Compound Heterozygous Mutations P336L and I1660V in the Human Cardiac Sodium Channel Associated With the Brugada Syndrome

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Background—Loss-of-function mutations in SCN5A have been associated with the Brugada syndrome. We report the first Brugada syndrome family with compound heterozygous mutations in SCN5A. The proband inherited 1 mutation from each parent and transmitted 1 to each daughter.

Methods and Results—The effects of the mutations on the function of the sodium channel were evaluated with heterologous expression in TSA201 cells, patch-clamp study, and confocal microscopy. Genetic analysis revealed that the proband carried 2 heterozygous missense mutations (P336L and I1660V) on separate alleles. He displayed a coved-type ST-segment elevation and a prolonged PR interval (280 ms). One daughter inherited P336L and exhibited a prolonged PR (210 ms). The other daughter inherited mutation I1660V and displayed a normal PR interval. Both daughters had a slightly elevated, upsloping ST-segment elevation. The parents had normal ECGs. Patch-clamp analysis showed that the P336L mutation reduced $I_{Na}$ by 85% relative to wild type. The I1660V mutation produced little measurable current, which was rescued by room temperature incubation for 48 hours. Sodium channel blockers also rescued the I1660V current, with mexiletine proving to be the most effective. Confocal immunofluorescence showed that I1660V channels conjugated to green fluorescent protein remained trapped in intracellular organelles.

Conclusions—Mutation P336L produced a reduction in cardiac $I_{Na}$, whereas I1660V abolished it. Only the proband carrying both mutations displayed the Brugada syndrome phenotype, whereas neither mutation alone produced the clinical phenotype. I1660V channels could be rescued pharmacologically and by incubation at room temperature. The present data highlight the role of compound heterozygosity in modulating the phenotypic expression and penetrance of Brugada syndrome. (Circulation. 2006;114:2026-2033.)

Key Words: arrhythmia ■ genetics ■ ion channels

Mutations in SCN5a, the α-subunit of the cardiac Na⁺ channel, have been linked to a variety of ion channelopathies such as Brugada syndrome, long-QT syndrome, and progressive conduction disease.¹ The Brugada syndrome is characterized by an ST-segment elevation in the right precordial leads and is unrelated to ischemic or structural heart disease.² This disorder has been linked to mutations in SCN5A, all of which have been shown to reduce the magnitude of the cardiac Na⁺ current by a variety of mechanisms.³ Loss of Na⁺ current by Brugada syndrome mutations has been linked to 3 primary mechanisms: (1) truncation of the Na⁺ channel α-subunit, which yields a nonfunctional channel; (2) alteration in channel gating, such as changes in activation, inactivation, or reactivation kinetics; and (3) altered trafficking of the channels for the endoplasmic reticulum/Golgi complex to the plasma membrane.⁴ The typical coved-type ST-segment elevation in the ECG is often concealed but can be unmasked by sodium channel blockers and vagal influences.⁵ The expression of the phenotype and penetrance of the disease is not well understood but...
appears to be related to sex differences, age, and other factors that alter the balance of outward and inward currents at the end of phase 1 of the epicardial ventricular action potential.

In the present study, we identified 2 compound heterozygous mutations, P336L and I1660V, in SCN5A in a proband with asymptomatic Brugada syndrome. The proband inherited 1 of these mutations in separate alleles and transmitted 1 to each daughter. The effects of these mutations were studied by heterologous expression of the mutated channels in TSA201 cells. Both mutations significantly reduced the amplitude of the sodium current compared with wild type (WT) and thus are expected to generate the substrate for the Brugada syndrome phenotype. Only the proband, who displayed compound heterozygosity, had the ST elevation in the right precordial leads.

