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A Novel Na_v1.5 Voltage Sensor Mutation Associated with Severe Atrial and Ventricular Arrhythmias

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Abstract

Background—Inherited autosomal dominant mutations in cardiac sodium channels (Na_v1.5) cause various arrhythmias, such as long QT syndrome and Brugada syndrome. Although dozens of mutations throughout the protein have been reported, there are few reported mutations within a voltage sensor S4 transmembrane segment and few that are homozygous. Here we report analysis of a novel lidocaine-sensitive recessive mutation, p.R1309H, in the Na_v1.5 DIII/S4 voltage sensor in a patient with a complex arrhythmia syndrome.

Methods and Results—We expressed the wild type or mutant Na_v1.5 heterologously for analysis with the patch-clamp and voltage clamp fluorometry (VCF) techniques. p.R1309H depolarized the voltage-dependence of activation, hyperpolarized the voltage-dependence of inactivation, and slowed recovery from inactivation, thereby reducing the channel availability at physiologic membrane potentials. Additionally, p.R1309H increased the “late” Na⁺ current. The location of the mutation in DIIIS4 prompted testing for a gating pore current. We observed an inward current at hyperpolarizing voltages that likely exacerbates the loss-of-function defects at resting membrane potentials. Lidocaine reduced the gating pore current.

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Conclusions—The p.R1309H homozygous Na_v1.5 mutation conferred both gain-of-function and loss-of-function effects on Na_v1.5 channel activity. Reduction of a mutation-induced gating pore current by lidocaine suggested a therapeutic mechanism.

Keywords

R1309H mutation; S4 segment; voltage sensor; Na_v1.5; gating pore current; ventricular arrhythmia; atrial arrhythmia

Introduction

The voltage-gated sodium (Na⁺) channel Na_v1.5 initiates the cardiac action potential. Dysfunction of Na_v1.5 channels causes cardiac arrhythmias such as Long QT syndrome type 3, Brugada syndrome, progressive cardiac conduction defect, and sick sinus syndrome [1]. The Na_v1.5 pore forming α subunit is composed of four homologous membrane domains (DI to DIV) that are connected by intracellular linkers. Each domain contains six transmembrane spanning segments (S1 to S6). The ion selective conducting pore is formed by S5 and S6. The S1 to S4 segments function as the voltage sensing domain (VSD), which controls Na_v1.5 activity. Central to the VSD is the S4 segment, which is characterized by regularly arranged positively charged arginine or lysine side chains at every third position [2]. Upon membrane depolarization these S4 segments move outward along a spiral pathway through a specialized gating pore formed by the S1 to S3 segments [3]. The consequence of this movement is that the arginine and lysine side chains in S4 move a positive gating charge outward. Along each step, the arginine and lysine side chains interact with conserved surrounding negatively charged amino acid residues in the S1 to S3 segments, thereby stabilize the gating charges in the intramembrane environment [3].

Investigations into the roles of these arginines or lysines in S4 have highlighted their critical roles in channel gating. Mutations of the arginines or lysines to uncharged residues reduce the steepness of voltage-dependent gating of Na⁺ channels. Additionally, these mutations generate gating pore currents, separate from the central ionic current, resulting from movement of protons and cations through the modified gating pore [4–8]. Such mutations in voltage-gated Na⁺, K⁺ and Ca²⁺ channels have been reported in a variety of inherited diseases including periodic paralysis, epilepsy, migraine, ataxia, peripheral nerve hyperexcitability [6–12], and, recently, in cardiac arrhythmias [13, 14]. Seven S4 mutations in the VSD of Na_v1.5 channels have been associated with development of severe arrhythmias and dilated cardiomyopathy [13–19]. Among them are five mutations located in DI/S4 and two in DII/S4. Thus, in contrast to the well-established paradigm in which mutations impair channel function due to alteration in control of ion conductance through the central pore of ion channels, there have been few reports characterizing mutations in S4 segments of Na_v1.5 channels, and only a limited understanding of how these mutants contribute to disease. Because studies demonstrated that the four homologous voltage sensors in Na⁺ channels are not identical [20], specific mutations in specific voltage sensors may have distinct effects upon channel function.

Here we report a novel homozygous mutation p.R1309H in DIII/S4 of Na_v1.5 in a patient with severe atrial and ventricular arrhythmias. Family members who are heterozygous for the p.R1309H have abnormal ECGs but no clinical phenotypes—unusual for an arrhythmogenic Na_v1.5 variant. We found that the R1309H mutation affected several biophysical parameters, including those predicted to lead to both loss- and gain-of-function phenotypes. Moreover, this mutation induced a gating pore leak current at hyperpolarization voltages. This gating pore current was ameliorated by lidocaine, which was therapeutic in the patient. Using voltage fluorometry, we found a slower movement of the S4 segment in response to changes in membrane potential. Together, these data provide new insight into a rare but illustrative class of Na_v1.5 arrhythmogenic channelopathies, revealing roles for the DIII/S4 voltage sensor.

Methods

Case Report

Genetic analysis was performed in a commercial laboratory after obtaining written informed consent from the family. Consent was also given for further analysis of the biophysical properties of the subsequently identified mutation. Specific written consent is not required by either the Research Ethics Board of the Children's Hospital of Eastern Ontario or the Institutional Review Board of Duke University Medical Center for presentation of a case report.

