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Striking In Vivo Phenotype of a Disease-Associated Human *SCN5A* Mutation Producing Minimal Changes in Vitro

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Background—The D1275N *SCN5A* mutation has been associated with a range of unusual phenotypes, including conduction disease and dilated cardiomyopathy, as well as atrial and ventricular tachyarrhythmias. However, when D1275N is studied in heterologous expression systems, most studies show near-normal sodium channel function. Thus, the relationship of the variant to the clinical phenotypes remains uncertain.

Methods and Results—We identified D1275N in a patient with atrial flutter, atrial standstill, conduction disease, and sinus node dysfunction. There was no major difference in biophysical properties between wild-type and D1275N channels expressed in Chinese hamster ovary cells or tsA201 cells in the absence or presence of β1 subunits. To determine D1275N function in vivo, the *Scn5a* locus was modified to knock out the mouse gene, and the full-length wild-type (H) or D1275N (DN) human *SCN5A* cDNAs were then inserted at the modified locus by recombinase mediated cassette exchange. Mice carrying the DN allele displayed slow conduction, heart block, atrial fibrillation, ventricular tachycardia, and a dilated cardiomyopathy phenotype, with no significant fibrosis or myocyte disarray on histological examination. The DN allele conferred gene-dose–dependent increases in *SCN5A* mRNA abundance but reduced sodium channel protein abundance and peak sodium current amplitudes (H/H, 41.0±2.9 pA/pF at −30 mV; DN/H, 19.2±3.1 pA/pF, $P<0.001$ vs H/H; DN/DN, 9.3±1.1 pA/pF, $P<0.001$ versus H/H).

Conclusions—Although D1275N produces near-normal currents in multiple heterologous expression experiments, our data establish this variant as a pathological mutation that generates conduction slowing, arrhythmias, and a dilated cardiomyopathy phenotype by reducing cardiac sodium current. **(*Circulation.* 2011;124:1001-1011.)**

Key Words: cardiomyopathy ■ electrophysiology ■ genetics ■ ion channels

Voltage-gated sodium channels play a critical role in the generation and propagation of the cardiac action potential, and mutations in *SCN5A*, the gene encoding the major pore-forming sodium channel α subunit in the heart (Nav1.5), cause multiple inherited cardiac arrhythmia syndromes, including long-QT syndrome, the Brugada syndrome, isolated cardiac conduction disease, sinus node dysfunction, and atrial fibrillation.[1–7] More recently, *SCN5A* mutations have been associated with dilated cardiomyopathy (DCM), and such DCM mutations have been associated with a similar range of arrhythmias.[8–15]

Editorial see p 993
Clinical Perspective on p 1011

The D1275N *SCN5A* mutation was initially reported in a Dutch family affected by atrial standstill, mild conduction disease, and atrial enlargement but no ventricular structural abnormality; only subjects who carried a variant in the connexin 40 promoter displayed the clinical phenotype.[16] Subsequently, D1275N was implicated in a large family affected by DCM and various arrhythmias such as sinus node dysfunction, atrial and ventricular tachyarrhythmias, and conduction disease.[8,9,17] Most recently, the mutation was reported in a family with atrial tachyarrhythmias, conduction disease, and ventricular enlargement without impaired contractility.[18]

Heterologous expression systems are conventionally used to assess function of ion channel mutations.[2,19] Most studies (including our own data described below) that have compared wild-type and D1275N channels in heterologous expression systems have not shown major differences in the biophysical

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properties of the variant channel.[16,20] Thus, although the mutation has been reported as a cause of unusual phenotypes in a number of kindreds, its relationship to the clinical phenotypes remains uncertain.

To address this discrepancy, we have used recombinase-mediated cassette exchange[21] to engineer mice expressing the mutant human channel (here called DN); we compared the functional properties of these animals with those expressing wild-type human alleles (H) that we previously generated in an identical fashion.[21] The data demonstrate that D1275N causes a severe defect in sodium channel function in vivo, consistent with the reported clinical phenotypes.

Methods

Study Subjects

The proband and family members were screened for mutations in *SCN5A* by polymerase chain reaction amplification of coding regions and flanking intronic sequences, followed by direct sequencing of amplicons on an ABI PRISM 3730 DNA Sequence Detection System (Applied Biosystems, Foster City, CA). Informed consent was obtained for presentation of the kindred.

Animal Model

All studies using animals were approved by the institutional animal care and use committees at Vanderbilt University and performed in accordance with National Institutes of Health guidelines. We have previously modified the *Scn5a* locus in mouse embryonic stem cells to enable the technique of recombinase mediated cassette exchange.[21–23] In our initial studies, we inserted the full-length human *SCN5A* cDNA into the targeted locus.[21] Mice homozygous for the exchanged allele (called H/H) expressed only the human allele and had normal ECGs and ventricular sodium current, supporting the hypothesis that expression of the exchanged allele was under control of endogenous *Scn5a* regulatory mechanisms.

For the present study, we used the same technique to generate DN mice in which the exchanged construct was identical to that previously used for the H/H mice with the exception of a c.3823G→A mutation resulting in p.D1275N and insertion of an FLAG epitope between residues 153 and 154 of the extracellular linker S1-S2 in domain I; the FLAG insertion into S1-S2 linker has previously been found to have no effect on channel gating or cell surface expression.[24,25] We also generated FG mice bearing the wild-type *SCN5A* allele with the FLAG tag. Initial matings between mice heterozygous for engineered alleles resulted in H/H, DN/H, and FG/H mice, and these mice were then bred into the 129/Sv background. H/H, DN/H, and DN/DN mice were generated from DN/H×DN/H matings, and H/H littermates were used as controls for all experiments. To genotype mice, genomic DNA was isolated from mouse tails, and the target *SCN5A* polymerase chain reaction amplicon (c.3688 to c.4082) was incubated with TaqI (New England Biolabs, Ipswich, MA) and then electrophoresed in agarose gels. TaqI digests the fragment containing p.D1275 but does not digest that with p.N1275.

Surface Electrocardiogram

Electrocardiograms were recorded during administration of isoflurane vapor titrated to maintain light anesthesia.[26] Baseline ECG (leads I and II) was recorded for 15 minutes. Heart rate was measured as the average during a 30-second interval at baseline when a steady state was reached during anesthesia. For measurement of all other ECG parameters, 30 seconds of data in each lead were signal averaged with a custom-built LabVIEW program (National Instruments, Austin, TX), and the resultant waveform was analyzed with an electric caliper by an electrophysiologist blind to the genotype.[27] The larger value from each lead was used. QRS duration was measured from the first deflection of the Q wave (or R wave when the Q wave was absent) and the end of the S wave defined as the point of minimum voltage in the terminal phase of the QRS complex. The QT interval was measured from the beginning of the QRS complex to the end of the T wave defined as the point where the T wave merges with the isoelectric line. Heart rate–corrected QT interval (QTc) was calculated from a formula developed for mice: $QTc=QT/(RR/100)^{1/2}$.[28]

Echocardiogram

Transthoracic echocardiograms were performed on resting conscious mice and analyzed by a sonographer blinded to the genotype. Signals were acquired with a 15-MHz transducer (Sonos 5500 system, Agilent, Santa Clara, CA) at the Murine Cardiovascular Core at Vanderbilt University as previously described.[29]

Histology

Hearts were fixed overnight in 10% formalin, paraffin embedded, sectioned at 5 μm, and stained with Masson trichrome.

mRNA Quantification

Real-time polymerase chain reaction was conducted with a 7900HT Real-Time Instrument (Applied Biosystems). mRNA was isolated from the left ventricles, and cDNA was synthesized from 2 μg of the RNA by use of the Transcriptor First Strand cDNA Synthesis Kit with random hexamer primers (Roche Applied Science, Indianapolis, IL) and used as a template. To generate a standard curve for absolute quantification, genes of interest were subcloned into the pGEM-T vector (Clontech, Mountain View, CA). cDNA and 5 different dilutions of the vector with target DNA were prepared with predesigned 6-carboxyfluorescein–labeled fluorogenic TaqMan probe and primers (Applied Biosystems) for *SCN5A* (Hs00165693 m1) or β-actin (Mm00607939 S1) in triplicate in the same 94-well plate for real-time polymerase chain reaction amplification. Data were collected with instrument spectral compensation and analyzed by use of absolute quantification and a standard curve with SDS 2.2 software (Applied Biosystems). Each value was normalized to that for β-actin.

Western Blotting

Protein was extracted from flash-frozen hearts that were pulverized into powder and homogenized in a Dounce apparatus with 1× radioimmunoprecipitation assay buffer. Lysates were centrifuged at 10 000*g* for 5 minutes, and protein content was analyzed with a bicinchoninic acid assay (Pierce Biochemicals, Rockford, IL). Protein (40 to 100 μg) from each cardiac sample was separated by running the sample on a NuPage 8% Tris-acetate gel (Invitrogen, Carlsbad, CA). The protein was transferred to 0.2 μm nitrocellulose membranes (Amersham Biosciences, Sweden), which were blocked overnight in 0.05% Tween-20 Tris-buffed saline (TTBS) plus 5% nonfat dry milk at 4°C and then incubated with antibodies targeting anti–Nav1.5 (polyclonal antibody, 1:200; Alomone Labs, Israel) or anti-calnexin (polyclonal antibody, 1:1000, Stressgen Bioreagents, Belgium) at room temperature for 2 hours. Membranes were washed 3 times with TTBS for 10 minutes each and incubated with secondary anti-mouse and anti-rabbit horseradish peroxidase-linked antibodies (Amersham Biosciences) in TTBS at room temperature for 1 hour. The blots were then washed 4 times for 10 minutes each in TTBS. We visualized antibody interactions with the ECL system (Amersham Biosciences).

