Mutations in the cardiac L-type calcium channel associated with inherited J-wave syndromes and sudden cardiac death

Elena Burashnikov, BS,* Ryan Pfeiffer, BS,* Héctor Barajas-Martinez, PhD,* Eva Delpón, PhD,† Dan Hu, MD, PhD,* Mayurika Desai, BS,* Martin Borggrefe, MD,‡ Michel Häissaguerre, MD,§ Ronald Kanter, MD,‖ Guido D. Pollevick, PhD,¶ Alejandra Guerchicoff, PhD,* Ruben Laiño, MD,** Mark Marieb, MD,†† Koonlawee Nademanee, MD,‡‡ Gi-Byoung Nam, MD, PhD,§§ Roberto Robles, MD,¶¶ Rainer Schimpf, MD,‡ Christian Wolpert, MD,††† Samuel Zimmerm, MD, §§§ Christian Veltmann, MD,¶ Charles Antzelevitch, PhD, FHRS*

From the *Masonic Medical Research Laboratory, Utica, New York, †Department of Pharmacology, School of Medicine, Universidad Complutense, Madrid, Spain, ‡1st Department of Medicine-Cardiology, University Medical Centre Mannheim, Mannheim, Germany, §Hospital Cardiologique Du Haut Leveque, Bordeaux-Pessac, France, ‖Duke University Health System, Durham, North Carolina, ¶PGxHealth, LLC, New Haven, Connecticut, **Instituto Cardiovascular de Buenos Aires, Buenos Aires, Argentina, ††Yale University, New Haven, Connecticut, ‡‡Pacific Rim Cardiac Electrophysiology and Research Institute, Inglewood, California, §§University of Ulsan, College of Medicine, Seoul, South Korea, ¶¶CEMIC, Buenos Aires, Argentina, ¶¶¶Guthrie Medical Group, Horseheads, New York, ⌧Tel Aviv Medical Center, Tel Aviv, Israel; †††Morristown Memorial Hospital, Morristown, New Jersey, ‡‡‡Department of Medicine, Cardiology, Nephrology and Internal Intensive Care Medicine, Posilipostr, Ludwigsburg, Germany, and §§§Sanger Heart and Vascular Institute, Charlotte, North Carolina.

BACKGROUND L-type calcium channel (LTCC) mutations have been associated with Brugada syndrome (BrS), short QT (SQT) syndrome, and Timothy syndrome (LQT8). Little is known about the extent to which LTCC mutations contribute to the J-wave syndromes associated with sudden cardiac death.

OBJECTIVE The purpose of this study was to identify mutations in the α1, β2, and α2δ subunits of LTCC (Ca,1.2) among 205 probands diagnosed with BrS, idiopathic ventricular fibrillation (IVF), and early repolarization syndrome (ERS). CACNA1C, CACNB2b, and CACNA2D1 genes of 162 probands with BrS and BrS+SQT, 19 with IVF, and 24 with ERS were screened by direct sequencing.

METHODS/RESULTS Overall, 23 distinct mutations were identified. A total of 12.3%, 5.2%, and 16% of BrS/BrS+SQT, IVF, and ERS probands displayed mutations in α1, β2, and α2δ subunits of LTCC, respectively. When rare polymorphisms were included, the yield increased to 17.9%, 21%, and 29.1% for BrS/BrS+SQT, IVF, and ERS probands, respectively. Functional expression of two CACNA1C mutations associated with BrS and BrS+SQT led to loss of function in calcium channel current. BrS probands displaying a normal QTc had additional variations known to prolong the QT interval.

CONCLUSION The study results indicate that mutations in the LTCCs are detected in a high percentage of probands with J-wave syndromes associated with inherited cardiac arrhythmias, suggesting that genetic screening of Ca, genes may be a valuable diagnostic tool in identifying individuals at risk. These results are the first to identify CACNA2D1 as a novel BrS susceptibility gene and CACNA1C, CACNB2, and CACNA2D1 as possible novel ERS susceptibility genes.

KEYWORDS Arrhythmia; Calcium; Electrophysiology; Genetics; Ion channels

ABBREVIATIONS BrS = Brugada syndrome; CHO = Chinese hamster ovary; ERS = early repolarization syndrome; IVF = idiopathic ventricular fibrillation; LQTS = long QT syndrome; LTCC = L-type calcium channel; PCR = polymerase chain reaction; SCD = sudden cardiac death; SNP = single nucleotide polymorphism; SQT = short QT; WT = wild type

(Hearth Rhythm 2010;7:1872–1882) © 2010 Heart Rhythm Society. All rights reserved.