A previously well 5-month old male child presented to the Children's Hospital of Eastern Ontario emergency room with an 8 hour history of lethargy, irritability and vomiting. His initial rhythm on electrocardiogram (ECG) was a non-sustained and sustained wide complex tachycardia at 225 beats/minute with varying QRS durations interspersed with some sinus tachycardia (Figure 1A). Intravenous amiodarone (5 mg/kg over 30 minutes) and intravenous magnesium (40 mg/kg over 30 minutes) were administered with termination of the tachycardia. The first ECG obtained in sinus rhythm showed a heart rate of 90 min⁻¹, first degree AV block (PR 220 ms), prolonged P wave duration (120 ms), ST elevation in lead V1, and RSr' pattern in V1 suggestive of a depolarization defect (Figure 1B).

Family history revealed that the parents were first cousins. In addition, a female sibling died at 2 months of age during a diarrheal illness, and a 6 year old male sibling died after a traumatic accident. A third sibling died from an autosomal recessive lethal multiple malformation syndrome characterized by severe dysmorphic features and central nervous system anomalies which has been linked to an inborn error of metabolism. There is no evidence that it is related to sodium channelopathy (Figure 1C). No other instances of sudden death have been identified in the extended pedigree. Maintenance therapy was intravenous amiodarone for 4 days with a transition to enteral propranolol at 5 mg/kg/day [21]. Family members were taught CPR, instructed on use of the AED that was provided, and given strict instructions regarding fever management.

Genetic testing for Brugada syndrome with a 7-gene GeneDx panel (GeneDx, Inc., Gaithersburg, MD) revealed a homozygous and previously unreported missense mutation in exon 22 of the SCN5A gene (c.3926 G>A; p.Arg1309His, Figure 1D), which was

considered to be “likely disease-causing”. Cascade screening was offered to both parents and the 6 living siblings (Figure 1C); the father declined genetic testing. Because the proband was homozygous for the p.R1309H variant, the mother was heterozygous for the variant, and one sibling was also homozygous, we assume that the father (who refused genotyping) was heterozygous. Although neither parent nor any of the heterozygous siblings have experienced syncope or known arrhythmias, the presence of the p.R1309H variant in the heterozygous state correlated with an abnormal ECG in those individuals for whom ECGs were able to be obtained (see Supplemental Figure 1A–C). In one homozygous sibling for whom an ECG was available, we observed multiple abnormalities including sinus bradycardia, P wave prolongation, first degree AV block and QRS prolongation (Supplemental Figure 1D).

The proband re-presented 6 months later with wide-complex tachycardia at 235 beats/minute that was terminated with intravenous amiodarone (2.5 mg/kg over 30 minutes). He was commenced on quinidine sulfate (30 mg/kg/day divided q6h) and maintained on the propranolol (4 mg/kg/day). A Reveal XT 9529 (Medtronic, Inc; St. Paul, MN) implantable loop recorder was placed in his epigastrium for monitoring. Subsequently, variable morphologic patterns were noted on the baseline 12 lead ECGs (Supplemental Figure 2A–C). Surveillance Holter recordings demonstrated brief episodes of wide complex rhythm as well as occasional episodes of definite atrial tachycardia with narrow complex QRS (Supplemental Figure 3A–B).

Because of a documented sustained wide complex arrhythmia noted on Holter (Supplemental Figure 4) and the pending lack of availability of the original quinidine sulfate formulation, he was admitted for transition to a powdered formulation of quinidine sulfate from a new supplier. Within 48 hours of stopping the quinidine, sustained atrial arrhythmias occurred (Supplemental Figure 5), followed by further episodes of sustained wide complex tachycardia. Intravenous adenosine failed to unmask an underlying atrial tachycardia during this episode of wide complex tachycardia. Bolus intravenous lidocaine terminated the tachycardia on 2 separate occasions (Supplemental Figure 6A–B). Due to a possibly limited quinidine supply and the response to lidocaine, a trial of oral mexiletine was instituted; it was not effective. The new formulation of the quinidine sulfate was started with immediate suppression of the sustained arrhythmias. The implantation of an epicardial defibrillator was postponed because of the clinical response to the quinidine sulfate.

The review of all his tracings indicates a complex mixed arrhythmia pattern of atrial tachycardia with and without conduction delay (not having classical bundle branch block morphologies; likely intramyocardial), ventricular tachycardia, first degree AV block and QRS pattern that varies despite being maintained on stable medication doses.

Molecular Biology and Cell Culture

SCN5A plasmid for the mutant sodium channels (p.R1309H) was derived from cloned human *SCN5A* in pcDNA3.1(-) vector (WT) with QuikChange Site-Directed Mutagenesis kit (Agilent Technologies, CA). In order to measure persistent (“late”) Na⁺ and gating pore leaking currents, *SCN5A* encoding cardiac sodium channels with C373Y mutation sensitive to tetrodotoxin (TTX) was made for WT (WT^{TTX}) or p.R1309H (R1309H^{TTX}) [22]. pMAX

plasmid containing R1309H-DIII-LFS and WT-DIII-LFS constructs were linearized with PacI and then purified with the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel) for patch clamp and Voltage Clamp Fluorometry (VCF) experiments in *Xenopus* oocytes. Capped mRNA was synthesized from linearized DNA (mMessage mMachine T7 Transcription Kit, Life Technologies)[23].