Methods

Subjects

Brugada-like ECG was diagnosed according to a recent consensus report.6,7 Institutional review board approval was obtained, and each patient included in the study provided informed consent. The proband was identified during a routine ECG screening. He had the typical Brugada syndrome ECG and no previous history of syncope or arrhythmias. He underwent electrophysiological study and was found to have asymptomatic Brugada syndrome. The proband included in the study provided informed consent. The proband in II-1 carried both heterozygous mutations P336L and I1660V, whereas the mother (I-2) had a substitution of the isoleucine for a valine at position 1660 (I1660V), whereas the mother (I-2) had a substitution of the isoleucine for a valine at position 1660 (I1660V), whereas the mother (I-2) had a substitution of the isoleucine for a valine at position 1660 (I1660V), whereas the mother (I-2) had a substitution of the isoleucine for a valine at position 1660 (I1660V).

Genetic Analysis

Informed consent was obtained before genetic analysis. Genomic DNA was extracted from peripheral blood leukocytes with a commercial kit (Puregene, Gentra Systems, Inc, Minneapolis, Minn). All exons of SCN5A from the proband were amplified by polymerase chain reaction. Polymerase chain reaction products were purified with a commercial reagent (ExoSAP-IT, USB Corporation, Cleveland, Ohio) and directly sequenced from both directions with the use of ABI PRISM 3100 Automatic DNA Sequencer (Applied Biosystems, Foster City, Calif). To determine the prevalence of the variations in the control population, we screened the mutated exons using the direct sequencing method in 200 controls. These 2 mutations were absent in the 200 unrelated control individuals with the same ethnic background.

Site-Directed Mutagenesis

P336L and I1660V mutations were constructed with the use of a Gene Tailor site-directed mutagenesis system (Invitrogen Corporation, Carlsbad, Calif) with plasmid pcDNA3.1 containing SCN5A cDNA. The primers for mutagenesis were the following:

P336LmutF: 5'-agctctgacgctgggaacatgtctggagggcta-3' 
P336LRev: 5'-gaactgctccagcgctagctggctccacaa-3' 
I1660VmutF: 5'-ctgctgtgcgctccatgcgtggctgct-3' 
I1660VRev: 5'-gttaagggaggcagggagcctgga-3'.

The mutated plasmids were sequenced to ensure the presence of either the P336L or I1660V mutation and the absence of spurious substitutions. Modified human embryonic kidney cells (TSA201) were cotransfected with 10 μg of α-subunit (either WT or mutant) and 4 μg of β-subunit using the calcium phosphate precipitation method as described previously.8 Cells were grown in 35-mm culture dishes (Cell +, Sarstedt, Newton, NC) and placed in a temperature-controlled chamber (Medical Systems, Greenvale, NY) for electrophysiological study 2 days after transfection.

Electrophysiology

Voltage clamp recordings were made with patch pipettes fabricated from borosilicate glass capillaries (1.5 mm OD, Fisher Scientific, Pittsburgh, Pa). The pipettes were pulled with a gravity puller (model PP-830, Narishige International USA, East Meadow, NY) and filled with pipette solution of the following composition (in mmol/L) KCl 10, CsF 105, NaCl 10, HEPES 10, ethylene glycol tetraacetic acid 10, and tetraethy lammonium chloride 5, pH 7.2 with CsOH. Pipette resistance ranged from 1.0 to 3.0 MΩ when pipettes were filled with the internal solution. The perfusion solution contained (in mmol/L) NaCl 130, KCl 5, CaCl 2 1.8, MgCl 2 1.0, sodium acetate 2.8, HEPES 10, glucose 10, pH 7.3 with NaOH. Current signals were recorded with a MultiClamp 700A amplifier (Axon Instruments, Foster City, Calif), and series resistance errors were reduced by approximately 60% to 70% with electronic compensation. Signals were acquired at 20 to 50 kHz (Digidata 1322, Axon Instruments) and analyzed with a microcomputer running pClamp 9 software (Axon Instruments). All recordings were made at room temperature (RT; 20°C to 22°C).

