A Mutation Causing Brugada Syndrome Identifies a Mechanism for Altered Autonomic and Oxidant Regulation of Cardiac Sodium Currents

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Background—The mechanisms of the electrocardiographic changes and arrhythmias in Brugada syndrome (BrS) remain controversial. Mutations in the sodium channel gene, SCN5A, and regulatory proteins that reduce or eliminate sodium current (I\textsubscript{Na}) have been linked to BrS. We studied the properties of a BrS-associated SCN5A mutation in a protein kinase A (PKA) consensus phosphorylation site, R526H.

Methods and Results—In vitro PKA phosphorylation was detected in the I-II linker peptide of wild-type (WT) channels but not R526H or S528A (phosphorylation site) mutants. Cell surface expression of R526H and S528A channels was reduced compared with WT. Whole-cell I\textsubscript{Na} through all channel variants revealed no significant differences in the steady-state activation, inactivation, and recovery from inactivation. Peak current densities of the mutants were significantly reduced compared with WT. Infection of 2D cultures of neonatal rat ventricular myocytes with WT and mutant channels increased conduction velocity compared with noninfected cells. PKA stimulation significantly increased peak I\textsubscript{Na} and conduction velocity of WT but not mutant channels. Oxidant stress inhibits cardiac I\textsubscript{Na}; WT and mutant I\textsubscript{Na} decreases with the intracellular application of reduced nicotinamide adenine dinucleotide (NADH), an effect that is reversed by PKA stimulation in WT but not in R526H or S528A channels.

Conclusions—We identified a family with BrS and an SCN5A mutation in a PKA consensus phosphorylation site. The BrS mutation R526H is associated with a reduction in the basal level of I\textsubscript{Na} and a failure of PKA stimulation to augment the current that may contribute to the predisposition to arrhythmias in patients with BrS, independent of the precipitants. (Circ Cardiovasc Genet. 2014;7:249-256.)

Key Words: death, sudden, cardiac • ion channel • mutation • reactive oxygen species

Mutations in the cardiac sodium channel gene, SCN5A, encoding Na\textsubscript{1.5} currents can produce number of heritable diseases of cardiac rhythm and contractile function. Loss-of-function mutations have been associated with sudden cardiac death and ST segment abnormalities in the right precordial leads of the ECG referred to as Brugada syndrome (BrS). This may be part of a constellation of functional and structural abnormalities of the heart associated with perturbations of the ST segment.

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BrS is characterized by ECG alterations in the right precordial leads in the absence of structural cardiac abnormalities, preponderance in men particularly of Asian descent, and a high rate of sudden cardiac death. The prevalence of type 1 ECG changes is estimated to be \(\approx 5\) of 10,000\(^{5,6}\) and more than double that in Japan and other parts of Asia.\(^{7,8}\) The updated expert consensus statement on heritable arrhythmias recommends that a diagnosis of BrS be made in the presence of a spontaneous or drug-induced type 1 ECG pattern in \(\geq 1\) right precordial lead (V\textsubscript{1} through V\textsubscript{3}).\(^{9}\)

The genetic mechanisms of BrS have provided insights into the links between metabolism, ion channel function, and cardiac arrhythmias. Approximately 20% of BrS is associated with mutations in SCN5A.\(^{10,11}\) In SCN5A-linked BrS, there is a reduction in Na current (I\textsubscript{Na}) density that may result from mutations in the channel subunits or in modifiers of Na channel function. Mutations in glycerol phosphate dehydrogenase 1-like (GPD1-L), which is \(> 80\%\) homologous with glycerol phosphate dehydrogenase 1 (GPD1),\(^{12}\) reduce I\textsubscript{Na} through...
nicotinamide adenine dinucleotide/reduced nicotinamide adenine dinucleotide (NADH)-dependent mechanisms\textsuperscript{13,14} via activation of protein kinase C (PKC).\textsuperscript{15} There is evidence to support a direct effect on Na\textsubscript{v}1.5 by PKC phosphorylation\textsuperscript{15} and an NADH/PKC-mediated overproduction of reactive oxygen species (ROS) leading to a reduction in expressed $I_{\text{Na}}$.\textsuperscript{14} These data suggest that inhibition of $I_{\text{Na}}$ by pyridine nucleotides is mediated by mitochondrial-produced ROS and that protein kinase A (PKA) activation may block ROS-induced reduction of the current and constitutes a treatment strategy for patients with BrS.\textsuperscript{13,16}

We studied a mutation in a patient with BrS presenting with a spontaneous type 1 ECG and history of syncope to elucidate the mechanism(s) of $I_{\text{Na}}$ reduction and its reversal by adrenergic activation. Our data suggest a mechanism for the ECG changes and arrhythmias in BrS initiated by altered metabolism and an increase in oxidant burden that is not mitigated by $\beta$-adrenergic stimulation.

### Materials and Methods

Detailed methods are provided in the Data Supplement. Molecular biological reagents including lentiviral vectors\textsuperscript{17–19} and ion channel plasmids were generated as previously described.\textsuperscript{20} All protocols followed US Department of Agriculture and National Institutes of Health guidelines and were approved by the Animal Care and Use Committee of the Johns Hopkins Medical Institutions.