Human embryonic kidney (HEK) cells were cultured in Dulbecco's modified Eagle's culture media with 10% fetal bovine serum in a 37°C incubator with 5% CO₂. Cells were grown in 60-mm culture dishes, and WT or mutant *SCN5A* (4 µg) was cotransfected with EGFP (0.3 µg) with Lipofectamine 2000 (Life Technologies). Whole-cell currents encoded by *SCN5A* were measured 48 hr after transfection at room temperature.

cRNA for WT or mutant human α -subunit Na_v1.5 and human β 1 subunit were injected at a 2:1 molar ratio into *Xenopus* oocytes for simultaneously recording current and measuring the movement of fluorescence labeled voltage sensor in domain III. Oocytes were incubated at 18°C for 4–6 d in solution with (mM) 93 NaCl, 5 KCl, 1.8 CaCl₂, 1 MgCl₂, 5 HEPES, and 2.5 Na-pyruvate, and 1% penicillin-streptomycin, pH 7.4. Before recording, oocytes were labeled with 10 µM methanethiosulfonate-carboxytetramethylrhodamine (MTS-TAMRA; Santa Cruz Biotechnology) in a depolarizing solution (mM: 110 KCl, 1.5 MgCl₂, 0.8 CaCl₂, 0.2 EDTA and 10 HEPES, pH 7.1) on ice for 20 min.

Electrophysiology and VCF

Sodium (Na⁺) currents in HEK cells were recorded in the whole-cell voltage patch-clamp configuration with an Axopatch 200B amplifier (Molecular Devices, CA) and sampled at 10 kHz and filtered at 2 kHz and data were analyzed with Axon Clampfit (Molecular Devices, CA). The pipette internal solution for Na⁺ and gating pore currents with tetraethylammonium chloride (TEA-Cl) Na⁺-free external solution contained (in mM): CsCl 50, CsF 30, L-aspartic acid 50, NaCl 10, tetraethylammonium hydroxide 11, EGTA 5, HEPES 10, pH 7.3 with CsOH. The external solution for Na⁺ current recordings of the central pore contained (in mM): NaCl 120, KCl 5.4, CaCl₂ 1.8, MgCl₂ 1, HEPES 10, glucose 10, tetraethylammonium chloride (TEA-Cl) 20, pH 7.4 with NaOH. For gating pore, we used two different external solutions for specific protocols. A TEA Na⁺-free external included (in mM): TEACl 140, KCl 5.4, CaCl₂ 1.8, MgCl₂ 1, HEPES 10, glucose 10, pH 7.4 with TEAOH. An alternative low Na⁺ external solution contained (in mM): N-methyl-D-glucamine (NMDG) 115, NaCl 20, CsOH 2, CaCl₂, Hepes 10, TEACl 10 and niflumic acid 1, pH 7.4 with methanesulfonic acid and was used together with an alternative internal solution that contained (in mM): CsF 135, NaCl 5, EGTA 10 and HEPES 10, pH 7.3 with CsOH, as previously described [13].

To observe the effect of lidocaine on the central pore gating kinetics or the gating pore currents, 0.1 mM lidocaine was added to external solution. To test the barium (Ba²⁺) sensitivity of the gating pore current, 6 mM Ba²⁺ was added to the TEA Na⁺-free external solution [6].

Na⁺ ionic currents in cut-open oocyte were recorded using an amplifier (CA-1B; Dagan Corporation) coupled to an A/D converter (Digidata 1440; Molecular Devices).

Simultaneously, fluorescence emission from the labeled domain was measured through the mounted photodiode. Recording temperature was maintained at 19°C with a temperature controller (HCC-100A; Dagan Corporation). Capacitance compensation and P/-8 leak subtraction were applied prior to recording. The internal recording solution was (mM): NMDG-Methanesulfonate (NMDG-MES) 105, Na-MES 10, HEPES 20, and EGTA 2, pH 7.4. The external recording solution was (mM): NMDG-MES 25, Na-MES 90, HEPES 20, and Ca-MES₂ 2, pH 7.4.

Whole-cell Na⁺ currents in HEK cells were initiated with a 50 ms depolarization step from -100 mV to +60 mV with 5 mV increment at holding potential of -120 mV. Steady-state inactivation were tested by a two-pulse protocol with the first pulse of 500 ms from -140 mV to -20 mV at 5 mV increment followed by a second pulse fixed at -40 mV. Another two-pulse (P1 and P2) protocol was adopted to test recovery from inactivation. Na⁺ channels were inactivated by P1 with 1000 ms depolarization pulse to -20 mV from the holding potential and the recovery currents from inactivation initiated by P2 at -20 mV were recorded at various intervals (1 to 200 ms) after P1. Currents induced by P2 over that by P1 (I_2/I_1 ratios) were plotted as function of the various intervals. A 200-ms one step depolarization to -10 mV was used to evaluate late Na⁺ current. After subtracting background currents obtained by applying TTX 1 μ M to block TTX-sensitive current, late current was measured at 150 ms and normalized to transient peak current. A protocol from -140 to +40 mV with 200 ms duration, 10 mV increment and holding at -80 mV was run to detect gating pore currents for both inward and outward in cells perfused with Na⁺-free TEA solution [14]. In order to observe the outward gating pore current, a protocol from -100 to +40 mV in 5-mV increment with 50 ms duration and a holding of -140 mV was applied to cells in low Na⁺ solution and in the same solution inward gating pore current was measured using a ramp protocol from -140 to 0 mV at increase rate of 0.72 mV/ms either with or without a 500 ms-predepolarization pulse to +40 mV from the holding of -140 mV [13]. To test use-dependent block of lidocaine, a depolarization step for 50 ms to -20 mV from a holding potential of -120 mV was applied at frequencies of 1, 5 and 10 Hz.