Immunostaining/Confocal Microscopy

Unfixed hearts were frozen in Tissue Tek and sectioned at 6 μm. Sections were washed in 1× Dulbecco phosphate-buffered saline and then incubated in 1× Dulbecco phosphate-buffered saline containing 0.3% fish gelatin and 0.1% Triton (block) for 1 hour at 4°C. Sections were immunostained with antibodies targeting anti–Nav1.5 (polyclonal antibody 1:50, Alomone Labs) diluted in block solution overnight. Samples were then washed 3 times and incubated with Alexa 488–conjugated goat anti-mouse IgG (1:400, Invitrogen) secondary antibody for 1 hour at room temperature. Then sections

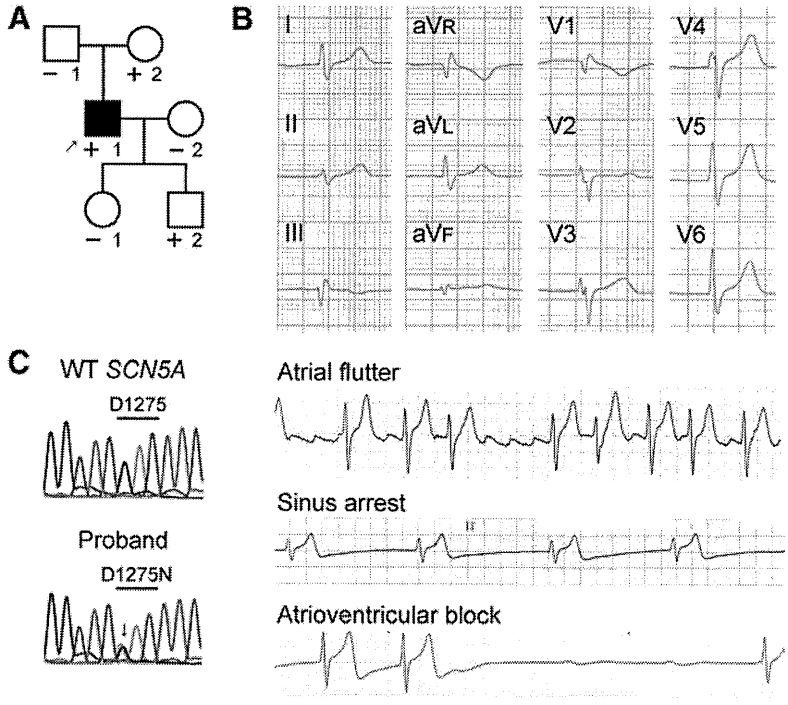

Figure 1. D1275N *SCN5A* mutation in a patient with sinus node dysfunction, atrial flutter, and conduction disease. **A,** Pedigree. The proband is indicated by the arrow. Individuals carrying the mutation are indicated (+). Individuals who tested negative for the mutation are indicated (−). Filled symbols indicate phenotype positive. **B,** Electrocardiogram and rhythm strips in the proband. **C,** Heterozygous single-nucleotide change in *SCN5A* (c.3823G→A) resulting in p.D1275N.

were washed and coverslips were applied with Vectashield (Vector Labs, Burlingame, CA). Images were collected with a Zeiss LSM510 Meta confocal imaging system with 20×1.3 NA lens (pinhole equals 1 airy disk) with 2× zoom and analyzed with LSM 4.0 software.

Sodium Current Recordings

Sodium current was recorded with the whole-cell voltage-clamp technique in single ventricular myocytes isolated by a modified collagenase/protease method or in Chinese hamster ovary (CHO) cells transiently expressing wild-type or D1275N *SCN5A*.[21,30,31] The *SCN5A* DNA (NM 198056) was subcloned into the pBK-CMV vector (Stratagene, La Jolla, CA), and the mutation was prepared with the QuickChange II XL site-directed mutagenesis kit (Stratagene), followed by verification by resequencing. *SCN5A* DNA (1 μg) was transfected with the plasmid encoding the enhanced green fluorescent protein (pEGFP-IRES, Clontech) by use of Fugene6 (Roche Applied Science, Indianapolis, IN) in CHO cells. Cells were grown for 48 hours after transfection before study. Similar methods were used to study the biophysical properties of wild-type and D1275N sodium channels transfected with the sodium channel β1 subunit in human embryonic kidney cells (tsA201). Late sodium current was measured at the end of 200-ms test pulses to −20 mV from a holding potential of −120 mV (interpulse duration, 5 seconds).

The extracellular bath solution contained (in mmol/L) 145 NaCl, 4.0 KCl, 1.0 $MgCl_2$, 1.8 $CaCl_2$, 10 glucose, and 10 HEPES, pH 7.4 (NaOH), for sodium current recording in CHO and tsA201 cells. Patch pipettes (≈1.5 mol/LΩ) contained (in mmol/L) 10 NaF, 110 CsF, 20 CsCl, 10 EGTA, and 10 HEPES, pH 7.4 (CsOH). To allow recording of sodium current in cardiomyocytes, the external Na^+ concentration was lowered to 5 mmol/L, electrodes with tip resistance <1 mol/LΩ were used, and experiments were conducted at 18°C. Data acquisition was carried out with an Axopatch 200B patch-clamp amplifier and pCLAMP software (version 9.2, Molecular Devices, Sunnyvale, CA). Currents were filtered at 5 kHz and digitized with an analog-to-digital interface (Digidata 1322A, Molecular Devices). To minimize capacitive transients, capacitance and series resistance were adjusted to 70% to 85%. Details of the pulse protocols are presented schematically in the figures.

Action Potential Recordings

Action potentials from isolated mouse ventricular myocytes were elicited with injection of brief stimulus current (1 to 2 nA, 2 to 6 ms) at 5 Hz in current clamp mode (Axopatch 200A amplifier, Molecular Devices). The extracellular bath solution contained (in mmol/L) NaCl 140, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1, HEPES 5, and glucose 10, pH 7.4 (adjusted by NaOH). Patch pipettes contained (in mmol/L) KCl 110, K_2-ATP 5, $MgCl_2$ 1, BAPTA 0.1, and HEPES 10, pH 7.2 (adjusted by KOH). Microelectrodes of 3 to 5 mol/LΩ were used. Data acquisition was carried out with an Axopatch 200B patch-clamp amplifier and pCLAMP. The action potential durations at 50% and 90% repolarization and the action potential amplitude were measured.

Data Analysis

Results are presented as mean±SEM. The unpaired *t* test was used for comparisons of electrophysiological characteristics between D1275N and wild-type channels expressed in heterologous expression systems. We used ANOVA followed by a post hoc analysis with Bonferroni correction for all of comparisons among the genotypes of mice, except for the linear mixed-effects models with Bonferroni correction for comparisons of in vitro electrophysiological characteristics of mice. All statistical analyses were performed with SPSS, version 12.0 (SPSS Inc, Chicago, IL). A 2-tailed value of $P<0.05$ was considered statistically significant.

Results

Clinical Case Presentation

A 19-year-old white man (II-1) presented with recurrent exertional syncope (Figure 1A, arrow). Physical examination and echocardiography were normal, and his ECG demonstrated unusually slow atrial flutter that was conducted 1:1 to the ventricles with hypotension during exertion (Figure 1B). After catheter ablation of the cavotricuspid isthmus for atrial flutter, he had atrial standstill, prolonged QRS duration, sinus node dysfunction, high-degree atrioventricular block, and normal QT interval. A cardioverter-defibrillator was implanted. He has been

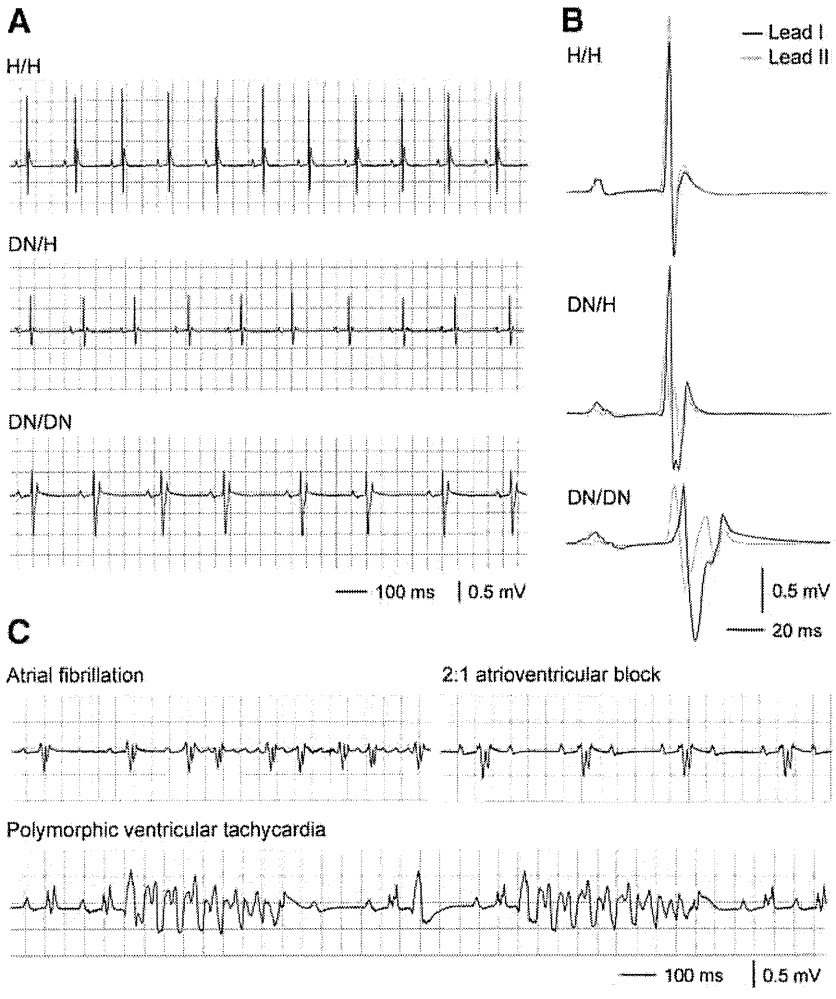

Figure 2. Electrocardiography in mice. **A,** Representative ECG traces in lead I at 3 weeks. **B,** Representative signal-averaged ECG traces in leads I (black) and II (gray) at 3 weeks. See Table 1 for detailed results. **C,** Arrhythmias recorded in DN/DN mice at 12 weeks.