Neonatal rat ventricular myocytes (NRVMs) were enzymatically isolated\textsuperscript{21} and transduced as previously described.\textsuperscript{18,22} Optical mapping was performed on plated monolayers of NRVMs, and data were analyzed using custom-written scripts in MATLAB (Mathworks, Natick, MA).\textsuperscript{22}

Whole-cell $I_{\text{Na}}$ was measured under voltage clamp at room temperature (22°C) as previously described.\textsuperscript{23} The bath and pipette solutions are described in the Data Supplement. Recombinant human Na\textsubscript{v},1.5 peptides were expressed in \textit{Escherichia coli}, purified, and incubated with PKA in the presence of $\gamma$P\textsubscript{32}-labeled ATP. HEK 293 cells were transformed with wild-type (WT) Na\textsubscript{v},1.5, RS26H, and SS28A Na\textsubscript{v},1.5 plasmids. Cell membrane proteins were labeled with biotin and captured on streptavidin beads. Total and membrane-bound Na\textsubscript{v},1.5 expression was determined by Western blotting using an anti-Na\textsubscript{channel} antibody (Sigma S8809).

The results are presented as mean±SD or SEM. Statistical comparisons were made using a 1-way ANOVA followed by Bonferroni/Dunn tests for multiple comparisons, and serial studies were assessed by repeated-measures ANOVA. Statistical significance was assumed at $P<0.05$.

### Results

The patient is a 33-year-old man who presented with 2 syncopal spells in rapid succession during micturition and without premonitory symptoms. In the field, he had a normal heart rate and blood pressure. He had experienced palpitations after vigorous exercise in the past but had never had syncope. There was a family history of a paternal aunt who died suddenly around age 40 of unknown causes and a maternal uncle with Down Syndrome who died suddenly at age 11 in the setting of a febrile illness. The mutation was transmitted maternally, and the proband’s mother had multiple episodes of syncope and near syncope associated with migraine headaches which were felt to be vasodepressor. Her baseline ECG exhibited 0.5- to 1-mm J-point elevation in leads V\textsubscript{1} and V\textsubscript{2}, and a saddleback segment in V\textsubscript{2}; after procainamide infusion, both V\textsubscript{1} and V\textsubscript{2} ST segments were changed into a coved type (Figure SI in the Data Supplement). The patient’s younger sister carries the mutation and has a baseline incomplete right bundle-branch block (RBBB), which did not change with procainamide infusion.

![Figure 1](http://circgenetics.ahajournals.org/)

**Figure 1.** A. Leads V\textsubscript{1} through V\textsubscript{3} of the patient’s ECG exhibit typical domed J-point elevation. B. A schematic of the Na\textsubscript{v},1.5 pore-forming $\alpha$-subunit and the $\beta$1 and $\beta$2 subunits. The disease-causing mutation (RS26H) is in the I-II interdomain linker of Na\textsubscript{v},1.5 in a canonical protein kinase A (PKA) phosphorylation recognition sequence. The residue 2 amino acids C-terminal to RS26, SS28 was mutated to alanine in this study. C. In vitro phosphorylation of I-II linker peptides. Purified fragments were incubated with PKA and calcium-calmodulin kinase II (CaMKII) in the presence of $\gamma$P\textsubscript{32}-labeled ATP. The labeled peptides were separated by polyacrylamide gel electrophoresis (PAGE). Mutant peptides RS26H and SS28A are not labeled by PKA but are phosphorylated as efficiently as wild type (WT) in the presence of CaMKII.
His 12-lead ECG revealed coved ST segments in leads V1 and V2 consistent with a Brugada type 1 ECG pattern (Figure 1A). A transthoracic echocardiogram was normal. A single-chamber defibrillator was implanted.

**BrS Mutation Disables PKA Phosphorylation**

DNA sequencing of SCN5A revealed a nucleotide transition at position 1577 encoding a missense mutation at codon 526 changing an arginine to histidine (R526H, Figure 1B). The minor allele frequency from the exome variant server is 0.016% and is a chemically and structurally conservative change. However, the mutation resides in a PKA recognition sequence in the I-II interdomain linker of the channel, with serine 528 (S528) as the phosphorylated residue.

The I-II linker of Na,1.5 contains consensus phosphorylation sites for several kinases including PKA and calcium calmodulin kinase II. We created mutant I-II linker peptides with the clinical mutation, R526H, and at the putative phosphorylation site, S528A. In vitro phosphorylation by PKA revealed complete elimination of 32P incorporation into the R526H and S528A peptides. By contrast, calcium calmodulin kinase II phosphorylated all channel peptides equally (Figure 1C).