Recording pipettes were pulled from borosilicate glass with Sutter P-97 Micropipette Puller (Sutter Instrument Co., CA). Pipette resistance ranged from 1.2 to 3.0 M Ω and series resistance was 8.8 ± 0.3 M Ω . The series resistance was compensated by 70~90%.

To obtain the voltage dependence of steady-state activation and the corresponding voltage dependence of voltage sensor movement (F-V), we followed the protocol as previously outlined [24]. Oocytes were depolarized in 20 mV increments from a holding potential of -100 mV. Depolarizing pulses were preceded by a 100-ms-long prepulse and 50-ms-long postpulse at -120 mV. The steady-state inactivation was evaluated by depolarizing to -20 mV after a 200 ms-prepulse at various voltage steps from -150 to +20 mV. To correct photobleaching of the fluorescence probe, fluorescence traces were subtracted by the fluorescence baseline, which was recorded with no change in voltage. The magnitude of fluorescence signals is expressed as F/F_0 , where F is the change in fluorescence in response to voltage change and F_0 is the magnitude of baseline.

Gating activation curves were obtained using a Boltzmann function: $G/G_{\max} = (1 + \exp(-(V - V_{1/2})/k))^{-1}$, where $G/G_{\max}$ is the conductance normalized to its maximal value, V is the membrane potential, $V_{1/2}$ is the membrane voltage at which the current amplitude is half-maximal, and k is the slope factor. For steady-state inactivation, Na^+ currents induced by the second pulse were normalized to the maximal current and plotted as the function of the voltages initiated by the first pulse, which was also fitted with Boltzmann function: $I/I_{\max} = (1 + \exp((V - V_{1/2})/k))^{-1}$, where $I/I_{\max}$ is the normalized value. Time constant τ of Na^+ channel recovery from inactivation was estimated with first order exponential decay $y = y_0 + Ae^{-x/t}$, where y_0 , y offset; A , amplitude; t , time constant. Time constant (τ) for activation and inactivation phases of Na^+ currents were obtained by standard one-exponential fit with Clampfit software (Molecular Devices, CA).

Data Analysis and Statistics

All data analyses were performed using Microsoft Excel 2010. Numerical averages are presented as mean $\pm$ SEM. Unless otherwise stated, statistical significance was calculated using the unpaired two-sample Student's t test and a p -value of < 0.05 was considered to be statistically significant.

Results

Arrhythmia and conduction defects in family members

The p.R1309H variant is rare, with an allele frequency of 0.001048% in the Exome Aggregation Consortium (ExAC). *In silico* analysis (PolyPhen-2) predicted p.R1309H to be probably damaging (score = 1.000) [25]. Although displaying abnormal ECGs (Supplemental Figure 1A–C), none of the relatives heterozygous for the p.R1309H variant had any known arrhythmic events. One sibling homozygous for the p.R1309H variant, had an atrial septal defect (ASD) and ventricular septal defect (VSD) requiring surgical closure. Pre-operative ECGs showed first degree AV block, left axis deviation and nonspecific intraventricular block (QRS duration 140 msec). Recurrent atrial flutter, which required cardioversion, occurred in the immediate postoperative period. There are persistent ECG abnormalities (Supplemental Figure 1D), however the atrial arrhythmias have not recurred in the 18 months since discharge. Although the genotype is not known, it is likely that the 2-month old sibling who died during a diarrheal illness was homozygous for the variant and experienced sudden cardiac death associated with the illness, analogous to the presenting arrhythmias in the proband during a febrile illness.

R1309H affects $\text{Na}_v1.5$ activation and inactivation

To examine the consequences of the p.R1309H variant, we expressed the wild type (WT) or p.R1309H mutant in HEK293 cells and recorded whole cell Na^+ currents with the patch clamp technique. Because the proband was homozygous and because none of the heterozygous family members experienced arrhythmias (although having abnormal ECGs), we elected to study the mutant expressed in the absence of a co-expressed WT channel to best mimic the consequences of the homozygous state. Families of exemplar traces are shown in Figure 2A. Current density for the p.R1309H mutant was mildly reduced compared to WT (Figure 2B). More strikingly, the p.R1309H mutation altered channel