asymptomatic for 10 years, and his echocardiography has been normal. We identified a missense mutation in *SCN5A*, c.3823G→A in exon 21 (Figure 1C), resulting in p.D1275N within a transmembrane domain of the protein (segment 3, domain III); the variant connexin 40 associated with atrial standstill in the reported Dutch kindred was absent.[16] Both his mother (I-2) and 1 son (III-2) share the mutation but have no clinical findings.

DN Mice Are Viable and Display Gene-Dose-Dependent Conduction Slowing and Arrhythmias

The distribution of pups from DN/H×DN/H matings was in Hardy-Weinberg equilibrium (52 H/H, 107 DN/H, 54 DN/DN). During a follow-up of 12 weeks, 1 DN/DN mouse died suddenly, but no DN/H or H/H mice died. ECG recordings revealed that the DN allele caused abnormal phenotypes in a gene-dose–dependent fashion at 3 weeks (Figure 2A and 2B, and Table 1). The DN allele was associated with slow heart rate and slow cardiac conduction (prolongation of the P-wave duration, PR interval, and QRS duration) at 3 weeks, and similar changes were observed at 12 weeks. In mice with ECGs recorded at both 3 and 12 weeks, the prolongation of the P-wave duration, PR interval, and QRS duration associated with the DN allele was progressive with age. In addition, spontaneous monomorphic and polymorphic ventricular tachycardia was observed in 7 of 9 DN/DN mice during 15-minute recording periods under light anesthesia at 12 weeks, but no arrhythmia was observed in 18 DN/H or 10 H/H littermates studied under the same conditions (Figure 2C). Sinus node dysfunction (n=3), atrioventricular block (second degree or higher; n=4), and atrial fibrillation/tachycardia (n=5) also occurred only in DN/DN mice, not in DN/H or H/H littermates.

Reduced Contractile Function in DN Mice

There was consistent and statistically significant end-diastolic and end-systolic left ventricular dilatation and calculated left ventricular fractional shortening reduction in a gene–dose-dependent fashion (Figure 3A and Table 2). Histological examination of mouse hearts revealed that the DN allele was associated with ventricular dilatation but was not associated with significant fibrosis or myocyte disarray (Figure 3B). One possibility is that the FLAG tag incorporated into the DN allele contributes to the phenotypes in the DN animals. However, we found no difference in ECG and echocardiographic phenotypes between H/H and FG/FG animals, indicating that the FLAG tag does not

Table 1. ECG Phenotype

	H/H	DN/H	DN/DN
At 3 weeks			
n	11	20	9
Heart rate, bpm	388±8	354±8*	335±15*
P-wave duration, ms	13.0±0.6	17.0±0.3*	19.4±0.5*†
PR interval, ms	33.7±0.7	35.6±0.7	44.1±0.9*†
QRS duration, ms	9.8±0.2	11.8±0.3*	22.3±2.2*†
QT interval, ms	48.5±1.9	50.2±1.1	67.6±4.2*†
QTc interval, ms	38.9±1.6	38.5±0.8	50.2±2.5*†
At 12 weeks			
n	10	18	9
Heart rate, bpm	387±8	368±11	317±18*
P-wave duration, ms	14±0.5	18.6±0.3*	27.1±1.2*†
PR interval, ms	37.8±0.8	39.7±0.7	57.1±3.1*†
QRS duration, ms	10.7±0.4	12.9±0.3	33.4±2.7*†
QT interval, ms	51.2±0.6	54.8±0.8	77.9±3.7*†
QTc interval, ms	41.1±0.6	42.8±0.9	55.9±1.8*†
Ratio of week 12 to week 3, %‡			
n	9	14	6
Heart rate, bpm	101±2	103±4	119±18
P-wave duration, ms	104±4	112±5	143±11*†
PR interval, ms	113±3	115±2	125±5*
QRS duration, ms	111±4	116±4	154±14*†
QT interval, ms	106±5	114±3	117±11
QTc interval, ms	106±5	115±3	123±6

QTc=QT/(RR/100)$^{1/2}$ (mouse-specific).
*P<0.05 vs H/H; †P<0.05 vs DN/H.
‡For animals with measurements at both time points.

contribute to the ventricular dysfunction or other phenotypes observed in DN animals.

Sodium Current Is Reduced in DN Myocytes

The manifest conduction slowing in DN mice is consistent with loss of sodium channel function. However, sodium current amplitudes and gating observed with heterologous expression of wild-type and D1275N channels in CHO cells were nearly indistinguishable (Figure 4A through 4C and Table 3). In CHO cells, there was also no difference in the voltage dependence of activation and inactivation or in the time course of inactivation. Similarly, only minor differences were observed between wild-type and D1275N channels coexpressed with β1 subunits in tsA201 cells; current amplitudes were nearly identical, but there were a slight shift in the voltage dependence of activation and an increase in late sodium current (percent to peak current: wild-type, 0.22±0.05%, n=7; D1275N, 1.34±0.11%, n=8; P<0.001; Figure 4D through 4F and Table 3).

In contrast, in ventricular cardiomyocytes, peak sodium current amplitude was markedly reduced in DN/H and DN/DN mice compared with H/H littermates (Figure 5A and 5B and Table 3). In addition, late sodium current was increased in DN/DN mice compared with DN/H and H/H littermates (Figure 5C). We also found that sodium current in

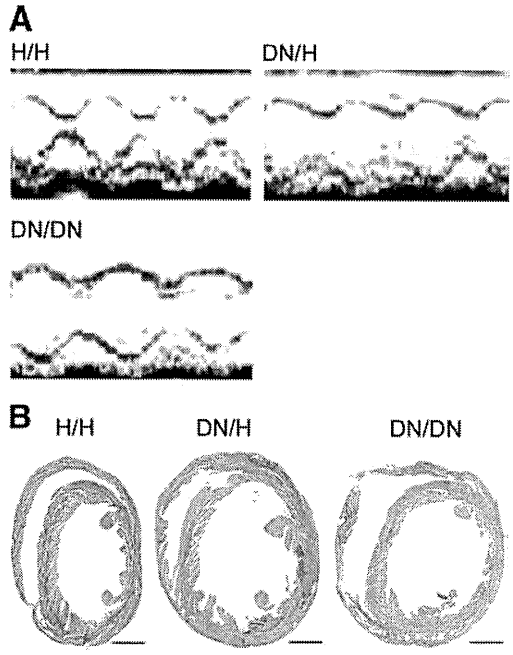

Figure 3. Dilated cardiomyopathy phenotype. **A,** Representative echocardiograms showing prominent increased end-systolic dimensions in DN/H and DN/DN mice at 12 weeks. See Table 2 for summary results. **B,** Masson trichrome staining in mice hearts. Scale bars indicate 1 mm.

DN/DN myocytes displayed consistent changes in gating. The voltage dependence of inactivation was positively shifted in DN/DN mice compared with DN/H and H/H mice (Figure 5D). The time course of inactivation was slower in DN/DN mice compared with DN/H and H/H littermates (time constant at −30 mV: DN/DN, 5.5±0.2 milliseconds; DN/H, 2.8±0.1 milliseconds; H/H, 2.7±0.2 milliseconds; Figure 5E and 5F). There was no difference in the voltage dependence of activation. The DN allele was associated with decreased action potential amplitude, consistent with the decrease in peak sodium current, and with prolonged action potential duration, consistent with the increase in late current (Figure 6).

Sodium Channel Protein Abundance Is Reduced in DN Myocytes

Western blotting showed a reduction in sodium channel protein abundance associated with the DN allele, and the changes were much more dramatic in DN/DN compared with DN/H hearts (Figure 7A and 7B). The abundance of

Table 2. Echocardiographic Phenotype at 12 Weeks

	H/H (n=9)	DN/H (n=19)	DN/DN (n=12)
Septal wall, mm	0.75±0.02	0.72±0.02	0.69±0.03
Posterior wall, mm	0.51±0.03	0.47±0.01	0.51±0.04
Left ventricle, mm			
End diastole	3.01±0.08	3.09±0.08	3.33±0.07*
End systole	1.49±0.09	1.76±0.05*	2.01±0.05*†
Fractional shortening, %	52.0±1.6	43.1±0.7*	39.9±0.7*†

*P<0.05 vs H/H; †P<0.05 vs DN/H.