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**Figure 2.** Protein kinase A (PKA) regulation of Na current variants. A, Representative families of current through wild-type (WT), R526H, and S528A channels expressed in HEK 293 cells in the presence and absence of PKA activation. B, I-V relationships in the presence (filled symbols) and absence (open symbols) of PKA stimulation. There is no increase in the current through the mutant channels. C, Activation (circles) and steady-state inactivation (triangles) curves in the presence (filled symbols) and absence (open symbols) of PKA stimulation. The data are fit to a Boltzman function as described in the Materials and Methods section. There are no significant differences between the WT and mutant channels in the basal voltage dependence and kinetics of gating.
Altered Channel Regulation in Response to Stress

We speculate that stressors that reduce \( \text{I}_{\text{Na}} \) density (oxidants, fever, and Na channel blocking drugs) may, in part, be offset by PKA stimulation of the channel. To study the functional effects of these mutations in response to PKA phosphorylation, we expressed WT and mutant Na\(_{\text{v}}\) currents in HEK 293 cells. Selected families of currents in standard recording solutions revealed current densities through the mutant channels that were only modestly smaller than WT but not different from each other in the absence of PKA stimulation (Figure 2A and 2B). The baseline whole-cell properties including the current–voltage (I–V) relationships, voltage dependence and kinetics of gating, and kinetics of current decay were not different between WT and the mutants (Figure 2C; Table SI in the Data Supplement). In the presence of PKA, WT Na\(_{\text{v}}\) currents were significantly upregulated with a hyperpolarizing shift in the peak I–V and activation curves (Figure 2) and hastened recovery from inactivation (Table SI in the Data Supplement). By contrast, neither the peak current, voltage dependence of gating, or recovery kinetics (Figure 2; Table SI in the Data Supplement) of R526H or S528A were significantly affected by the addition of PKA.

Oxidative stress and an increase in glycolysis will lead to increased levels of cytosolic NADH, which rapidly decreases \( \text{I}_{\text{Na}} \). We recorded currents in a metabolic stabilizing solution (see Methods in Data Supplement, inhibiting kinases and phosphatases) to examine the effect of intracellular NADH on the WT and mutant currents. The baseline peak density of the WT current was significantly larger than either R526H or S528A mutant currents (Figure 3A–3C). The addition of 100 \( \mu \)mol/L NADH to the pipette solution reduced the average \( \text{I}_{\text{Na}} \) by >50% in all channel variants (Figure 3A and 3B). The addition of NADH had no significant effect on the decay kinetics or voltage dependence of gating of WT Na\(_{\text{v}}\),1.5 or the mutant channels (Figure 3E; Table SII in the Data Supplement). We speculated that the current density might be restored by PKA in the setting of an increase in NADH and that this restoration might be compromised in the mutant channels. To study the effect of activation of PKA signaling on NADH-induced suppression of Na\(_{\text{v}}\),1.5 currents, cells were incubated with forskolin and 8-Br cAMP for 20 minutes

![Figure 3. Regulation of Na channel variants by reduced nicotinamide adenine dinucleotide (NADH) and protein kinase A (PKA). The currents are recorded with fluoride in the pipette. A, Representative families of current through wild-type (WT), R526H, and S528A channels expressed in HEK 293 cells (top) in the presence of 100 \( \mu \)mol/L NADH (middle) and NADH plus PKA activation with forskolin (FSK) and 8-Br cAMP (bottom). B, I–V relationships in the absence (open squares) and presence of NADH (gray squares) and NADH+PKA stimulation (filled squares). NADH reduces the current through all channel variants, whereas PKA activation does not increase the current density through the mutant channels. C, Bar plots of peak current densities of WT (black), R526H (blue), and S528A (red) under basal conditions. D, Summary bar plots for WT, R526H, and S528A channel peak current density at baseline (open bars) with NADH (gray bars) and NADH+PKA activation (black bars). E, Activation and steady-state inactivation curves in the absence (open circles activation, open triangles inactivation) and presence of NADH (gray circles activation, gray triangles inactivation) and NADH + PKA stimulation (filled circles activation, filled triangles inactivation) for each of the channel variants; there are no significant differences in any of the variants under any of the conditions.](http://circgenetics.ahajournals.org/Downloaded from)
prior to patch clamp recording. Incubation with forskolin and 8-Br cAMP completely reversed the downregulation of WT NaV1.5 current by intracellular NADH (Figure 3A, 3B, and 3D) and produced a modest but significant hyperpolarizing shift in the voltage dependence of activation gating (Table SII in the Data Supplement). The reductions in R526H and S528A currents by NADH were not affected by the addition of forskolin and 8-Br cAMP (Figure 3D; Table SII in the Data Supplement).

**Trafficking of Mutant Channels**

The reduction in basal current density of R526H and S528A channels without a change in biophysical characteristics compared with WT suggested the possibility of an alteration in trafficking to the surface membrane. Surface membrane expression of WT and mutant NaV1.5 channels in HEK 293 was quantified by labeling intact cells with biotin and purifying the protein on immobilized streptavidin. Overall expression of the channel proteins did not differ; however, Figure 4A illustrates that even with less WT NaV1.5 protein in the total lysate, a greater proportion of the WT channel was biotinylated compared with both the R526H and S528A mutants (Figure 4B).

To study trafficking in a cardiac cell background, NRVMs were infected with lentiviruses containing the WT or mutant channels fused to enhanced green fluorescent protein (eGFP) at the carboxyl terminus. The channel-infected NRVMs were cotransfected with an endoplasmic reticulum marker dsRED-ER. Consistent with the biotinylation experiments, NRVMs infected with the viruses encoding the R526H and S528A mutants exhibited more channel protein in subcellular membranes, colocalized with dsRED-ER compared with WT channels (Figure 4C and 4D).