Localization of Na⁺ Channels

To identify trafficking defects, which may be caused by mutant Na⁺ channels, we localized Na⁺ channels conjugated to green fluorescent protein (GFP) by confocal microscopy. Briefly, cells were grown on polylysine-coated 35-mm culture dishes and studied 2 days after transfection. Experiments were performed on an Olympus Fluoview laser-scanning confocal microscope (Olympus, Melville, NY), and images were acquired with a FluoView acquisition software program on a microcomputer. GFP-labeled cells were analyzed in the XYZ configuration as described previously.9 An argon laser provided the excitation wavelength.
light at 488 nm, and the emission light was collected at 520 nm in photomultiplier tube #1. The transmission image was acquired in photomultiplier tube #2. Fluorescence signals were collected with either a 40× or a 60× oil-immersion objective lens. The Z axis was changed in 0.5-μm increments by computer control through the entire volume of the cell. Analysis of GFP-labeled cells was performed with both Fluoview and Image J software.

### Statistics and Data Analysis

The modified Hodgkin and Huxley model equation for sodium current

\[
I(V) = n_p o I_e \left(1 + \exp \left(-\frac{V_m - V_h}{k_m}\right)^{1} \times \left(1 + \exp \left(-\frac{V_m - V_h}{k_i}\right)^{1}\right) \times (V_m - E_r)
\]

was used to predict the effects of changes in steady state gating parameters on the maximum amplitude of the sodium current.

Parameters \(n, p, I, E_R,\) and \(V_m\), respectively, correspond to the number of channels, open channel probability, unitary current, reversal potential, and membrane test potential. Values for midpotentials and slopes factor for activation \((V_a, k_a)\) and inactivation \((V_i, k_i)\) were calculated from a Boltzmann distribution function fitted on steady state inactivation and activation data obtained experimentally. The product \((n_p I_e)\) was kept constant to estimate the changes in current amplitude brought solely by the changes in steady state gating parameters.

Electrophysiological data are presented as mean±SEM, and statistical comparisons were made with ANOVA or Student t test, as appropriate. Significance was defined as \(P<0.05\).

The authors had full access to the data and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

### Results

Genetic analysis of the cardiac sodium channel gene (SCN5A) revealed that the proband carries mutations in exons 9 and 28 on separate alleles. No other synonymous or nonsynonymous variations in SCN5A were detected. The grandparents’ DNA revealed that the paternal allele had a substitution of an adenine for a guanine at position 4975, exon 28, in the coding sequence of SCN5A (hH1a). The nucleotide change caused a substitution of isoleucine for a valine at position 1660 (I1660V). Exon 9 of SCN5A on the maternal allele displayed substitution of a cytosine for a thymine at position 336 (P336L). Analysis of the daughters’ chromosomal DNA revealed that each inherited a single missense mutation.

The proband, with both mutations, displayed a type 1 ST-segment elevation (Figure 1) and first-degree atrioventricular block with a very prolonged PR interval (280 ms). One of the daughters (III-2), who inherited mutation P336L, displayed first-degree atrioventricular block; it was less severe than in the proband (210 ms), however. The daughter who inherited I1660V (III-1) displayed a normal PR interval. QT intervals were within normal limits in all individuals.

Because of the presence of the mutations in different alleles, we separately determined the contribution of each mutation to the Brugada syndrome phenotype. We coexpressed each of the mutated SCN5A channels in a 1.33:1 molar ratio with the sodium channel β1-subunit in TSA201 cells and performed patch-clamp experiments. The effect of β-subunits on SCN5A expression and NaV1.5 gating remains

![Figure 2](http://circ.ahajournals.org/)

Figure 2. Representative whole-cell current recordings for WT (A) and P336L mutant (B) in transfected TSA201 cells. Current recordings were obtained at test potentials between −100 and 0 mV in 5-mV increments from a holding potential of −120 mV. C, I-V relation for WT (n=11) and P336L (n=15) channels showing a reduction in current for P336L. D, Steady state-activation relation for WT and P336L. Chord conductance was determined with the ratio of current to the electromotive potential for the cells shown in C. Data were normalized and plotted against their test potential.
The same approach was used to electrophysiologically characterize I1660V channels. Figure 4A shows that I1660V channels did not generate electrical currents of significant amplitude (−0.18±0.07 nA at −40 mV) compared with WT (−5.98±0.55 nA at −40 mV) in response to our I-V protocol. Previous studies have reported that many nonfunctional channels can be rescued by a variety of methods, including incubation at low temperature and addition of pharmacological agents to the culture media.12–14 We next tested whether I1660V channels could be rescued by culturing our transfected cells at RT before electrophysiological recordings.