kinetics. The p.R1309H mutation slowed the τ of activation at voltages between -60 and -30 mV and caused a minor depolarization in the $V_{1/2}$ of channel activation (-40.0 ± 1.1 mV vs. -44.5 ± 0.8 for WT, $p = 0.02$), driven mainly by a reduction in the slope, k , as shown in Figure 2E and in Table 1. A reduced steepness of k is similar to what has been observed in previous S4 mutations [13]. Because the patient's arrhythmia burden was reduced with lidocaine, we examined the drug's effects on the p.R1309H mutant channel kinetics. First, we examined whether the p.R1309H mutation affected the use dependent block of lidocaine by applying repeated test pulses to -20 mV at 1, 5, and 10 Hz. We employed a concentration of lidocaine (0.1 mM) that did not completely block $Na_v1.5$, as shown in Figure 2D. Lidocaine induced a use dependent block and the magnitude of block increased with the frequency of the test pulses for both WT and the p.R1309H mutant channels (Supplemental Figure 7). There were no apparent differences in the efficacy of the use dependent block between the WT and the p.R1309H mutant channels, however. Returning our attention to channel kinetics, we found that lidocaine depolarized the $V_{1/2}$ of activation for the p.R1309H but not for the WT channel (Table 1). However, lidocaine did not affect the activation time constants (Figure 2F), suggesting that the therapeutic effects of lidocaine were elsewhere.

Inactivation kinetics were also affected by the p.R1309H mutation. The τ of inactivation was slower at voltages between -60 and -30 mV (Figure 2C and 3A) and the $V_{1/2}$ of steady-state inactivation for the p.R1309H mutant was hyperpolarized (-99.7 ± 1.0 mV vs. -93.5 ± 0.7 mV for WT, $p < 0.01$), as shown in Figure 3B and in Table 1. Because the differences in the $V_{1/2}$ of activation and steady-state inactivation were mainly driven by changes in the slope, the window current was minimally affected (not shown). Lidocaine (0.1 mM) slowed the τ of inactivation for the p.R1309H mutant at voltages more hyperpolarized to -30 mV, but had no effect on the WT channel (Figure 3A). Lidocaine also hyperpolarized the $V_{1/2}$ of steady-state inactivation for the both the p.R1309H and WT channels (Figure 3C–D).

The p.R1309H mutation also slowed the rate of recovery of inactivation, as queried with a two-pulse protocol in Figure 3E–F ($\tau = 22.3 \pm 1.2$ ms vs. 15.7 ± 1.1 ms for WT, $p < 0.01$). Because of the use-dependent properties of lidocaine (see below), we did not test the effects of the drug on recovery from inactivation. The p.R1309H mutation also increased the late Na^+ channel current (Figure 3G–H and Table 1), which we assessed using a TTX-sensitive version of WT or p.R1309H to reduce background and allow closer scrutiny of the low amplitude late current.

R1309H affects voltage sensor movement

Because of the p.R1309H mutation's location within the DIII voltage sensor, we analyzed directly whether the mutation affected the S4 sensor's movement function by using voltage-clamp fluorometry. The R1309H mutation was placed in the background of a M1296C mutation (R1309H-DIII-LFS), which allowed covalent attachment of a fluorophore that reports movement of the DIII voltage sensor, and expressed in *Xenopus* oocytes. Simultaneous ionic (Na^+) current and fluorescence signals were recorded, and exemplar traces are shown in Figure 4A. Steady-state activation and inactivation curves (Figure 4B) show that the ionic currents and kinetics of activation and inactivation for R1309H-DIII-

LFS in *Xenopus* oocytes mimicked the findings observed in cultured mammalian cells for p.R1309H (see Figure 2D and 3B)—there was essentially no change in activation and there was a hyperpolarizing shift in steady-state inactivation—suggesting that the fluorophore did not affect channel function. The trivial differences in the effects upon the $V_{1/2}$ of activation between the two expression systems likely resulted from different stoichiometry of accessory subunits. With this assurance, we then measured the voltage-dependence of steady state fluorescence signal change (F-V) as shown in Figure 4C–D. The activation and deactivation time constants for exponential fits of the fluorescence signal of the DIII voltage sensor were slowed for both activation and deactivation (Figure 4E).

R1309H induces a gating pore current

Because recent analyses of mutations within the D1 S4 voltage sensor of $Na_v1.5$ have revealed a mutation-generated gating pore current that was thought to contribute to the disease mechanism [13, 14], we investigated whether p.R1309H resulted in a gating pore current. Previous studies reported that mutation of S4 charges may cause either a hyperpolarization-activated or a depolarization-activated non-specific cation leak called the omega or gating pore current [26]. Using two different protocols, we detected an inward gating pore current at hyperpolarized potentials but did not detect an outward gating pore current at depolarized potentials for the p.R1309H mutant but not the WT channel (Figure 5A–H). Previous investigations of gating pore currents [6] have demonstrated a requirement for certain monovalent cations and blockade with divalent cations such as Ba^{2+} . We therefore tested whether the gating pore current in the p.R1309H mutant was sensitive to Ba^{2+} . Indeed, the addition of Ba^{2+} eliminated the gating pore current observed in p.R1309H mutant (Figure 5C). Moreover, lidocaine (0.1 mM) reduced the gating pore current (Figure 5D).