**Effect of Mutants on Conduction Velocity**

The functional consequences of the reduced surface expression and altered regulation of the mutant channels were studied using optical mapping of 2D cultures of NRVMs. To control for variability in baseline conduction velocities (CVs), cultures that were transduced with Na channel variants were compared with naïve nontransfected cultures. The cultures were stimulated over a range of pacing cycle lengths (175–500 ms) to determine the CVs; isochronal maps at baseline and in the presence of isoproterenol are shown for WT, R526H, and S528A transfected cultures (Figure 5A). The baseline CVs of NRVM cultures infected with WT NaV1.5 and the mutant channels were similar and ≈20% to 50% (5–10 cm/s) faster than nontransfected cultures over a range of pacing cycle lengths (Figure 5B).
At more rapid pacing rates, accumulation of ROS is anticipated, which would decrease $I_{Na}$. The CVs of the WT and mutant channel–transduced cultures are greater than that of nontransfected control cultures at slower pacing rates; at faster rates, the difference is diminished and no longer significant at a pacing cycle length of 180 ms (Figure 5B). The CVs of the WT-infected cultures are significantly increased by exposure to isoproterenol while that of the mutant-infected cultures are no more responsive than nontransfected control cultures (Figure 5C).

**Discussion**

Mutations in the Na channel, $SCN5A$, or regulatory proteins such as $GPD1-L$ that reduce current have been associated with BrS and sudden infant death syndrome (SIDS). In both conditions, arrhythmias are more prevalent under conditions of various types of stress (oxidant stress, fever, ischemia, and Na channel blocking drugs). We describe an $SCN5A$ mutation in a patient with BrS that produces both a chronic reduction in $I_{Na}$ and absence of augmentation of the current by adrenergic stimulation. Interestingly, the reduction in the basal current produced by the R526H and S528A mutations compared with WT Na$_v$1.5 is more pronounced when fluoride is included in the patch pipette to inhibit protein phosphatase, consistent with increased basal phosphorylation of the WT channel. The elimination of a PKA site seems to be central to both the signaling and trafficking deficits. The disease-causing mutation, R526H, which is chemically and structurally conservative, eliminates the basic priming residue in a consensus PKA phosphorylation recognition sequence. Replacement of the phosphorylation target residue, S528A, alters channel trafficking and regulation by PKA in a manner that is comparable to the disease-causing mutation and similar to previously described defects in PKA-mediated $I_{Na}$ potentiation. The mutations reduce channel expression at the membrane surface as assessed by biotinylation and immunocytochemistry.

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**Figure 5.** Optical mapping of Na channel variants. **A**, Representative isochronal maps from cultures of neonatal rat ventricular myocytes infected with the Na channel variants and stimulated at the left side of the culture. **B**, Plots of the average conduction velocity (CV±SEM) of wild-type (WT), R526H, and S528A transduced cultures and noninfected (NT) control cultures over a range of pacing cycle lengths (PCLs). CV is consistently faster in WT, R526H, and S528A NaV1.5 infected cultures compared with the NT controls for the entire range of PCLs. *P*<0.05, all vs NT; #P<0.01, all vs NT. **C**, Plots of the change in average CV±SEM of WT, R526H, and S528A transduced cultures and NT control cultures after application of 1 μmol/L isoproterenol. The change in CV of WT-transduced cultures is significantly larger than CV changes of NT cultures and mutant transduced cultures after isoproterenol application. *P*<0.05, R526H vs WT; #P<0.05, S528A vs WT. The number of cultures studied at all PCLs in both the absence and presence of isoproterenol were NT (14), WT (13), S528A (5), and R526H (11).
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studies (Figure 4) and are reflected in a lower expressed current density in HEK cells (Figure 3).

The reduction in \( I_{Na} \) is further complicated by the absence of a response of the channel to adrenergic stimulation. Neither the direct activation of adenyl cyclase nor \( \beta \)-adrenergic stimulation with isoproterenol had a significant effect on current density or CV mediated by the mutant channel variants. These findings suggest a mechanism of stress-induced arrhythmias that may be due to changes of cellular redox balance with alterations in the ratio of NAD/NADH and consequent reduction in \( I_{Na} \). Such metabolic stressors are typically accompanied by activation of the sympathetic nervous system that may partially offset the redox-induced reduction in \( I_{Na} \) and would dampen stress-induced ECG changes in BrS and alterations in CV. In such cases, arrhythmias would not be predicted to be induced by sympathetic activation but instead would be mitigated by an increase in sympathetic tone. This concept is consistent with the utility of isoproterenol in the treatment of arrhythmic storm in patients with BrS.12-15 