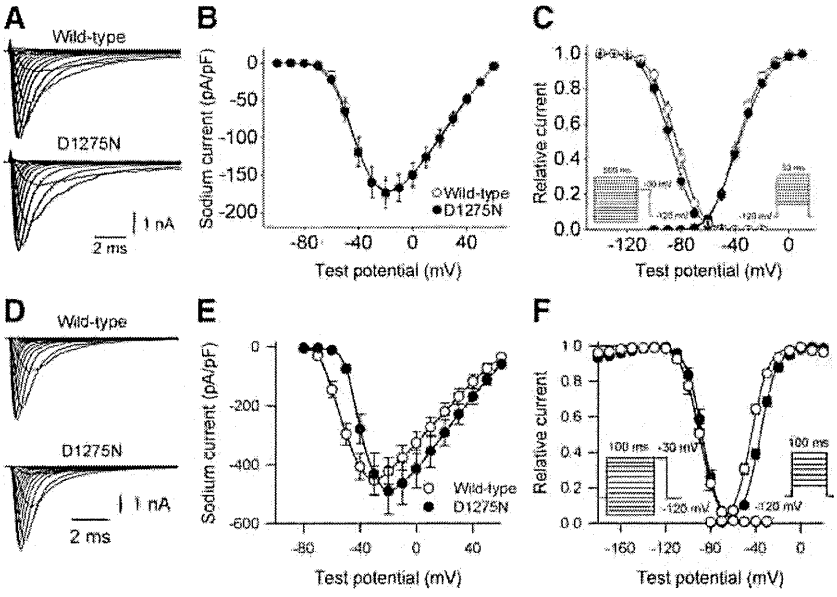

Figure 4. Wild-type and D1275N sodium current in Chinese hamster ovary cells (**A** through **C**) and tsA201 cells (**D** through **F**). Wild-type or D1275N channels were coexpressed with β1 subunits in tsA201 cells. **A** and **D,** Representative current traces. See Table 3 for summary results. **B** and **E,** Current voltage relationships. **C** and **F,** Voltage dependence of activation and inactivation. The pulse protocols are shown in the inset.

the control calnexin protein was similar among H/H (reference, 100±6%), DN/H (103±4% of H/H), and DN/DN mice (100±5% of H/H) (P=NS for each). Although sodium current and sodium channel protein were reduced in DN/DN and DN/H mice compared with H/H littermates, real-time polymerase chain reaction showed that *SCN5A* transcript levels were elevated in mice with the DN allele (Figure 7C). Expression levels of β-actin transcripts were similar among H/H (reference, 100±1%), DN/H (100±1% of H/H), and DN/DN mice (102±5% of H/H) (P=NS for each). Immunostaining experiments were conducted in heart sections at 3 weeks (Figure 8). The DN allele was associated with reduced levels of cell surface expression. Notably, staining was obvious on the lateral myocyte aspects in H/H hearts but was nearly absent in DN/DN hearts stained under identical conditions.

Discussion

The D1275N mutation has been associated with sinus node dysfunction, conduction abnormalities, tachyarrhythmias, and contractile dysfunction.[8,9,16–18] However, in previous studies, the evidence implicating D1275N as the causative mutation has been weak: for example, in the large Dutch kindred, the contribution of an additional connexin variant was invoked to explain why only a minority of subjects displayed a clinical phenotype, but that variant was absent in the proband reported here. In addition, D1275N does not generate major changes in sodium channel function in heterologous expression studies.[8,9,16–18] Thus, despite the previous and the present clinical case reports, the formal possibility remained that D1275N does not actually contribute to the abnormal phenotypes. To address the role of this (and other) variants in mediating sodium channel-

Table 3. Sodium Channel Gating in Heterologous Expression Systems and Ventricular Cardiomyocytes

	Peak Current Density at −30 mV		Voltage Dependence of Activation		Voltage Dependence of Inactivation	
	pA/pF	n	$V_{1/2}$, mV	n	$V_{1/2}$, mV	n
CHO cells						
Wild type	−160±20	25	−35.4±0.6	25	−84.5±1.0	24
D1275N	−159±21	28	−34.7±0.6	28	−88.4±0.8	27
tsA201 cells						
Wild type	−454±48	16	−47.7±1.1	16	−89.4±0.7	19
D1275N	−432±71	13	−35.7±1.1†	13	−88.0±1.6	18
Cardiomyocytes*						
H/H	−40.9±2.9	10	−44.1±1.0	10	−84.1±1.0	10
DN/H	−19.2±3.1‡	12	−44.3±1.4	12	−81.2±1.1	12
DN/DN	−9.3±1.1§‖	12	−45.6±0.9	12	−76.5±0.8§‖	12

CHO indicates Chinese hamster ovary; n, number of cells. Study conditions differ for heterologous expression systems and cardiomyocytes as described in Methods.

*Cardiomyocytes from 3 mice for each genotype.

†P<0.001 versus wild type; ‡P<0.05 versus H/H; §P<0.01 versus H/H; ‖P<0.01 vs DN/H.

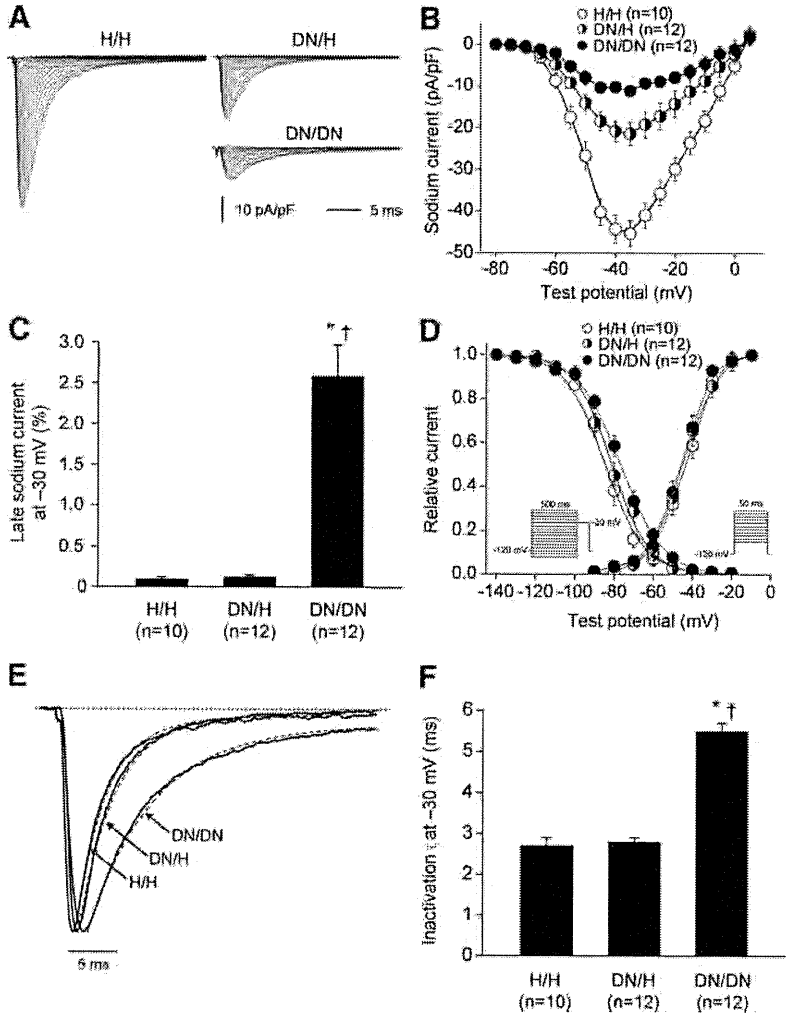

Figure 5. Sodium current in male ventricular cardiomyocytes at 3 weeks showing altered sodium channel function by the DN allele. **A,** Representative current traces in H/H, DN/H, and DN/DN cells. See Table 3 for summary results. **B,** Current voltage relationships. **C,** Late sodium current at −30 mV. Late current amplitude was normalized to peak current amplitude. **D,** Voltage dependence of activation and inactivation. **E** and **F,** Inactivation time constant (τ) at −30 mV. *$P<0.001$ vs H/H; †$P<0.001$ vs DN/H. n Indicates the number of cardiomyocytes from 3 mice.

linked clinical phenotypes, we generated a series of mouse lines in which the murine cardiac sodium channel was ablated and human alleles were substituted in the murine *Scn5a* locus. The technique of recombinase-mediated cassette exchange allowed us to place wild-type or mutant human sodium channel cDNAs in the murine cardiac sodium channel locus.[21] We have previously reported that this approach eliminates expression of the murine channel and that sodium currents from unmodified wild-type murine ventricular myocytes and those expressing wild-type human *SCN5A* are indistinguishable, indicating that expression of the exchanged sequence is determined by endogenous sodium channel regulatory mechanisms.[21]

DN Mice Display Sodium Channel Dysfunction

Sodium current amplitude was similar between D1275N and wild-type channels when expressed in heterologous expression systems in the present study in either the absence or presence of β1 subunit.[16] This is in agreement with most results previously reported, although 1 group has found that D1275N channels generate significantly less current than wild-type channels in tsA201 cells; the reason for this discrepancy is unknown.[20] In our mouse model, D1275N was associated with decreased levels of sodium channel protein by Western analysis of total ventricular protein, decreased expression of sodium channels at the ventricular myocyte surface, and marked reduction of sodium current. In addition, we observed increased late current and altered voltage-dependence of channel inactivation. Thus, channel dysfunction conferred by D1275N becomes evident in the myocyte environment. The major change, reduction in peak sodium current, could represent decreased cell surface expression and/or altered gating of the channel protein. One possible explanation in either case is altered interactions with sodium channel partners, present in myocytes and absent in CHO and tsA201 cells.[32,33] There is precedent for such a hypothesis; the E1053K *SCN5A* mutation, which is associated with a loss of sodium channel function phenotype, has no effect on current density when studied in heterologous expression systems but abolishes binding of the channel to ankyrin-G and reduces cell surface expression and sodium current in cultured cardiomyocytes.[34] However, although E1053K affects channel gating under heterologous expression,[34] altered channel gating by D1275N was found only in the mice, not in heterologous systems, in our study. This is clearly not a general rule because channel dysfunction observed with heterologous expression of other mutants