Supported by Grant HL47678 from the National Heart, Lung, and Blood Institute, New York State and Florida Masonic Grand Lodges to Dr. Antzelevitch, and Grant SAP2008-04903 from the Spanish Ministry of Sciences to Dr. Delpón. Address reprint requests and correspondence: Dr. Charles Antzelevitch, Masonic Medical Research Laboratory, 2150 Bleecker Street, Utica, New York 13501. E-mail address: ca@mmrl.edu. (Received August 16, 2010; accepted August 30, 2010.)

doi:10.1016/j.hrthm.2010.08.026
Burashnikov et al. L-Type Calcium Channel Mutation, J-Wave Syndromes, and SCD

Introduction
Sudden cardiac death (SCD) is often associated with inherited cardiac arrhythmia syndromes.1 Twenty-five percent of all unexplained sudden deaths may be due to inherited cardiac diseases such as Brugada syndrome (BrS), idio-pathic ventricular fibrillation (IVF), and long QT syndrome (LQTS).2 BrS, early repolarization syndrome (ERS), and some forms of IVF represent a continuous spectrum of phenotypic expression that differ with respect to the magnitude and lead location of abnormal J-wave manifestations, which we and others have proposed be termed J-wave syndromes.3

The past decade has witnessed a veritable explosion of information linking inherited cardiac arrhythmia syndromes to cardiac ion channel mutations. BrS has been associated with mutations in seven genes classified as BrS1 through BrS7.4 Mutations in SCN5A, which encodes the Na1.5 protein forming the α subunit of the sodium channel, have been associated with 11% to 28% of BrS probands by different groups.5 A genotype has not yet been identified in the majority of BrS probands. ERS has thus far been associated with one mutation in KCNJ8, a gene encoding the pore-forming subunit of the I$_{K_{ATP}}$ channel.6 Expression studies suggesting a functional effect of this mutation has recently been reported.7

Little is known about the contribution of calcium channel gene variations to the etiology of inherited cardiac arrhythmia syndromes. Splawski et al.8,9 first described gain-of-function mutations in CACNA1C, a gene encoding Ca1.2 protein that forms the α subunit of the L-type calcium channel (LTCC), associated with a multiorgan dysfunction causing long QT intervals, arrhythmias, and autism known as Timothy syndrome (LQT8). Our group first described loss-of-function mutations in the α and β subunits of the cardiac LTCC associated with BrS and shorter than normal QT intervals and SCD.10,11

The LTCC is composed of four subunits: the main pore-forming α1 (Ca1.2) subunit, which determines the main biophysical and pharmacologic properties of the channel, and three auxiliary subunits, including a cytoplasmic β subunit, encoded by CACNB, α2δ encoded by CACNA2D, and a γ subunit, which is present in skeletal, but not cardiac, muscle.12–14 Although a number of isoforms for the auxiliary subunits have been identified, in this study we focused on β2 (CACNB2), the dominant isoform known to play an essential role in the voltage dependence of LTCC,15,16 and the extracellular α2 and transmembrane δ1 (CACNA2D1), which are linked to each other via disulfide bonds.

Few data are available on the extent to which mutations in the various subunits of LTCC contribute to SCD, the extent to which they are associated with ST-segment elevation and QT abbreviation giving rise to the BrS and BrS+SQT phenotypes, and their pathogenicity. The present study sought to identify genetic variations in the α1, β2, and α2δ1 subunits of LTCC among probands diagnosed with BrS, ERS, and IVF and the extent to which they contribute to pathogenesis of these syndromes. We tested the hypothesis that mutations in LTCC genes are relatively common among probands diagnosed with these syndromes. We also examined the hypothesis that LTCC mutation-mediated BrS associated with a normal QTc is attributable to additional genetic variations known to prolong the QT interval.