There are several mechanisms by which metabolism has been proposed to alter the \( I_{Na} \). Direct oxidation of the channel can alter channel conductance,3,11-13 gating,3,11,13 and trafficking.14 Mutations associated with BrS have informed other mechanisms of metabolic regulation of Na channels. NADH is generated from NAD during glycolysis, and NAD must be regenerated for glycolysis to continue. \( GPD1 \) reduces dihydroxyacetone phosphate to glycerol-3-phosphate, causing oxidation of NADH and regeneration of NAD with the electrons released from this reaction entering the electron transport chain. \( GPD1-L \) is highly homologous to \( GPD1 \) and harbors mutations associated with BrS.12 When mutant \( GPD1-L \) is coexpressed with \( Na_{1.5}, I_{Na} \) is significantly reduced.12-15 The BrS mutations have been proposed to reduce the enzymatic function of \( GPD1-L \) and would be expected to increase intracellular NADH levels. An increase in NADH possibly via activation of PKC,13 enhancing phosphorylation of complex III resulting in an increase in ROS release,14 reduces channel function. Alternatively, inactivating mutants of \( GPD1-L \) have been proposed to increase PKC phosphorylation of the Na channel in the III-IV linker, reducing current density.15 Increased mitochondrial ROS release, elevated levels of NADH, and PKC activation have been implicated in \( I_{Na} \) downregulation in models of nonischemic cardiomyopathy and in CV slowing in diseased human ventricles.16-18 Our work supports another mechanism of coupling of metabolism to channel function through altered PKA activation.22 Increased NADH causes a reduction in current through WT and mutant R526H- and S528A-expressed channels. The absence of the PKA phosphorylation site in the mutant channels precludes current augmentation in the setting of sympathetic activation.

The mechanism of arrhythmias in this \( SCN5A \)-mediated BrS seems to involve a substrate that is characterized by decreased basal current expression as a result of altered mutant channel trafficking. Although it is difficult to infer general mechanisms from a single family, this disease-causing mutation produces the requisite basal reduction in \( I_{Na} \) by compound by defective current augmentation that generates the substrate for a potentially lethal arrhythmias. A trigger that further reduces \( I_{Na} \) through any number of mechanisms such as alterations in glycolysis, ROS levels, or PKC activation, cannot be mitigated by PKA activation, leading to a further reduction in current. The absence of a PKA-mediated reversal of NADH-induced current reduction (Figure 3) is consistent with the lack of effect on conduction in infected NRVMs. The reduction of \( I_{Na} \) could alter either regional dispersion of repolarization or conduction in the heart; one or both may contribute to the genesis of the potentially lethal ventricular arrhythmias in patients harboring this mutation.

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Disclosures

None.

References

general mechanisms of arrhythmias involving $\alpha$ the gene encoding the potentially arrhythmogenic decrease in the sodium current ($I_{\text{Na}}$) and cardiac arrhythmias. Oxidant stress, as may occur with fever, ischemia, or even an increase in heart rate, can produce mechanisms of Brugada syndrome have provided general insights into the links between metabolism, ion channel function, $I_{\text{Na}}$. We have described a mutation in a consensus protein kinase A phosphorylation site in a patient with Brugada syndrome that does not respond to sympathetic stimulation. Wild-type and Brugada syndrome successfully treated using isoprenaline. Eur. J Pharmacol. 2010;7:248–249.


**CLINICAL PERSPECTIVE**

Brugada syndrome is an inherited cardiac arrhythmia characterized by coved-type ST-segment elevation in the right precordial ($V_1$ and $V_2$) leads of the ECG and an increased risk of sudden cardiac death. In some cases, the cause is a mutation in SCN5A, the gene encoding the $\alpha$-subunit of cardiac sodium channels, typically resulting in a loss of function. The genetic and cellular mechanisms of Brugada syndrome have provided general insights into the links between metabolism, ion channel function, and cardiac arrhythmias. Oxidant stress, as may occur with fever, ischemia, or even an increase in heart rate, can produce a potentially arrhythmogenic decrease in the sodium current ($I_{\text{Na}}$). We have described a mutation in a consensus protein kinase A phosphorylation site in a patient with Brugada syndrome that does not respond to sympathetic stimulation. Wild-type and Brugada syndrome mutant $I_{\text{Na}}$ are reduced by oxidant stress and subsequent protein kinase A stimulation mitigates the reduction of the wild-type $I_{\text{Na}}$ but not the current through mutant channels. This disease-causing mutation may have relevance to more general mechanisms of arrhythmias involving $I_{\text{Na}}$. A trigger that reduces $I_{\text{Na}}$ through any number of mechanisms that cannot be mitigated by protein kinase A activation, could produce a reduction in $I_{\text{Na}}$ with an increased risk of cardiac arrhythmias.
A Mutation Causing Brugada Syndrome Identifies a Mechanism for Altered Autonomic and Oxidant Regulation of Cardiac Sodium Currents

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In the article by Ma and Adelstein, “A Point Mutation in Myh10 Causes Major Defects in Heart Development and Body Wall Closure,” which appeared in the June 2014 issue of the journal (Circulation: Cardiovascular Genetics, 2014;7:249–256), there was an error in the keywords.

The last key word, “nonmuscle myosin type IIA”, in the list at the bottom of the abstract on the first page of the article should have been separated with a bullet point from “hernia, diaphragmatic”.