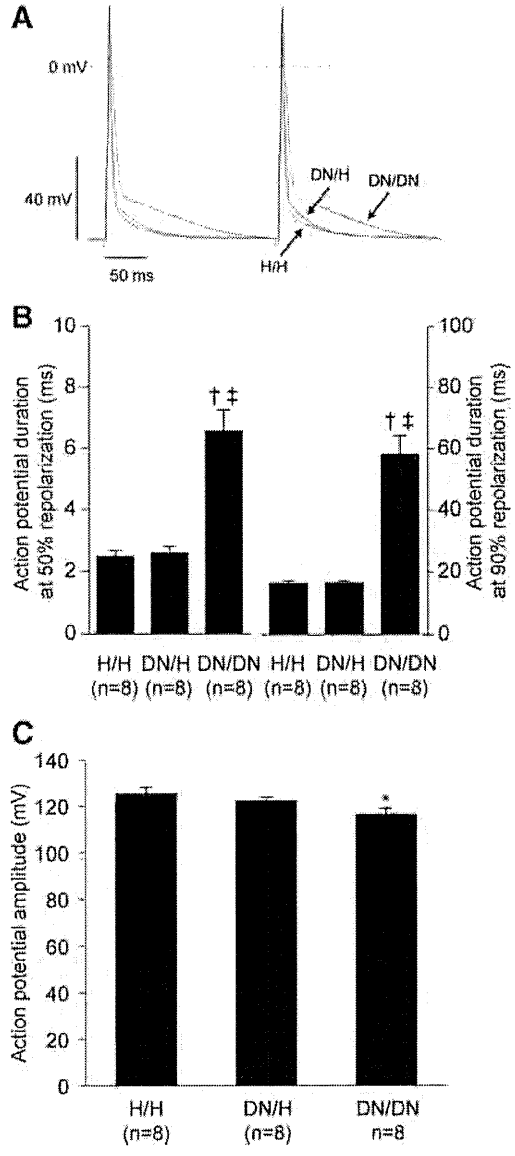

Figure 6. Action potential in male ventricular cardiomyocytes at 3 weeks. **A,** Representative action potential traces. **B,** Action potential duration at 50% and 90% repolarization. **C,** Action potential amplitude. *$P<0.05$ vs H/H; †$P<0.01$ vs H/H; ‡$P<0.01$ vs DN/H. n Indicates the number of cardiomyocytes from 3 mice.

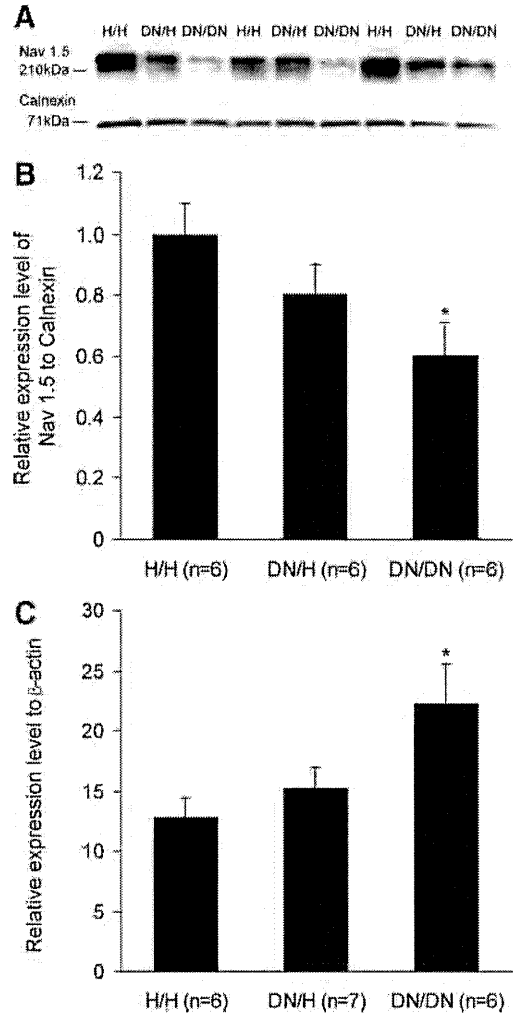

Figure 7. Sodium channel expression levels at 3 weeks. **A,** Representative Western blots in ventricles. **B,** Sodium channel expression levels normalized to those of H/H. Calnexin was used as the loading control. **C,** Relative expression levels of *SCN5A* transcript normalized to those of β-actin in ventricle. *$P<0.05$ vs H/H.

(delKPQ1505–1507 and 1795insD) recapitulates phenotypes observed clinically and in genetically modified mice.[7,35–37]

Association of Sodium Channel Mutations With Cardiomyopathy

In addition to arrhythmias, *SCN5A* mutations have been associated with cardiomyopathy.[8–15] To date, 12 rare variants in *SCN5A* have been identified in cardiomyopathy, and all of the variants have been associated with arrhythmia phenotypes that result from loss of sodium channel function.[8–15] In our mouse model, the loss of sodium channel function by D1275N is consistent with biophysical properties of other *SCN5A* mutations associated with DCM,[10,11,38] and findings in clinical and experimental studies suggest that a marked reduction in sodium current is critical for the development of cardiomyopathy.[13,14,39,40] In prior studies, mice with 90% reduction of *Scn5a* expression level developed cardiac dysfunction,[39] whereas heterozygous *Scn5a* knockdown mice ($Scn5a^{+/-}$) display normal cardiac function.[40] In our study, mice expressing D1275N, one of the initially reported *SCN5A* mutations in a cardiomyopathy kindred,[8,9] showed a reduction in sodium current with disrupted channel gating and developed evident cardiomyopathy at 12 weeks. This is consistent with other reports describing that both R814Q occurring homozygously and the compound heterozygous occurrence of the W156X and R225W are associated with cardiomyopathy.[13,14] In these settings, the cardiomyopathy phenotype is generally absent in heterozygotes.[13,14]

Among 12 rare variants in *SCN5A* associated with cardiomyopathy, 7 are located in transmembrane domains, and 6 of them, including D1275N, are predicted to change

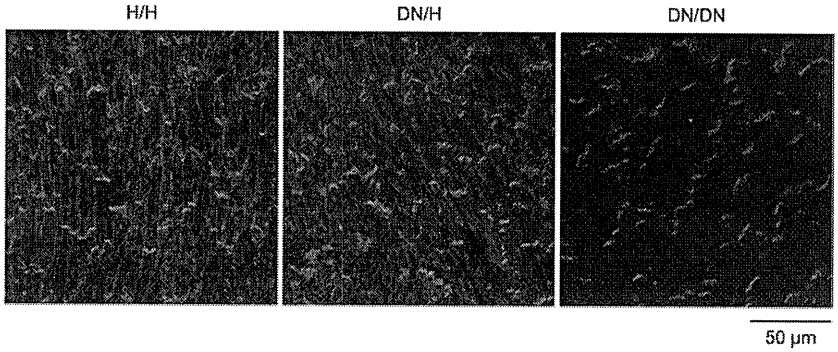

Figure 8. Immunostaining for sodium channel (Nav1.5) at 3 weeks. Heart sections from the ventricles were stained with anti-Nav1.5 (green). Note the obvious lateral staining in the H/H heart and its absence in the DN/DN heart.

the electric charge of substituted amino acids.[8-15] These substitutions may lead to changes in channel structure, resulting in altered channel gating and/or reduced channel expression levels directly or by disrupted interaction with sodium channel accessory proteins.

Although this study and previous work strongly imply that loss of sodium channel function has a critical role for development of cardiomyopathy,[10,41] the mechanisms remain controversial. The surface ECG tracings in DN mice (Figure 2) not only demonstrate gene-dose–dependent conduction slowing but also suggest altered activation sequence (with ECG complex splintering); thus, electromechanical dyssynchrony, a well-recognized cause of cardiac contractile dysfunction,[42] may be sufficient to explain the DCM phenotype. Another possibility raised by a recent report that suggests 2 pools of sodium channel protein in heart is that the mutant channel does not target the appropriate subcellular domain to support normal cell propagation.[43] Among causative genes for DCM, cytoskeletal components such as syntrophins and dystrophins have been associated with SCN5A channel, and disrupted interaction with such proteins may result in cardiomyopathy.[44-46] Although *SCN5A* mutations have been associated with cardiac fibrosis,[3,40,47] we did not observe fibrosis when the mice carrying the DN allele developed cardiac dysfunction. It has been reported that *SCN5A*-related DCM phenotype usually develops later (>10 years) than the onset of arrhythmia phenotypes, suggesting a possibility that the DCM phenotype is secondarily mediated by arrhythmia.[8-10,48] In our study, however, the cardiomyopathy phenotype was evident relatively early, in the absence of sustained arrhythmia. Taking these results together, we propose that sodium channel dysfunction and electromechanical dyssynchrony represent the primary pathophysiology for DCM in this setting.