Material and methods
Diagnosis
The probands and their family members were diagnosed as having BrS, BrS with shorter than normal QT (BrS/SQT), IVF, or ERS based on established criteria.17–20 Diagnosis was made based on 12-lead ECG, personal history of syncope, seizures, or aborted cardiac death, and family history of SCD or arrhythmic events. BrS patients displayed a coved-type ST-segment elevation in at least one right precordial lead under baseline conditions or after sodium channel block challenge with ajmaline or procainamide. Criteria for BrS with shorter than normal QT included QTc ≤360 ms for males and QTc ≤370 ms for females. An early repolarization pattern was defined as J-point (QRS–ST junction) elevation >0.1 mV manifested as QRS slurring or notchting or a distinct J wave. Patients were categorized as having IVF when no clear established phenotype was discernible in individuals experiencing one or more episodes of ventricular fibrillation. Most, but not all, patients underwent a sodium block challenge to rule out BrS and/or an isoproterenol challenge to rule out catecholaminergic polymorphic ventricular tachycardia.

Mutation analysis
A total of 205 BrS, BrS/SQT, ERS, and IVF probands who tested negative for SCN5A mutations were included in the study. After obtaining informed consent, blood was collected from the probands and family members. Genomic DNA was extracted from peripheral blood leukocytes using a commercial kit (Puregene, Gentra Systems, Inc., Minneapolis, MN, USA) and amplified by polymerase chain reaction (PCR) on GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA). All exons and intron borders of the CACNA1C, CACNB2, and CACNA2D1 genes were amplified and analyzed by direct sequencing. PCR products were purified with a commercial reagent (ExoSAP-IT, USB, Cleveland, OH, USA) and directly sequenced from both directions using an ABI PRISM 3100 Automatic DNA Analyzer (Applied Biosystems). Electropherograms were visually examined for heterozygous peaks and compared with reference sequences for homozygous variations (GenBank accession number NM_000719, NM_201590, NM_000722.2) using CodonCode Aligner Version 2.0.4 (CodonCode Corporation, Dedham, MA, USA). Fifty-five primer pairs were used to screen 55 exons, including splice alternative variants of CACNA1C, 20 primer pairs were used for CACNB2, and 39 primer pairs were used for CACNA2D1. Probands with calcium channel mutations and rare variants were also screened for KCNH2, KCNQ1, KCNJ8, KCNE1, KCNE2, KCNE3, KCNE4, SCN1B, and...
To be considered a mutation, a variation must have changed or disrupted the open reading frame (missense, nonsense, insertion/deletion mutation) and been absent in a minimum of 400 reference alleles obtained from more than 200 healthy individuals of similar ethnicity whenever possible. Possible single nucleotide polymorphisms (SNPs) were confirmed in the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/projects/SNP). Numbering of residue variations was based on the NCBI database nucleotide reference sequence. The degree to which variations uncovered are conserved among species was determined using VISTA browser (http://pipeline.lbl.gov/cgi-bin/gateway2).

**Mutagenesis and functional expression**

The human wild-type (WT) CACNA1C cDNA [(EYFP)Na1c,77] in pcDNA vector was a gift from Dr. Nikolai Soldatov. cDNA of CACNB2b and CACNA2D1 genes, both cloned in pcDNA3.1 vector (Invitrogen, Carlsbad, CA, USA), were a gift from Dr. Michael Sanguinetti. Site-directed mutagenesis was performed with QuikChange II XL mutagenesis kit (Stratagene Agilent Technologies Co., La Jolla, CA, USA).

Mutated genes were functionally expressed in either Chinese hamster ovary (CHO) cells or human embryonic kidney (TSA201) cells as previously described. CHO cells were transfected with the cDNA encoding WT or p.V2014I CACNA1C subunits (3 μg) together with the cDNA encoding CACNB2b (12 μg), CACNA2D1 (5.1 μg), and the CD8 antigen (0.5 μg) using FuGENE 6 (Roche Diagnostics, Pittsburgh, PA, USA). The other two calcium variants were expressed in TSA201 cell line as previously described. C DNA of the three LTCC subunits were transfected in a 1:1:1 molar ratio using FuGENE 6 (Roche Diagnostics). In addition, CD8 cDNA was cotransfected as a reporter gene for the experiment involving p.D601E CACNB2b. Because p.E1829_Q1833dup CACNA1C was already tagged with YFP, no extra reporter gene was added. Before experimental use, cells were incubated with polystyrene microbeads precoated with anti-CD8 antibody (Dynabeads M450, Invitrogen Dynal, Carlsbad, CA, USA) for the experiment of p.V2014I CACNA1C and p.D601E CACNB2b. For protocols involving p.E1829_Q1833dup CACNA1C, cells were directly identified by epifluorescence.