The correction has been made online. The publisher regrets the error.
SUPPLEMENTAL MATERIAL

Supplemental Methods

Table S1. Current features baseline solution
Table S2. Current features metabolic stabilizing solution

Supplemental Figure S1

SUPPLEMENTAL METHODS

METHODS AND MATERIALS

Molecular Biology

Third-generation lentiviral vectors based on the human immunodeficiency virus Type 1 were generated as previously described. The lentiviral constructs used in this study include the self-inactivating (SIN) long terminal repeat (LTR), the central polypurine tract (cPPT) and the woodchuck hepatitis virus post-transcriptional regulatory element (Wpre). The human Na\textsubscript{v}1.5-eGFP fusion construct was cloned from a previously described plasmid DNA and then cloned into the lentivirus vector (LV) plasmid, \textit{pRRLsin18.cPPT.CMV.eGFP.Wpre} after removal of the enhanced green fluorescence protein (eGFP) sequence. To obtain the Na\textsubscript{v}1.5 mutant plasmids R526H and S528A, site-directed mutagenesis was performed using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer’s instructions. Lentiviruses encoding the Na\textsubscript{v}1.5 variants were produced by Lipofectamine 2000 (Invitrogen, Life Technologies, Carlsbad, CA) transfection of the four lentiviral plasmids into human embryonic kidney (HEK) 293T cells as previously described. Briefly, the supernatant from HEK293T cell flasks containing virus was collected 48 and 72 hours after transfection, filter sterilized through 0.2 μm cellulose acetate (Corning, Cambridge, MA) and concentrated by ultra-
filtration (100,000 MWCO, Centricon Plus-70, Millipore, Milford, MA). Transduction titer was assigned on concentrated viral stock by assessing transgene expression in HEK 293T cells using a limiting dilution assay in the presence of 8 µg/mL of Polybrene (Sigma-Aldrich, St. Louis, MO) three days after transduction.

The Na\textsubscript{V}1.5 mutations for expression in human embryonic kidney cells (HEK293) were made by site-directed mutagenesis in the previously described Na\textsubscript{V}1.5-GFP cDNA background\textsuperscript{3}. Approximately 0.75x10\textsuperscript{6} HEK293 cells (American Type Culture Collection, Manassas, VA) were cultured in 6-well tissue culture dishes in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), L-glutamine (2 mmol/L), penicillin (100 U/mL), and streptomycin (10 mg/mL). The cells were co-transfected with plasmids encoding the appropriate Na\textsubscript{V}1.5-GFP and Na\textsubscript{V}\textbeta\textsubscript{1} subunits as previously described.\textsuperscript{3} Cells were transfected using Lipofectamine\textsuperscript{TM} 2000 (Invitrogen) according to the manufacturer’s instructions and were studied 48 to 72 hours post-transfection. The total amount of DNA for all transfections was kept constant.

Neonatal rat ventricular myocytes (NRVMs) were enzymatically dissociated from the ventricles of 2-day-old Sprague-Dawley rats (Harlan Laboratories, Inc., Indianapolis, IN) with trypsin and collagenase (Worthington Biochemical Corp., Lakewood, NJ) as previously described.\textsuperscript{6} For trafficking studies, NRVMs were maintained in a humidified incubator at 37°C and 5% CO\textsubscript{2} and transfected with plasmids containing the Na\textsubscript{V}1.5-GFP channel variants (WT, R526H or S528A) and pDsRed2-ER (Clontech Laboratories, Inc., Mountainview, CA, catalog #632409) using Lipofectamine 2000 (Invitrogen) according to manufacturer’s specifications.

For optical mapping, NRVMs were infected with lentiviral vectors with modifications of a method described previously.\textsuperscript{1,5} For all transduction experiments, the concentrated lentivirus
stock was applied at the indicated multiplicity of infection (MOI) in the presence of 8 µg/mL of Polybrene (Sigma-Aldrich). Freshly isolated NRVMs were suspended in culture medium containing 10% FBS at a concentration of 10^6 cells/ml. The specified lentivirus was added to the cell suspension and subsequently plated on fibronectin-coated (25 µg/mL in water), UV-treated coverslips (day 0). After 24 hours at 37°C the medium was changed and fresh medium with 10% FBS was added. The culture medium was changed every two days.

**Optical mapping**

Optical mapping was performed on plated monolayers of NRVM 6-7 days post viral transduction as previously described. Cover slips with cultured NRVMs were placed in a custom chamber and stained with 10 µM di-4-ANEPPS (Molecular Probes, Life Technologies, Eugene OR) for 5 min. at 37°C under continuous superfusion with oxygenated Tyrode solution. Point stimulation via platinum electrodes was applied just above one edge of the monolayer. To determine the conduction velocity (CV), cells were stimulated with monophasic, 10ms pulses at 1.5 times the diastolic threshold over a range of stimulation frequencies (2 - 10 Hz). A 2-s recording was taken after a twenty beat drive train. The data were analyzed using custom-written scripts in MATLAB (MathWorks, Natick, MA). All mapping studies were performed with transfected cultures compared to non-transfected (NT) cultures. The CV in NT cultures due to endogenous Na currents varied as did the CV response of NT cultures to application of isoproterenol. In order to account for this variability in NT cultures we normalized CV of the transfected cultures to the CV of naïve NT NRVM cultures. Notably, all of the transfected cultures exhibited about a 15-50% increase in CV compared to NT controls depending upon the pacing cycle length. In paired experiments isoproterenol increased the CV in WT channels.
compared to NT control cultures whereas the mutants exhibited no change in CV when
normalized to NT controls.