Conclusions

We found that the D1275N *SCN5A* mutation was associated with cardiomyopathy and multiple arrhythmias in vivo, in line with clinical findings in our and other studies.[8,9,16-18] Although D1275N did not generate serious channel dysfunction when studied in heterologous expression systems, the mutation produced extensive channel dysfunction, notably marked reduction in peak current amplitude, and a cardiomyopathy phenotype in our mouse model. Further experiments along the lines outlined above are required to elucidate the precise mechanisms for channel dysfunction and how this leads to the DCM phenotype. Defining the mechanisms underlying the disconnect between the results in heterologous expression systems and those in myocytes will contribute to furthering our understanding of the variable phenotypes and penetrance of D1275N and other *SCN5A* mutations.

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Disclosures

None.

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CLINICAL PERSPECTIVE

A conventional approach to characterize the function of ion channel mutations is to compare wild-type and variant channel function by heterologous expression in mammalian, noncardiac cells like Chinese hamster ovary or human embryonic kidney cells. The cardiac sodium channel mutation D1275N has been reported in multiple individuals and families with a range of phenotypes, including arrhythmias and dilated cardiomyopathy; however, conventional heterologous expression studies have not identified major differences between wild-type and D1275N function. Thus, it has even been uncertain whether this mutation causes the clinical phenotypes with which it has been associated. In this study, we addressed this issue by studying mice in which the cardiac sodium channel locus had been disrupted and replaced with full-length human wild-type or D1275N mutant sodium channels. We observed slowed and disordered cardiac conduction and decreased contractile function in mice bearing the mutation; mice with 2 D1275N alleles displayed worse phenotypes than those with 1 variant allele. In vitro electrophysiological studies identified reduced peak cardiac sodium current as a key defect, and this is consistent with the observed reduced conduction velocity. The major clinical implication of these findings is that heterologous expression may be insufficient to assess mutant channel function. In addition, the data lend support to the concept that sodium channel mutations are associated not only with arrhythmias but also with dilated cardiomyopathy phenotypes. The mutant mice will be an invaluable tool to dissect mechanisms underlying these findings.

Electrocardiographic Characteristics and *SCN5A* Mutations in Idiopathic Ventricular Fibrillation Associated With Early Repolarization

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Background—Recently, we and others reported that early repolarization (J wave) is associated with idiopathic ventricular fibrillation. However, its clinical and genetic characteristics are unclear.

Methods and Results—This study included 50 patients (44 men; age, 45±17 years) with idiopathic ventricular fibrillation associated with early repolarization, and 250 age- and sex-matched healthy controls. All of the patients had experienced arrhythmia events, and 8 (16%) had a family history of sudden death. Ventricular fibrillation was inducible by programmed electric stimulation in 15 of 29 patients (52%). The heart rate was slower and the PR interval and QRS duration were longer in patients with idiopathic ventricular fibrillation than in controls. We identified nonsynonymous variants in *SCN5A* (resulting in A226D, L846R, and R367H) in 3 unrelated patients. These variants occur at residues that are highly conserved across mammals. His-ventricular interval was prolonged in all of the patients carrying an *SCN5A* mutation. Sodium channel blocker challenge resulted in an augmentation of early repolarization or development of ventricular fibrillation in all of 3 patients, but none was diagnosed with Brugada syndrome. In heterologous expression studies, all of the mutant channels failed to generate any currents. Immunostaining revealed a trafficking defect in A226D channels and normal trafficking in R367H and L846R channels.

Conclusions—We found reductions in heart rate and cardiac conduction and loss-of-function mutations in *SCN5A* in patients with idiopathic ventricular fibrillation associated with early repolarization. These findings support the hypothesis that decreased sodium current enhances ventricular fibrillation susceptibility. **(*Circ Arrhythm Electrophysiol.* 2011;4:874-881.)**

Key Words: arrhythmia ■ sodium channel ■ electrophysiology ■ genetics ■ mutations

Early repolarization or J-wave is characterized by an elevation at the junction between the end of the QRS complex and the beginning of the ST-segment (J-point) in a 12-lead ECG and generally has been considered benign for decades.[1] However, early repolarization can be observed under various negative biological conditions, such as low body temperature and ischemia,[2–4] and there is increasing evidence that early repolarization is associated with an increased risk of ventricular fibrillation and sudden cardiac death.[5–7]

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In previous studies, including our own, early repolarization in the inferior or lateral leads was associated with pathogenesis in idiopathic ventricular fibrillation.[5,6] Moreover, early repolarization in the right precordial leads also has been associated with idiopathic ventricular fibrillation.[8] Heritability of early repolarization has been shown in a recent population-based study,[9] and as in other arrhythmia syndromes such as long QT syndrome and Brugada syndrome,[10] ion channel genes are responsible for idiopathic ventricular fibrillation associated with early repolarization.[11–13] A mutation in *KCNJ8*, which encodes a pore-forming subunit of the ATP-sensitive potassium channel, has been identified in idiopathic ventricular fibrillation with early repolarization.[11,14] Mutations in L-type calcium channel genes, including *CACNA1C*, *CACNB2B*, and *CACNA2D1*, also have been associated with idiopathic ventricular fibrillation with early repolarization.[12]

In this study, we compared electrocardiographic parameters between patients with idiopathic ventricular fibrillation and healthy controls and found that heart rate and cardiac conduction were slow in patients with idiopathic ventricular fibrillation. Furthermore, we screened patients with idiopathic ventricular fibrillation for mutations in *SCN5A*, which encodes the predominant cardiac sodium channel α subunit and is critical for cardiac conduction. Here, we present the clinical and in vitro electrophysiological characteristics in idiopathic ventricular fibrillation associated with early repolarization.

Methods

Study Populations

This study included patients with idiopathic ventricular fibrillation and early repolarization who were referred to our institutions. Patients were diagnosed with idiopathic ventricular fibrillation if they had no structural heart disease as identified using echocardiography, coronary angiography, and left ventriculography. Baseline electrophysiological studies without antiarrhythmic drugs were performed based on the indication of each institution. Early repolarization was defined as an elevation of the J-point, either as QRS slurring or notching $\geq$0.1 mV $\geq$2 consecutive leads in the 12-lead ECG.[5] Patients were excluded if they had a short QT interval (corrected QT interval using Bazett formula <340 ms) or a long QT interval (corrected QT interval >440 ms) in the 12-lead ECG.[15,16] All patients received sodium channel blocker challenge, and patients with Brugada type ST-segment elevations at baseline or after sodium channel blocker challenge were excluded.[17] Twelve-lead electrocardiograms recorded in the absence of antiarrhythmic drugs were compared between patients with idiopathic ventricular fibrillation and control subjects who were matched to patients with idiopathic ventricular fibrillation based on gender and age (patient: control ratio, 1:5). Control subjects were selected from 86 068 consecutive electrocardiograms stored in the ECG database in Niigata University Medical and Dental Hospital from May 7, 2003 to July 2, 2009.[18] Control subjects who had a normal QT interval (corrected QT interval, 360 to 440 ms) and no cardiovascular disease or medication use were included. Control subjects with Brugada type ST-segment elevations or early repolarization were excluded.

Genetic Analysis

All probands and family members who participated in the study gave written informed consent before genetic and clinical investigations in accordance with the standards of the Declaration of Helsinki and local ethics committees. Genetic analysis was performed on genomic DNA extracted from peripheral white blood cells using standard methods. The coding regions of *KCNQ1*, *KCNH2*, *SCN5A*, *KCNE1*, *KCNE2*, and *KCNJ8* were amplified by PCR using exon-flanking intronic primers,[19–21] and direct DNA sequencing was performed using ABI 310, 3130, and 3730 genetic analyzers (Applied Biosystems, Foster City, CA).[22]

Generation of Expression Vectors and Transfection in Mammalian Cell Lines

Full-length human *SCN5A* cDNA was subcloned into the mammalian expression plasmid pcDNA3.1+ (Invitrogen, Carlsbad, CA).[22] Mutant constructs were prepared using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. The human cell line tsA201 was transiently transfected with wild-type or mutant *SCN5A* plasmid using Lipofectamine LTX (Invitrogen), in combination with a bicistronic plasmid (pCD8-IRES-hβ1) encoding CD8 and the human sodium channel β1 subunit (hβ1) to visually identify cells expressing heterologous hβ1 using Dynabeads M-450 CD8 (Invitrogen).[22] Electrophysiological measurements were performed 24 to 72 hours after transfection.

Electrophysiology

Sodium currents were recorded using the whole-cell patch clamp technique as previously described.[22] Electrode resistance ranged from 0.8 to 1.5 mol/LΩ. Data were acquired using an Axopatch 200B patch clamp amplifier and pCLAMP8 software (Axon Instruments). Sodium currents were filtered at 5 kHz (−3 dB, 4-pole Bessel filter) and were digitally sampled at 50 kHz using an analog-to-digital interface (Digidata 1322A; Molecular Devices, Sunnyvale, CA). Experiments were performed at room temperature (20 to 22°C). Voltage errors were minimized using series resistance compensation (generally 80%). Cancellation of the capacitance transients and leak subtraction were performed using an online P/4 protocol. The time from establishing the whole-cell configuration to the onset of recording was consistent (5 minutes) between cells to exclude possible time-dependent shifts of steady-state inactivation. The pulse protocol cycle time was 10 s. The data were analyzed using Clampfit 10 (Molecular Devices) and SigmaPlot 9 software (Aspire Software International, Ashburn, VA). The holding potential was −120 mV. The bath solution contained the following (in mmol/L): 145 NaCl, 4 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, and 10 glucose, pH 7.35 (adjusted with NaOH). The pipette solution (intracellular solution) contained the following (in mmol/L): 10 NaF, 110 CsF, 20 CsCl, 10 EGTA, and 10 HEPES, pH 7.35 (adjusted with CsOH).