Discussion

We analyzed a novel p.R1309H mutation in DIII/S4 of $Na_v1.5$ found in a homozygous patient with complex atrial and ventricular arrhythmias. Overall, analysis of the central conduction pore gating kinetics suggested both loss-of-function and gain-of-function effects. A depolarized $V_{1/2}$ of channel activation along with slowed activation kinetics and a hyperpolarized $V_{1/2}$ of steady-state channel inactivation joined with a delayed recovery from inactivation are consistent with loss-of-function effects. On the other hand, an increased Na^+ late current indicated a gain-of-function effect of the mutation. Voltage clamp fluorometry experiments, which uncovered sluggish movement both for activation and especially deactivation of the mutant DIII/S4 helix, may explain both the loss-of-function (e.g., slowed activation kinetics) and the gain-of function (increased late Na^+ current from slowed deactivation) that we measured in a heterologous expression.

It appears that the various biophysical defects in the p.R1309H mutant $Na_v1.5$ channel may all contribute to the complex arrhythmia syndrome in this patient. The ST elevation in leads V1-V2 and inverted T waves (Figure 1B) are suggestive of a Brugada syndrome-like pattern. In that regard, the ventricular and atrial arrhythmias, which are reported to be associated with Brugada syndrome in up to 15% of adult cases, and—especially relevant in

this case—are associated with Na_v1.5 loss-of-function mutations in infants [27], indicate a prominent role for the observed loss-of-function effects (e.g., slower kinetics of activation and reduced channel availability). In addition to these loss-of-function effects and their likely contribution to arrhythmogenesis, we observed an increased late Na⁺ current, which is also likely a factor in the complex nature of this patient's arrhythmias. Although the patient never exhibited a prolonged QT interval that often results from *SCN5A* mutations associated with an increase the late Na⁺ current, the contribution of the increased late current caused by the p.R1309H mutation may be analogous to that conferred by the well-characterized p.S1103Y, for which the arrhythmogenic contribution only manifests under certain circumstances, such as in the setting of drug-induced block of HERG [28], left ventricular hypertrophy [29], or heart failure [30].

While the available genetic and clinical information do not allow a definitive identification of the homozygous p.R1309H variant as causative for the arrhythmia, there are extensive correlative data that support such a hypothesis. First, heterozygous family members displayed abnormal ECGs, but no arrhythmias. The one sibling who was found to be homozygous displayed an abnormal ECG with abnormalities that were more severe than her heterozygous relatives and had atrial flutter immediately following congenital heart surgery. Thus, these data suggest a dose-dependent effect of the variant. Second, the functional data demonstrate that the variant alters channel behavior in ways consistent with the arrhythmias observed in the proband. Third, the proband's striking response to lidocaine may be explained by the observed effects of lidocaine upon the p.R1309H mutant channel's biophysical properties. Specifically, we found that lidocaine slowed the τ of inactivation for the p.R1309H mutant; decreased channel availability; and decreased the gating pore current. The decrease in channel availability for the p.R1309H mutant but not the WT channel is interesting. Since it was previously demonstrated that application of lidocaine reduces the probability of channel opening [31], we speculate that the decreased availability for the p.R1309H mutant but not the WT channel reflects an increased sensitivity for the mutant at the low dose of lidocaine (0.1 mM) that we employed here. Indeed, this is consistent with the known actions of lidocaine on stabilizing the DIII/S4 (the location of the p.R1309H mutation) in the “up” depolarized position [32]. That is, our activation kinetics data (Figure 2E) suggest that DIII/S4 in the p.R1309H mutant channels shifts more favorably to “up” position compared to the WT channels.

Given the therapeutic efficacy of lidocaine, it is particularly intriguing that the S4 of the VSD in DIII is the main target of lidocaine, for which use-dependent block occurs through stabilization of the activated depolarized state [32]. The specific location in DIII/S4 may explain some of the the nature of the p.R1309H mutation's biophysical defects as well as their arrhythmogenic consequences. Previous kinetic and steady-state analyses have indicated that the S4 charges from all four Na⁺ channel domains are involved in activation, albeit to a variable extent [33]. DIII/S4 moves at the most hyperpolarized potentials, whereas the S4s in domains I and II move at more depolarized potentials. DIV/S4 movement is a later step in the activation sequence and may not even be a prerequisite for channel opening [20]. On the inactivation side, the S4 segments of domains III and IV of Na_v1.4 are immobilized by fast inactivation, whereas those of domains I and II are unaffected [34].

Similarly, the DIII and DIV VSDs of Na_v1.5 also display kinetics that are consistent with inactivated state interaction[23]. Thus, these previous studies provide a rationale for the kinetic changes in central pore induced by the p.R1309H mutation in DIII/S4 in this report. Further, expression of the periodic paralysis mutation in the skeletal muscle Na_v1.4, p.R1135H, homologous to p.R1309H in Na_v1.5 tested here, revealed enhancement of fast and slow inactivation, prolonged recovery and impaired deactivation [35], also consistent with our results. The impaired deactivation induced by the homologous p.R1135H in Na_v1.4 may explain the increased late Na⁺ current in homologous p.R1309H channels in Na_v1.5 found in this study, an interesting hypothesis for future study.