Electrophysiology and Recording Solutions

Whole cell $I_{Na}$ was measured under voltage-clamp with an Axopatch 200A patch-clamp
amplifier (Molecular Devices Corp., Sunnyvale, CA) at room temperature (22°C) as previously
described. Voltage command protocols were generated by custom-written software and
PCLAMP 10 (Molecular Devices Corp). Capacitance compensation was optimized and series
resistance was compensated by 40-80%. Membrane currents were filtered at 5 kHz and digitized
with 12-bit resolution through a DigiData-1200 interface (Molecular Devices Corp.).

The patch pipettes had 1-2 MΩ tip resistances when filled with a pipette solution containing
(in mM): 35 NaCl, 105 CsCl, 10 Cs-HEPES, pH 7.3 with CsOH. The bath solution contained (in
mM) 150 NaCl, 2 KCl, 2 CaCl$_2$, 1 MgCl$_2$, 10 glucose, 10 HEPES (pH 7.4 with NaOH). The
metabolic stabilizing solution (inhibiting kinases and phosphatases) contained (in mM): 10 NaF,
100 CsF, 20 CsCl$_2$, 5 BAPTA, 4 CaCl$_2$ and 10 HEPES pH 7.35 with CsOH. The bath solution
contained (in mM): 145 NaCl, 4 KCl, 1.8 CaCl$_2$, 1 MgCl$_2$, 10 glucose, and 10 HEPES pH 7.4
with NaOH with an estimated liquid junction potential = +7.5 mV. The NADH solution was
similar to the intracellular control solution except that 100µM of NADH was added. The PKA
stimulating solution was obtained by adding 20µM of forskolin and 200µM of 8-Br-cAMP to the
extracellular solution. The pH and osmolality were adjusted to 7.4 (±0.02) and 330 (±2) mOsm
Kg$^{-1}$ with the addition of NaOH or CsOH and glucose, respectively. In all experiments,
recording was begun 10 minutes after establishment of the whole-cell mode to permit
stabilization of the voltage dependence and kinetics of gating.
Standard protocols were used for assessment of the voltage-dependence of activation and inactivation, recovery from inactivation, and rates of entry into inactivation and are provided as insets in the relevant figures. To determine the $V_{1/2}$ and slope factor $k$, steady state inactivation data were fit with a Boltzmann function of the form: $I/I_{\text{max}} = \{1+\exp[(V-V_{1/2})/k]\}^{-1}$. Recovery from inactivation data were fit with a bi-exponential function of the form: $I(t)/I_{\text{max}} = A_1 \cdot \exp(-t/\tau_1) + A_2 \cdot \exp(-t/\tau_2) + A_3$, using a nonlinear least squares error minimization. The decay phase of the current during a voltage step was fit with biexponential function of the form: $I(t) = A_f \cdot \exp(-t/\tau_f) + A_s \cdot \exp(-t/\tau_s)$, where $A_f$ and $A_s$ are the fractions of fast and slow inactivating components, respectively. The persistent inward (late) $\text{Na}^+$ current was the tetrodotoxin (TTX)-sensitive current (30μM) measured at 100-500 ms after the depolarizing voltage step.

**Protein Chemistry: In Vitro phosphorylation Western blotting, cell surface expression**

PCR amplified cDNAs encoding the intracellular regions of human NaV1.5 were cloned into the EcoRI site of pGEX-6P1 (Genbank ID: U78872). The resulting glutathione-S-transferase (GST) fusion constructs were confirmed for proper orientation and sequence by automated DNA sequencing. These plasmids were used to transform BL21 *E. coli*. The intracellular peptides were expressed by isopropylthiogalactoside (IPTG) induction of bacterial cultures grown to an absorbance at 600 nm ($A_{600}$) between 0.6-1.0, followed by culture for another 1-2 hours. Cells were pelleted and resuspended in ice-cold phosphate buffered saline (PBS) with protease inhibitors and then lysed by sonication. The sonicate was centrifuged, and the soluble fraction was passed over a GSTrap column (GE Healthcare). The column was washed with >10 column volumes of PBS and eluted with 10mM glutathione. The purified fragments were separated by electrophoresis on 4-12% polyacrylamine gel (PAGE). The purified fragments were incubated using a 1:20 molar ratio of PKA (~5000 U/µg protein, NEB, cat# P6000) to I-II linker fragment.
in the presence of $\gamma P^{32}$ labeled ATP. The labeled peptides were run on the same percentage gel and exposed to film.