Immunocytochemistry

For immunocytochemistry, the FLAG epitope was inserted between residues 153 and 154 of the extracellular linker S1-S2 in domain I. The FLAG insertion into the S1-S2 linker previously has been shown to have no effect on channel gating or cell surface expression.[22,23] Immunocytochemistry was performed in HEK293 cells transfected with wild-type or mutant *SCN5A* plasmid as described previously.[22,24] After 48 hours of transfection, the cells were washed with phosphate-buffered saline, fixed in 4% paraformaldehyde, and permeabilized with 0.15% Triton X-100 in phosphate-buffered saline with 3% bovine serum albumin. Then the cells were stained with anti-FLAG polyclonal antibody (F7425; Sigma-Aldrich, St Louis, MO; 1:100) for 1 hour at room temperature. Protein reacting with antibody was visualized with Alexa Fluor 568–labeled secondary antibody (A-11011, Invitrogen, 1:1000). Images were collected using a Zeiss LSM 510 laser confocal microscope and analyzed using LSM 4.0 software.

Data Analysis

Differences in parameters between patients with idiopathic ventricular fibrillation and control subjects were analyzed using conditional logistic regression models. To exclude the effects of multicollinearity among electrocardiographic parameters, each electrocar-

Table 1. Electrocardiographic Parameters

	IVF Patients N=50	Controls N=250	OR (95% CI)/ 10 Unit Increase	*P* Value
Male sex, N (%)	44 (88)	220 (88)	...	...
Age, y	45±17	45±16	...	...
Heart rate, beats/min	62±9	70±14	0.62 (0.47–0.81)	<0.001
PR interval, ms	175±34	147±20	1.32 (1.22–1.43)	<0.001
QRS interval, ms	96±14	89±8	1.63 (1.31–2.02)	<0.001
QTc, ms	388±25	397±22	0.85 (0.75–0.98)	0.02

IVF indicates idiopathic ventricular fibrillation; OR, odds ratio; QTc, corrected QT interval.

diographic parameter was separately tested in the logistic models. All statistical analyses were performed with SPSS, version 12.0 (SPSS Inc, Chicago, IL). A 2-sided $P<0.05$ was considered statistically significant. Values are expressed as mean±SD. The study protocol was approved by the ethics committee of each institution.

Results

We identified 50 patients with idiopathic ventricular fibrillation and early repolarization (44 men [88%]; mean age, 45±17 years). All of the patients had experienced arrhythmia events, and 8 (16%) had a family history of sudden death.

Electrocardiographic parameters were compared between 50 patients with idiopathic ventricular fibrillation and 250 healthy control subjects without cardiovascular disease and not taking medication who were matched with gender and age (Table 1). The heart rate was slower, and the PR interval and QRS duration were longer in patients with idiopathic ventricular fibrillation compared with control subjects. The corrected QT interval was shorter in patients with idiopathic ventricular fibrillation than control subjects. No patient with idiopathic ventricular fibrillation showed type I Brugada electrocardiograms in repeated recordings.[25] Sodium channel blockers were administered in all patients, and Brugada type electrocardiograms were not provoked in any of these patients.[25] Electrophysiological study was performed in 29 patients. His-ventricular interval was 48±9 ms, and 4 patients had prolonged His-ventricular time ≥55 ms.[26] Ventricular fibrillation was inducible by programmed electric stimulation in 15 patients (52%).

We screened for mutations in *SCN5A* in 26 unrelated patients with idiopathic ventricular fibrillation and identified 3 mutations (A226D, R367H, and L846R) in 3 patients (Figure 1, Table 2). R367H and L846R are predicted to be located in the pore region. These mutations were not found in the genomes of 200 healthy control individuals. Two of the patients exhibited prolongation of the PR interval, and sodium channel blocker challenge was negative for Brugada syndrome in all of them. Alignment of the amino acid sequences from multiple species demonstrated that the amino acids substituted by mutations are highly conserved, supporting the importance of these amino acids. A226D and L846R, but not R367H, are predicted to change the electric charge of substituted amino acids.

A missense mutation, A226D (Figure 1A), was identified in a 36-year-old man (patient 1) resuscitated from ventricular fibrillation. He had experienced multiple episodes of syncope. The physical examination and echocardiography were normal. His ECG showed prolongation of the PR interval and early repolarization in leads II, III, and aVF, and J-point/ST-segment elevation in lead V1 (Figure 2A). Administration of pilsicainide augmented early repolarization in the inferior leads and induced ventricular fibrillation, but did not produce a type I Brugada ECG in the right precordial leads (Figure 2B). Electrophysiological study revealed prolongation of His-ventricular interval (68 ms), and ventricular fibrillation was induced by programmed electric stimulation. The patient's family history was negative for syncope, sudden cardiac death, and epilepsy.

A missense mutation L846R (Figure 1B) was identified in a 27-year-old man (patient 2). He was admitted after multiple episodes of syncope, and polymorphic ventricular tachycardia was documented when he lost consciousness. The physical examination and echocardiography were normal. His ECG

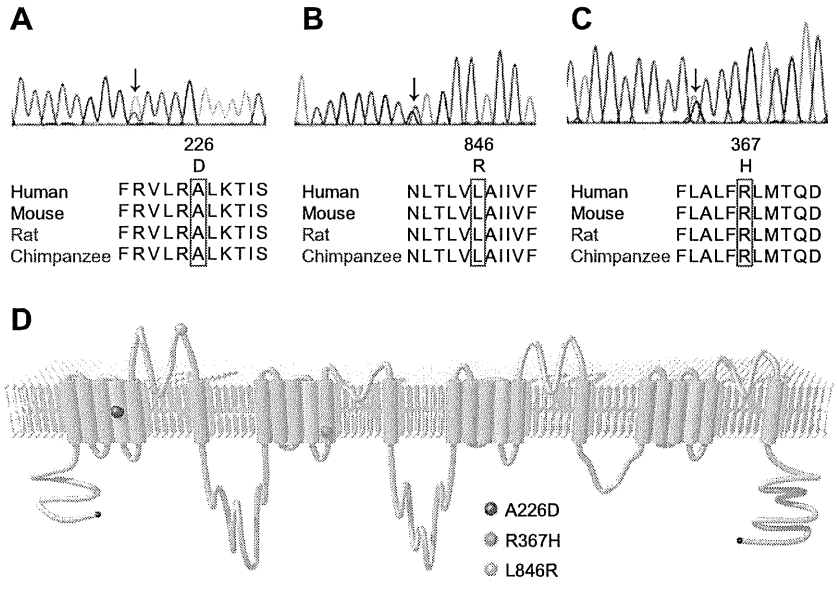

Figure 1. Mutations in *SCN5A* identified in patients with idiopathic ventricular fibrillation associated with early repolarization. **A**, The c.677C→A mutation in *SCN5A* resulting in p.A226D found in patient 1. **B**, The c.2537T→G mutation in *SCN5A*, resulting in p.L846R found in patient 2. **C**, The c.1100G→A mutation in *SCN5A*, resulting in p.R367H found in patient 3. We previously reported the R367H mutation (modified from Takehara et al[27]). **D**, Predictive topology of the SCN5A channel. Circles indicate the locations of the mutations.

Table 2. Characteristics of Idiopathic Ventricular Fibrillation Patients With *SCN5A* Mutations

Patient No.	Sex	Age at Onset (y)	Family History of SCD	Presenting Symptom	Location of J Wave	Other ECG Abnormalities	Response to Sodium Channel Blocker	Amino Acid Substitution
1	M	36	N	Aborted SCD	II, III, aVF, V1	PR prolongation	Augmentation of J-point amplitude and VF	A226D
2	M	27	Y	Aborted SCD	I, II, III, aVF	PR prolongation	Marked QRS prolongation and VF	L846R
3	F	37	N	Aborted SCD	II, III, aVF, V2	N	Augmentation of J-point amplitude and marked QRS prolongation	R367H

ECG indicates electrocardiogram; SCD, sudden cardiac death.

showed prolongation of the PR interval and early repolarization in lead III (Figure 2C). During the recovery phase of exercise testing, the amplitude of the J-point/ST-segment was augmented in leads I, II, III, and aVF, and ventricular fibrillation was induced. Pilsicainide caused marked prolongation of QRS duration and augmented the J-point/ST-segment amplitude in leads V1 and V2, followed by the development of ventricular fibrillation (Figure 2C and 2D). Pilsicainide did not produce a type I Brugada ECG. During electrophysiological study, His-ventricular interval was 55 ms. His uncle died suddenly.