Moreover, the location of p.R1309H mutation within the DIII/S4 voltage sensor, by analogy to other channelopathies resulting from voltage sensor mutations, suggests that the generation of a gating pore current may contribute to arrhythmogenesis. The roles of gating pore currents in periodic paralysis have well been characterized in skeletal muscle calcium or sodium channels [6, 8–10]. Additionally, several mutations in gating-charge-carrying arginine residues within Na_v1.5 S4 domains have been associated with the development of severe arrhythmias and dilated cardiomyopathy, and the underlying biophysical defects attributable to the mutations have recently been explored [13, 14]. In these prior studies, gating pore currents were present in Na_v1.5 channels bearing DI/S4 mutations of R219H, R222Q and R225W. Using similar protocols we, for the first time, recorded a hyperpolarization-activated gating pore current in a DIII/S4 mutation. Because each of the voltage-sensing domains functions independently, it is not surprising that the DIII/S4 mutation studied here has different consequences than the DI/S4 Na_v1.5 mutations previously studied. Specifically, we did not record outward gating pore currents that were present in homologous Na_v1.4 DIII/S4 mutations previously studied [35, 36]. This discrepancy is not clear but may be because the outward gating pore currents resulting from DIII/S4 mutations are relatively small and below a level of detection in the mammalian heterologous expression system used here compared to the *Xenopus* oocyte system employed previously [36]. On the other hand, the inward gating pore current induced by hyperpolarized voltages in p.R1309H mutant channels might contribute to the specific nature of this patient's arrhythmias by exacerbating the overall loss-of-function effects on the central conduction pore. Because the inward gating pore current occurs at hyperpolarized potentials (Figure 5), the resultant effects would be most prominent close to the resting membrane potential (~−90 mV), causing a slight depolarization of the resting membrane potential. Since the $V_{1/2}$ of the steady-state inactivation curve for the p.R1309H mutant is hyperpolarized compared to the wild type channel, one consequence would be decreased availability of the mutant Na_v1.5, contributing to the observed loss-of-function effects. Further, the inward gating would contribute to the arrhythmogenesis by destabilizing the resting membrane potential through compensatory activation of several membrane exchangers such as the Na⁺/K⁺ ATPase, and Na⁺/Ca²⁺ exchanger to alter Ca²⁺ homeostasis, as described previously [13]. In this context, the marked reduction in the p.R1309H mutant channel's gating pore current with lidocaine provides a rationale for lidocaine's therapeutic efficacy and provides additional support for assigning causality to the p.R1309H mutation. The previous demonstration that lidocaine stabilizes the DIII/S4 in the depolarized “up” position suggests a mechanism for the reduction in the gating pore current. Pulling R1309H

away from the cytoplasm and into the membrane likely eliminates a key component of the ion conducting path.

Finally, the homozygous nature of the arrhythmia syndrome is unusual, likely suggesting that the various contributions of one mutant allele encoding p.R1309H are not sufficient to promote arrhythmogenesis, as indicated by a minimal clinical phenotype (manifest by only minor ECG changes as shown in Supplemental Figure 1 and a complete absence of any clinical arrhythmia). In this regard, the lack of arrhythmias in the heterozygous state is analogous to the aforementioned p.S1103Y in *SCN5A* or the growing list of K⁺ channel mutations that increase susceptibility to drug-induced long QT syndrome [37] for which arrhythmias manifest only under specific stressors. The addition of the biophysical defects from the second mutant allele in the proband may be a “stressor-equivalent”. On the other hand, the correlation of the arrhythmia syndrome with the homozygous state of this mutation provides additional evidence that the p.R1309H mutant is, indeed, causal.

Conclusions

In summary, here we report that a single DIII/S4 mutation p.R1309H induced both loss- and gain-functions of central pore and an inward leak gating pore current. Studies of the biophysics of VSD, especially the gating pore formed by the mutations in positive-charged residues, have provided new insights into the pathophysiology of associated familial diseases such as periodic paralysis [6, 8–10, 36] and cardiac arrhythmias. S4 has become a potential target to discover new therapeutic approaches for the familial diseases [11, 38]. The biophysical effects described are likely pathologic because they would affect the resting membrane potential, alter voltage-gated Na⁺ currents, and affect the action potential through indirect influence of multiple conductances. As a result, these disturbances likely lead to the underlying arrhythmias and other clinical manifestations reported here.

Highlights

1. We identified a novel, homozygous, recessive p.R1309H mutation in Na_v1.5 associated with a complex arrhythmia syndrome.
2. The mutation, within the domain III / S4 voltage sensor, induces a gating pore current.
3. Biophysical analysis of the p.R1309H mutant also revealed several other features that likely contribute to arrhythmogenesis.
4. Therapeutic success of lidocaine in the proband was consistent with amelioration of the gating pore current for the p.R1309H mutant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

Na_v1.5	sodium channels
VCF	voltage clamp fluorometry
F-V	voltage sensor movement
VSD	voltage sensing domain
ECG	electrocardiogram
CPR	cardiopulmonary resuscitation
AED	automated external defibrillators
HEK	human embryonic kidney
EGFP	enhanced green fluorescent protein
WT	wild type
R	arginine
H	histidine
C	cysteine
Y	tyrosine
M	methionine
Q	glutamine
W	tryptophan
S	serine

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Figure 1.