HEK 293 cells were transformed with WT Na\textsubscript{V}1.5, R526H and S528A Na\textsubscript{V}1.5 plasmids. Cell surface membrane proteins with accessible lysine residues were labeled with a sulfo-NHS-SS-Biotin (Sulfosuccinimidyl-2-(biotinamido)-ethyl-1,3’-dithiopropionate, Pierce Protein, Thermo Scientific, Rockford, IL). Total protein concentrations in the cell lysates were analyzed using the Bradford method, and the same amount of total protein for each transformation was added to the immobilized beads. Total and membrane bound Na\textsubscript{V}1.5 expression was determined by SDS-PAGE follow by Western blotting and ImageJ quantification of band density of cell lysate and streptavadin-purified biotinylated membrane proteins (anti-Na\textsubscript{Ch} antibody, Sigma S8809). Membrane associated Na\textsubscript{V}1.5 is expressed as a fraction of total channel protein in the lysate.

**Statistics**

The results are presented as mean ± SD or SEM. Statistical comparisons were made using a one-way ANOVA followed by Bonferroni/Dunn tests for multiple comparisons. Differences in serial studies were assessed by repeated measures ANOVA. In some cases, an unpaired Student's $t$ test was used to evaluate the significance of the difference between means. Statistical significance was assumed at $P<0.05$.

**References**


SUPPLEMENTAL FIGURE LEGEND

Figure S1. ECGs recorded from the proband’s mother at the time of provocative testing. (A) Baseline ECG leads V1-V3 prior to drug infusion. (B) The same ECG leads after 750 mg intravenous infusion of procainamide.
Table S1. – Current features in baseline solution

<table>
<thead>
<tr>
<th>Current density Peak (pA/pF, SE, (n))</th>
<th>Baseline</th>
<th>PKA</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>-207 ± 40 (7)</td>
<td>-346 ± 49 (9)#</td>
</tr>
<tr>
<td>R526H</td>
<td>-256 ± 52 (8)</td>
<td>-291 ± 42 (6)</td>
</tr>
<tr>
<td>S528A</td>
<td>-214 ± 57 (4)</td>
<td>-261 ± 64 (4)</td>
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<table>
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<tr>
<th>Steady State Activation (mV, SE, (n))</th>
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<th>PKA</th>
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<tbody>
<tr>
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<td>-43.3 ± 1.8 (7)</td>
<td>-52.8 ± 2.0 (6)#</td>
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<tr>
<td>R526H</td>
<td>-43.5 ± 2.3 (9)</td>
<td>-48.6 ± 1.4 (5)</td>
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<tr>
<td>S528A</td>
<td>-45.5 ± 1.9 (4)</td>
<td>-44.1 ± 1.5 (4)</td>
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<tr>
<th>Steady State Inactivation (mV, SE, (n))</th>
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<tr>
<td>R526H</td>
<td>-80.6 ± 1.3 (8)</td>
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<td>S528A</td>
<td>-79.7 ± 2.1 (5)</td>
<td>-81.5 ± 2.2 (4)</td>
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<th>Recovery from Inactivation (ms, SE, (n))</th>
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<td>WT</td>
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<td>3.2 ± 1.9 #</td>
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<td></td>
<td>76.8 ±30.0 (5)</td>
<td>35.3 ± 14.6 (7)</td>
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<td>R526H</td>
<td>3.8 ± 1.1</td>
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<td>45.2 ± 16.3 (4)</td>
<td>47.9 ± 27.0 (4)</td>
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<td>S528A</td>
<td>5.1 ± 1.4</td>
<td>4.5 ± 0.6</td>
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<tr>
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<td>52.1 ± 24.2 (4)</td>
<td>54.5 ± 16.5 (4)</td>
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</table>

*p<0.05, compared to WT; #p<0.05 compared to baseline; §p<0.001 compared to NADH
Table S2 Current features in metabolic stabilizing solution

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<th>Current density Peak (pA/pF, SE, (n))</th>
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<td>100 µM NADH</td>
<td>100 µM NADH + 20 µM FSK + 200 µM 8 Br cAMP</td>
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<td>WT</td>
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<td>R526H</td>
<td>-222±24 (6)*</td>
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<td>S528A</td>
<td>-175±27 (9)*</td>
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<tr>
<td>WT</td>
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<td>-45.9±2 (7)</td>
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<tr>
<td>R526H</td>
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<td>-40.6±3 (4)</td>
<td>-38.7±2 (5)</td>
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<td>S528A</td>
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<td>-42.9±3 (6)</td>
<td>-41.8±4 (6)</td>
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<td></td>
<td>Baseline</td>
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<td>100 µM NADH + 20 µM FSK + 200 µM 8 Br cAMP</td>
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<td>WT</td>
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<tr>
<td></td>
<td>Baseline</td>
<td>100 µM NADH</td>
<td>100 µM NADH + 20 µM FSK + 200 µM 8 Br cAMP</td>
</tr>
<tr>
<td>WT</td>
<td>5.6±0.6 (3)</td>
<td>5.8±1.5 (3)</td>
<td>5.8±0.6 (4)</td>
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<td>R526H</td>
<td>6.2±0.4 (3)</td>
<td>6.6±1.6 (4)</td>
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*p<0.05, compared to WT; #p<0.05 compared to baseline; §P<0.001 compared to NADH
Figure S1. ECGs recorded from the proband’s mother at the time of provocative testing. (A) Baseline ECG leads V1-V3 prior to drug infusion. (B) The same ECG leads after 750 mg intravenous infusion of procainamide.