebels 2003; Sisodiya et al., 2007). *SCN3A* (which encodes sodium channel Nav1.3) is located on the same chromosomal cluster as *SCN1A* and *SCN2A*, but there is little evidence linking it with epilepsy or other inherited channelopathies. $Na_V1.3$ is expressed predominantly in the embryonic and neonatal rodent CNS, and is almost undetectable in the adult rodent CNS (Beckh et al., 1989; Black et al., 1996; Felts et al., 1997; Waxman et al., 1994). However, relatively higher levels of this channel are retained in the adult human brain (Whitaker et al., 2001). Until recently, $Na_V1.3$-related pathogenesis has been limited to pain, a role for Nav1.3 which has been inferred from rodent models of nervous system injury (Waxman and Hains, 2006), and from the accumulation of $Na_V1.3$ at blind endings of axons from human painful neuromas (Black et al., 2008). Recently, a charge-neutralizing K354Q mutation in $Na_V1.3$, which substitutes an evolutionarily conserved lysine located within the channel pore region with a glutamine, has been reported in a patient with cryptogenic childhood epilepsy (Holland et al., 2008). A genetic link of $Na_V1.3$ mutation and epilepsy would be of special interest, because it would support a potential pathogenic role for this channel in the human CNS and would increase the repertoire of genetic loci that may underlie cryptogenic epilepsies.

Pathogenicity of the $Na_V1.3$/K354Q mutation has been suggested on the basis of conservation of this residue in all sodium channels, its absence from control alleles, and from functional analysis of the corresponding substitution in the cardiac muscle sodium channel $Na_V1.5$ (Holland et al., 2008). However, since identical mutations may produce different functional changes in different channel isoforms (Lampert et al., 2006a; Lossin et al., 2002), a more direct demonstration of pathogenicity

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requires functional analysis of the K354Q mutation within $Na_V1.3$, and assessment of the contribution of the mutant $Na_V1.3$/K354Q channels to excitability of CNS neurons. Here we report gain-of-function properties of $Na_V1.3$/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 $Na_V1.3$ and the $Na_V1.3$/K354Q mutant, we studied both by patch-clamp using a rat $Na_V1.3$ construct. The construct, converted to a tetrodotoxin-resistant form (TTX-R, $rNa_V1.3_R$) by the Y384S substitution, permits the current produced by $rNa_V1.3$ 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% CO_2, 37 °C) in Dulbeccos's Modified Eagle's Medium supplemented with 10% fetal bovine serum, were transiently-transfected with plasmids containing β1-IRES-CD4 and β2-IRES-GFP (Lossin et al., 2002) and either $rNav1.3_R$ (referred to as WT hereinafter) or $rNa_V1.3_R$/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 characterization of heterologously expressed $Na_V1.3$ channels.

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, Springfield, IL) supplemented with 2% B-27 (Invitrogen) and 0.5 mM Glutamax (Invitrogen)], sliced into 300 µm sections using a McIlwain tissue chopper (Warner Instruments, Hamden, CT) and digested (30 min at 30 °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 settling 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 (1.5×10^7cells/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 EGFP). 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 PDGFbb (Invitrogen)] was added. Cells were plated on 12 mm glass coverslips coated with poly-D-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 40–50 h 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 $MgCl_2$, 1 $CaCl_2$, 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 $MgCl_2$, 0.1 $CaCl_2$, 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 filter 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, $G = I/(V_m - E_{Na})$, where G is the conductance, I is the peak inward current, V_m is the membrane potential step used to elicit the response and E_{Na} 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 $G = G_{min} + (G_{max} - G_{min})/(1 + \exp[(V_{1/2} - V_m)/k])$, where $V_{1/2}$ 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 $I = I_{min} + (I_{max} - I_{min})/(1 + \exp[(V_m - V_{1/2})/k])$, where V_m represents the inactivating pre-pulse membrane potential and $V_{1/2}$ 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 $CaCl_2$ and $MgCl_2$ 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 h–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