In cells cultured at 37°C (Figure 4B), I1660V channels expressed minimal current compared with WT channels (Figure 4A). When the transfected TSA201 cells were incubated at room temperature (20°C to 22°C) for 48 hours before recordings were made, I1660V channels expressed robust inward currents. Incubation of WT channels at RT did not affect the magnitude of their currents (Figure 4C). Thus, temperature-dependent rescue of I1660V channels yielded a similar I-V relationship to WT (Figure 4D). Incubation of P336L channels at RT, however, did not rescue the current (data not shown). Previous reports showed that addition of mexiletine, a class I antiarrhythmic agent, to culture media could correct trafficking problems and thus restore expression of the channels.12–14 We next tested whether I1660V channels could be rescued by incubation with sodium channel blockers.

TSA201 cells transfected with I1660V were separately exposed to quinidine (100 μmol/L), ajmaline (100 μmol/L), mexiletine (300 μmol/L), or ranolazine (100 μmol/L) for 48 hours. Each drug was washed out 30 minutes before patch-clamp recordings (Figure 5A). Incubation with the antiarrhythmic agents at concentrations close to their respective EC50 rescued I1660V to various degrees, with mexiletine proving to be the most effective agent (Figures 5B and 5C).

Our results thus far suggest that the loss of current caused by mutation I1660V is due to trafficking defect, because RT incubation and drugs could rescue the channels. To confirm this hypothesis, we tagged to the WT and I1660V channels with the GFP and localized the channel proteins by confocal microscopy. XYZ scans of WT channels revealed both a central and peripheral pattern of staining that suggested that a pool of these channels exists in intracellular organelles and that WT proteins translocated normally to the cell membrane (Figure 6A). In contrast, the fluorescence distribution of I1660V channels was essentially localized in intracellular organelles (Figure 6B), which suggests that channels remained trapped inside the cells, likely in the endoplasmic reticulum, as previously shown for WT channels.15 After incubation of the I1660V transfected cells at RT for 48 hours (Figure 6C), a central and peripheral staining pattern appeared, which suggests that normal trafficking of the channels to the cell membrane was restored.

**Discussion**

To the best of our knowledge, the present study is the first report of a case of Brugada syndrome in which the disease phenotype is observed only as a result of the additive effects from 2 heterozygous missense mutations. Carriers of the single mutation, both parents and daughters, failed to display an ECG phenotype diagnostic of the Brugada syndrome.
Pedigree analysis revealed that the proband inherited the mutations from separate alleles and transmitted 1 mutation to each of his daughters.

Patch-clamp recordings of the mutant channels in TSA201 cells revealed that P336L yields an 85% amplitude reduction in peak Na$^+$ current compared with WT. The I1660V mutant channels produced little measurable $I_{Na}$ that could be rescued by incubation of transfected cells at RT or exposure to sodium channels blockers during expression of the channels in culture. Mexiletine was the most effective agent to rescue I1660V current. I1660V rescued channels exhibited a similar current magnitude as WT channels (Figure 4). These observations suggest that I1660V channels are functional but remain trapped in the ER/Golgi complex, a phenomenon previously described for other mutations.4,12

Using channels conjugated to GFP and localized by confocal microscopy, we confirmed that I1660V channels are not properly trafficked to the plasma membrane but remain trapped in intracellular organelles, likely the ER. In contrast, WT channels displayed both an intracellular and sarcolemmal fluorescence pattern, as expected with normal trafficking of the channels.

The mechanisms by which these channels remain trapped within the cell or those responsible for rescue by temperature and drugs are not well understood. Previous reports have demonstrated rescue of channels carrying mutations M1766L16 and G1743R12 by the sodium channel $\beta$1-subunit or by mexiletine, respectively. Association of the $\alpha$-subunit of SCN5A with the $\beta$-subunit also alters gating and enhances expression of WT channels by itself.17,18 Temperature rescue of M1766L channels remained marginal, however, in sharp contrast with the marked recovery we observed with the I1660V channels.