We previously reported a missense mutation R367H in patient 3 as a case with Brugada syndrome (Figure 1C).[27] However, idiopathic ventricular fibrillation associated with early repolarization was diagnosed at a later time because a type 1 Brugada ECG has never been seen spontaneously or after the administration of sodium channel blocker in more than 1 right precordial lead, and thus the diagnostic criteria for Brugada syndrome were not fulfilled.[25] When the patient admitted to the hospital after recurrent episodes of syncope, early repolarization was present in the inferior and right precordial leads (Figure 2E). After sinus pause, early repolarization was augmented in leads II, III, and aVF, followed by the development of ventricular fibrillation after a few hours of the admission (Figure 2F). Procainamide further exaggerated early repolarization but did not produce a type I

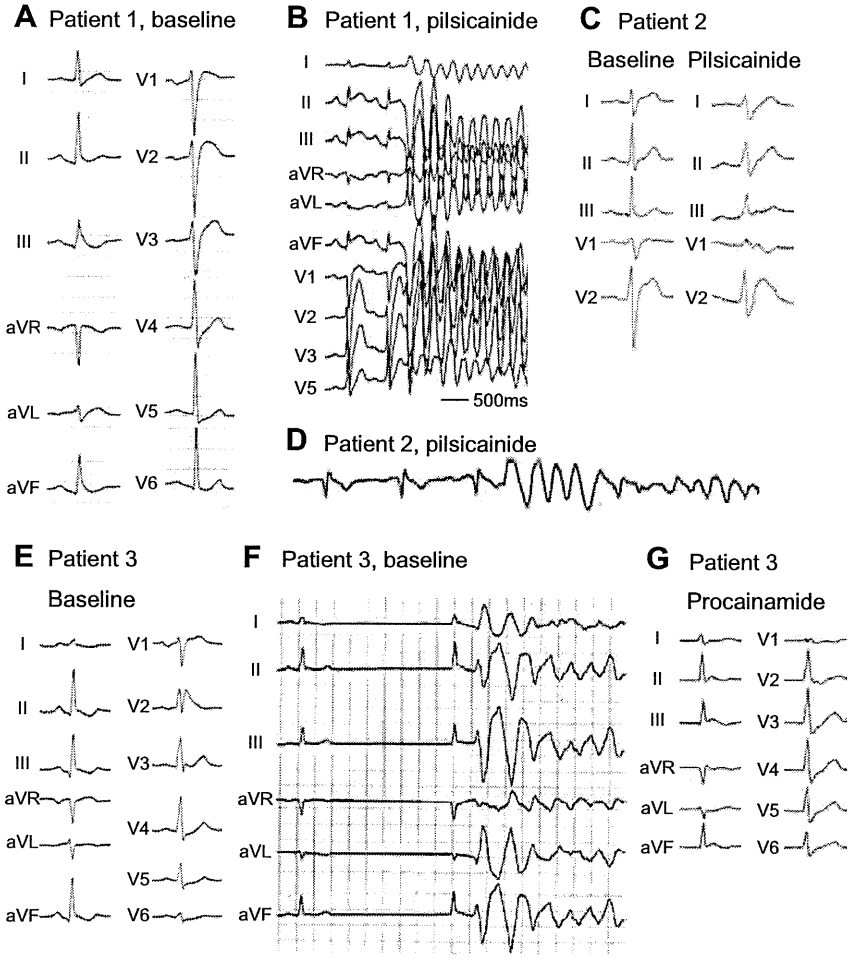

Figure 2. Electrocardiograms of patients with idiopathic ventricular fibrillation and a mutation in *SCN5A*. **A**, Early repolarization was present in the inferior and right precordial leads in patient 1. **B**, After administration of pilsicainide, early repolarization was augmented and ventricular fibrillation developed. **C** and **D**, Pilsicainide caused marked prolongation of QRS duration and J-point elevation in the right precordial leads, followed by the development of ventricular fibrillation in patient 2. **E**, Early repolarization was present in the inferior leads and right precordial leads in patient 3. **F**, The augmentation of early repolarization after sinus pause, followed by ventricular fibrillation. **G**, After the administration of procainamide, early repolarization was augmented in the inferior. In all patients, sodium channel blockers did not provoke a type I Brugada ECG. **E**, **F**, and **G** were modified from Takehara et al.[27]

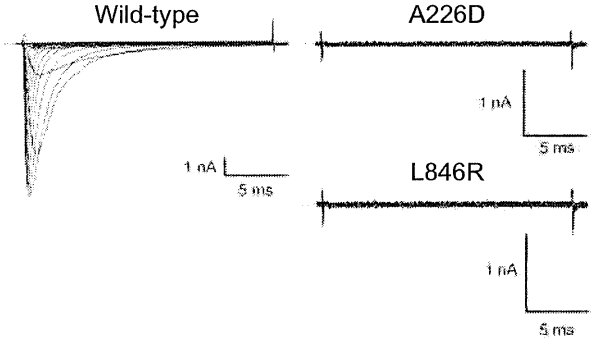

Figure 3. Electrophysiological characteristics of the *SCN5A* mutants. Representative traces of sodium current demonstrating that all of the mutant channels failed to generate any currents. We previously reported that R367H mutant fails to generate any currents.[27]

Brugada ECG (Figure 2G). During electrophysiological study, His-ventricular time was prolonged (65 ms) and ventricular fibrillation was not induced. The patient's family history was negative for syncope, sudden cardiac death, and epilepsy.

The electrophysiological characteristics of the mutant sodium channels were assessed in transfected mammalian cells using the whole-cell patch-clamp technique. Figure 3 shows representative current traces in cells expressing wild-type or mutant SCN5A channels. There was no detectable current in A226D, R367H,[27] and L846R mutant channels. Immunostaining revealed that cells expressing A226D channels showed cytoplasmic fluorescence, while cells expressing wild-type channels showed marked peripheral fluorescence, suggesting that the mutation results in trafficking defect (Figure 4). Cells expressing R367H channels and those expressing L846R channels showed a similar fluorescence pattern to wild-type channels, suggesting that these mutations do not affect trafficking.

Discussion

In this study, patients with idiopathic ventricular fibrillation associated with early repolarization exhibited slower heart rate and slower cardiac conduction properties than did controls. We found rare, nonsynonymous variants in *SCN5A* in patients who had idiopathic ventricular fibrillation associated with early repolarization. These variants affect highly conserved residues, and all of the mutant SCN5A channels failed to generate any currents when expressed in heterologous expression systems. Immunostaining experiments suggested 2 possible mechanisms for the sodium channel dysfunction by the *SCN5A* mutations, a defect of channel trafficking to cell surface in A226D and critical alterations of the structures required for the sodium ion permeation or gating in R367H and L846R that are predicted to be located at the pore region.

Loss-of-function mutations in *SCN5A* are associated with a wide range of inherited arrhythmia syndromes, including Brugada syndrome, progressive cardiac conduction disease, and sick sinus syndrome.[28–30] Furthermore, our results suggest that *SCN5A* is a causative gene of idiopathic ventricular fibrillation associated with early repolarization. Evidence supporting disease causality of the mutations includes the

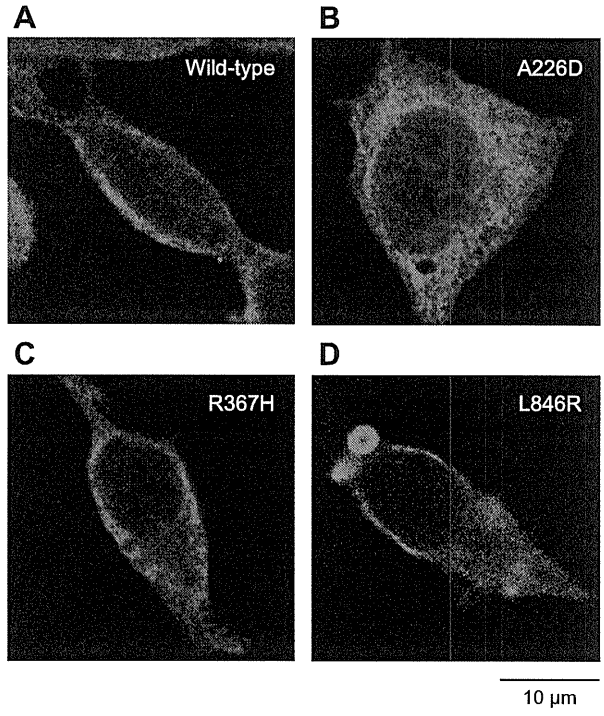

Figure 4. Representative confocal microscopy images. **A**, Cells expressing wild-type SCN5A channels showed marked peripheral fluorescence. **B**, Cells expressing A226D channels showed cytoplasmic fluorescence. **C** and **D**, Cells expressing R367H channels and those expressing L846R channels showed a similar fluorescence pattern to wild-type channels.

identification of 3 mutations in 3 unrelated probands who shared similar clinical phenotypes and the loss of sodium channel function effects in heterologous expression systems in all of the mutant channels.

Although our findings suggest that loss of sodium channel function plays a role in idiopathic ventricular fibrillation associated with early repolarization, the mechanisms of early repolarization are not understood well. In wedge preparations of canine ventricles, early repolarization results from increased action potential notches at the ventricular epicardium by either a decrease in inward currents or an increase in outward currents.[31] A mutation in *KCNJ8*, which encodes the ATP-sensitive potassium channel, recently has been identified in idiopathic ventricular fibrillation associated with early repolarization.[11] The *KCNJ8* mutation has shown gain-of-function effects in ATP-sensitive potassium channels in heterologous expression studies,[14] and augmentation of ATP-sensitive potassium currents results in the development of ventricular fibrillation in wedge preparations.[32] Decreased calcium currents also have been proposed as a mechanism for idiopathic ventricular fibrillation associated with early repolarization.[33] Mutations in L-type calcium channel genes, including *CACNA1C*, *CACNB2B*, and *CACNA2D1*, recently have been identified; however, functional studies are not yet available.[12] Our findings that mutant *SCN5A* channels displayed loss of sodium channel function, resulting in a decrease of inward currents, are consistent with findings in prior studies and with the proposed mechanism.[11,12,14,33]