Presenting ECGs and identification of *SCN5A* p.R1309H variant. **A**, Presenting rhythm strip ECG showing wide complex tachycardia with varying QRS durations interspersed with sinus tachycardia. **B**, The ECG shows sinus bradycardia at 90 beats/min, first degree AV block (PR interval 220 ms), prolonged P wave duration (120 ms), ST elevation in lead V1 and RSr' pattern in lead V2. **C**, Family pedigree (arrow indicates proband). **D**, Schematic structure of Na_v1.5 in which the positive charged mutant arginine in DIII/S4 is highlighted in red.

Figure 2.

Kinetics of Nav1.5 channel activation. **A**, Exemplar current traces recorded using a depolarization protocol from -100 to +60 mV with 5 mV increment at holding potential of -120 mV. **B**, Summary current density. **C**, Exemplar current traces showing activation and inactivation phases of the Nav1.5 currents induced by a depolarization step at -25 mV. **D**, Exemplar peak currents of WT (left) and p.R1309H mutant channels showing reduction in current amplitude by 0.1 mM lidocaine. **E**, Voltage dependence of activation fitted with a Boltzmann function. **F**, Relationship between voltage and τ for activation.

Figure 3.

Inactivation kinetics. **A**, Relationship between voltage and τ for inactivation. **B**, Normalized currents induced by the second pulse were plotted as function of the first pulse and fitted by Boltzmann function. **C** and **D**, Effect of 0.1 mM lidocaine on the inactivation kinetics.

Dashed lines indicate the data (in the absence of lidocaine) repeated from panel **B**. **E**, Exemplar traces from a standard two-pulse protocol testing recovery from inactivation with 7.5, 10, 20, 30 or 40 ms interval between P1 and P2. **F**, Summarized data for recovery from inactivation (WT, n=34; p.R1309H, n=37). **G**, Examples of transient peak (upper) and late

(lower) Na^+ currents induced by a 200 ms depolarization to -10 mV. **H**, Ratio of late current to transient peak current.

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Figure 4.

Voltage fluorometry analysis. **A**, Exemplar current traces recorded at -140 mV, -80 mV, -20 mV, and 40 mV. **B**, Voltage dependent activation and inactivation relationships. **C**, Exemplar fluorescence signals recorded simultaneously with current traces shown in **A**. Percentage of fluorescence change was calculated as F/F_0 . **D**, Voltage dependence of steady state fluorescence signal change ($F-V$). The amplitude of fluorescence signal was determined as the mean of the signal in a 5 ms period between starting and end of the depolarizing voltage pulse. **E and F**, Activation and deactivation time constants (τ) of fluorescent signal showing movement of the S4. Activation time constants were obtained by fitting fluorescence trace activation during channel activation in response to voltage pulse of -80 mV and deactivation time constants were calculated by fitting fluorescence trace deactivation at voltage pulse of 40 mV.

Figure 5.

Inward gating pore currents. **A** and **B**, Exemplar current traces and summarized data (WT, n=33; p.R1309H, n=38) of gating pore currents recorded with Na⁺-free TEA external solution (see ref. [14]). **C** and **D**, Gating pore currents with Na⁺-free TEA external solution were blocked by 6 mM Ba²⁺ (WT, n=5; p.R1309H, n=6) (**C**) and reduced by 0.1 mM lidocaine (WT, n=5; p.R1309H, n=9) (**D**). **E** and **F**, Absence of outward gating pore currents recorded with a low Na⁺ external solution (see ref. [13]) (WT, n=11; p.R1309H, n=10). **G** and **H**, Summary of inward gating pore currents recorded with low Na⁺ solution [13] (WT, n=11; p.R1309H, n=10).

Table 1Kinetics of activation and inactivation and late current of *Nay1.5*

	WT				p.R1309H			
	Mean	s.e.m.	N	P	Mean	s.e.m.	N	P
<u>No lidocaine</u>								
$V_{1/2}$ of activation (mV)	-44.5	0.8	36		-40.0	1.1	40	0.02*
K of activation (pA/mV)	5.6	0.2	36		8.0	0.2	40	<0.01*
G_{max}	3.69	0.4	36		2.72	0.2	40	0.04*
$V_{1/2}$ of inactivation (mV)	-93.5	0.7	34		-99.7	1.0	38	<0.01*
K of inactivation (pA/mV)	5.2	0.1	34		6.2	0.2	38	<0.01*
Late current (% of transient peak)	0.2	0.04	22		0.6	0.1	21	<0.01*
<u>Lidocaine</u>								
$V_{1/2}$ of activation (mV)	-45.8	1.0	8	0.63	-36.1	1.2	9	0.03#
K of activation (pA/mV)	4.9	0.4	8	0.20	9.3	0.7	9	0.1
G_{max}	3.01	0.6	8	0.31	1.31	0.3	9	<0.02*#
$V_{1/2}$ of inactivation (mV)	-100.2	0.9	9	<0.01#	-108.8	1.9	9	<0.01#
K of inactivation (pA/mV)	5.8	0.2	9	<0.01#	7.2	0.2	9	<0.01#

* WT vs. pR1309H;

no lidocaine vs. lidocaine.