Several amino acids in the COOH portion of segment S6 in domain IV of the cardiac sodium channel are known to be involved in the binding of class I antiarrhythmic drugs such as lidocaine and mexiletine.19 Channels with mutations M1766L and G1743R in that region appear to be capable of being rescued by mexiletine.12,16 One likely explanation is that mexiletine may stabilize the protein and attenuate the mutation-induced changes in the conformation of the protein. As a corollary, the receptor site for mexiletine may already be formed in the ER. In this context, the location of the mutated isoleucine 1660 in the NH3 termini part of the putative fifth transmembrane segment of domain 4 (IVS5) may affect the $\beta$-helix conformation of that segment and play a significant role in the trafficking and expression of the channel. Lowering temperature or adding mexiletine may therefore act as a stabilizer of this conformation and facilitate translocation of the channel. The reduction in current amplitude produced by mutation P336L cannot be explained by the changes in steady state activation and inactivation that we observed, which suggests that changes in the conductance of the channel may be involved. This hypothesis is
supported by the fact that a nearby cysteine at position 373 within the selectivity filter is a key residue for tetrodotoxin and zinc binding and for modulation of single-channel current amplitude.20,21

Correlation to the Clinical Observations

Mutations in SCN5A have been linked to 3 major cardiac arrhythmia syndromes, namely, Brugada syndrome, progressive conduction disease, and long-QT syndrome.1 All of these syndromes show highly variable penetrance and phenotypic expression within families carrying mutations in SCN5A.

Our patch-clamp analysis of the autosomal dominant I1160V mutation showed that these channels carry little current when expressed in TSA201 cells, a result that predicts a 50% loss in I_{Na} in vivo. Surprisingly, the mother of the proband and the daughter carrying this mutation exhibited a normal ECG. P336L mutation reduced the peak Na^{+}/H^{+} current by 85% versus WT, but the daughter carrying this mutation only displayed a slight prolongation of the PR interval on her ECG, whereas the father of the proband showed a normal ECG. Although 4 family members carried 1 mutation, none of them has developed Brugada syndrome to date. These observations suggest that the allele carrying the mutation is effectively “silent,” whereas the normal allele is upregulated. In contrast, the proband who inherited both defects displayed a prominent, coved-type ST-segment elevation diagnostic of the Brugada syndrome, which suggests an additive effect of the 2 heterozygous mutations.

A similar example of compound heterozygosity and low penetrance was recently reported by Bezzina et al22 in a case of conduction disturbance. As with our proband, their patient carried separate SCN5A mutations on different alleles, whereas none of his parents showed ECG abnormalities. In the present study, several factors appear to influence the degree of phenotypic expression and penetrance of this channelopathy. One surprising fact was that only the proband developed a severe Brugada syndrome phenotype. Because the proband carried each of the mutated channels, this likely explains the more severe phenotype. In addition, among the members affected by only 1 mutation (either I1660V or P336L), 3 are female and 2 are younger. In Brugada syndrome, sex is known to play a determining role. Despite equal genetic transmission of the mutation, the clinical phenotype is 8 to 10 times more prevalent in males than in females. Among the hypothesis brought forward to explain such preponderance of the disease is that females tend to display a smaller transient outward current (I_{to}) and therefore exhibit ventricular action potentials that have a smaller “notch” at the end of phase 1 repolarization.23,24 Such sex-based electrophysiological differences in the contribution of I_{to} to the action potential may explain why the female offspring of the proband exhibit minimal alterations of their ECGs. This variable does not explain the lack of a Brugada phenotype in the proband’s father, however.
Another major factor associated with the development of Brugada syndrome is age. Although patients inherit the defective gene at birth, Brugada syndrome on average does not manifest until about the fourth decade of life. Because the daughters are still in the second decade of their life, it remains to be determined whether the Brugada syndrome phenotype will appear later. However, the absence of a Brugada phenotype in the parents (I-1 and I-2 of Figure 1A) suggests that age may not be a determining factor here. The present results clearly show that I1660V has a variable level of expression that can be modulated by several factors, including temperature and exposure to class I antiarrhythmic agents. These results suggest that I1660V may be also be subject to high variability in its in vivo expression. Such variability may have played a role in the father’s lack of Brugada syndrome symptoms.

**Study Limitations**

The results of the present study show a dramatic loss of current by P336L and I1660V compared with WT channels. However, caution should be exercised when making the translation to the clinical setting. Several factors may explain the disconnection between the strong loss of function observed in the expression system and the minimal phenotypic expression observed in individuals carrying only 1 mutation. The TSA201 cells used in the present study expressed only the α- and β1-subunits, whereas cardiac myocytes likely have additional proteins that are not present in TSA201 cells. Some of these proteins include 4 different β-subunits (β1 through β4), as well as anchoring proteins, all of which combine to form the Na+ channel macromolecular complex. It is likely that TSA201 cells are missing several key elements present in the native myocytes.

In the present experiments, both mutations I1660V and P336L reduced the amplitude of current in the hH1a (Q1077del) variant. We did not test the effects of these mutations in a background where the Q1077 was present. However, previous studies have shown that coexpression of several polymorphisms in the Q1077 splice variant produce a reduction in Na+ current magnitude that is not present when the same polymorphisms are expressed in the Q1077del variant. Although it is clear that the magnitude of Na+ current can be dramatically different depending on which splice variant (hH1a, hH1b, or hH1c) is used in the patch-clamp studies, the use of Q1077del appears to underestimate the loss of function of genetic variations.

The concentration of mexiletine used in the present rescue experiments was supratherapeutic. In addition, the use of mexiletine, a sodium channel blocker, is not indicated in the treatment of Brugada syndrome, and therefore, the experiments presented are not intended to show the therapeutic potential of the drug. The experiments merely demonstrate that mutated channels trapped in intracellular organelles in diseases such as Brugada syndrome are capable of being pharmacologically rescued. This may open new therapeutic approaches for the treatment of this disease. The rationale would be to develop a drug/molecule with little or no Na+ channel blocking ability that is capable of rescuing channels that do not translocate properly.

In summary, we have found 2 heterozygous Na+ channel mutations present in several members of a family. Although patch-clamp analysis revealed that each mutation produces either a severe reduction (P336L) or a complete loss (I1660V) of Na+ current, neither mutation alone produced profound changes in the ECG of the patient. Only the combined effect of the 2 mutations resulted in a sufficient loss of Ina to reveal the Brugada syndrome phenotype. The results of the present study also show that the interaction between genetic mutations, environmental factors, and phenotypic expression is complex.

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Disclosures

None.

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CLINICAL PERSPECTIVE

The Brugada syndrome is characterized by an ST-segment elevation in the right precordial leads. Inheritance of the disease is through an autosomal dominant mode of transmission, and the only gene linked to the syndrome is SCN5A, the gene encoding the α-subunit of the cardiac sodium channel. The basis for the low penetrance of the disease in many families is poorly understood. In this study, we identified a family that carried 2 different mutations (I1660V and P336L) on separate alleles of the SCN5A gene. Carriers of the single mutation (the parents and 2 daughters of the proband) did not display an ECG phenotype diagnostic of the Brugada syndrome. Pedigree analysis revealed that the proband inherited the 2 mutations on separate alleles and displayed a prominent, coved-type ST-segment elevation diagnostic of the Brugada syndrome. Expression studies showed a loss of function of sodium channel current with either mutated channel. Genotype-phenotype correlation indicated low penetrance of the disease with either mutation alone. In contrast, the proband who inherited both genetic defects exhibited the Brugada syndrome, which suggests an additive effect of the 2 heterozygous mutations, leading to a reduction in sodium channel current sufficient to manifest the Brugada syndrome phenotype. These findings highlight the role of compound heterozygosity in modulating the phenotypic expression and penetrance of the Brugada syndrome.
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