CHO cells were perfused with an external solution containing the following (in mmol/L): NMDG 130, KCl 5, MgCl\(_2\) 1, and HEPES 10 (pH 7.35 with HCl). Recording pipettes were filled with internal solution containing the following (in mmol/L): CsCl 120, MgCl\(_2\) 2, MgATP 2, HEPES 10, CaCl\(_2\) 5, and EGTA 10 (pH 7.25 with CsOH). Voltage-clamp recordings on TSA201 cells were made with patch pipettes filled with a solution containing the following (in mmol/L): CsCl 110, CaCl\(_2\) 0.1, HEPES 10, EGTA 10, MgATP 2, and TEA 10 (pH 7.3 with CsOH). Extracellular solution contained the following (in mmol/L): glucose 10, CaCl\(_2\) 2, MgCl\(_2\) 1, HEPES 10, and TEA 150 (pH 7.35 with CsOH). Currents were filtered with a four-pole Bessel filter at 5 kHz and digitized at 50 kHz. Series resistance was electronically compensated at 70% to 80%.

**Electrophysiology**

Calcium currents were recorded in CHO or TSA201 cells using whole-cell, patch-clamp techniques at 21°C to 23°C with Axon-200B patch-clamp amplifiers and pCLAMP9 software (Axon Instruments, Chicago, IL, USA). Mean maximum current amplitude and cell capacitance were 0.5 ± 0.1 nA and 12.2 ± 1.5 pF, respectively (n = 16). Capacitance and series resistance were optimized, and ≈80% compensation was usually achieved, leading to uncompensated access resistances of 1.5 ± 0.7 MΩ.

Current–voltage relationships were constructed by applying 500-ms pulses from a holding potential of −70 mV to potentials ranging −50 and +70 mV. Voltage dependence of inactivation was determined using a two-step voltage-clamp protocol with a 500-ms conditioning pulse from −70 mV to potentials between −90 and +50 mV, followed by a test pulse to +20 mV. Inactivation curves were constructed by plotting the current amplitude elicited by the test pulse as a function of the voltage command of the conditioning pulse. Calcium channel conductance (G) was determined from the following relationship:

\[
G = \frac{I_{ip}}{(V_m - V_R)},
\]

where \(I_{ip}\) = peak current amplitude at \(V_m\), and \(V_R\) = reversal potential (−67.4 ± 1.1 mV, n = 16). A Boltzmann function was fitted to the conductance–voltage and inactivation curves, yielding the midpoint (\(V_b\)) and slope (k) value of the curves.

**Statistical analysis**

Results are expressed as mean ± SEM. Data were compared using analysis of variance followed by the Newman-Keuls test. \(P < .05\) was considered significant.

**Results**

**Clinical characteristics**

A total of 205 unrelated probands enrolled at the Masonic Medical Research Laboratory (MMRL) inherited cardiac arrhythmia registry over the past 5 years diagnosed with BrS, BrS/SQT, IVF, or ERS and their families were included in the study. Figure 1 shows representative 12-lead ECGs from BrS, ERS, and BrS/SQT phenotypes. The cohort consisted of 152 probands diagnosed with BrS, 10 with BrS/SQT, 19 with IVF, and 24 with ERS. Demographic characteristics are given in Table 1. Average age ranged between 30 ± 11 and 43 ± 16 years, and all four categories were male dominated (68%–90%).

**Identification of mutations**

Among all diagnostic groups, 25 probands were identified with one or more mutations in CACNA1C, CACNB2, or...
CACNA2D1 genes encoding the three subunits of the L-type Ca channel: 15 BrS, 5 BrS/SQT, 1 IVF, and 4 ERS. Clinical characteristics and demographics of the probands with mutations are summarized in Table 1. Mean age at time of diagnosis and gender among the probands identified with mutations were similar to those of the entire cohort. A majority of probands in all four diagnostic groups were symptomatic, and there was a high incidence of syncope, ventricular tachycardia/ventricular fibrillation, and family history of SCD in all groups. An early repolarization pattern was observed in one or more of the inferior or lateral leads of 26% of BrS probands. Corrected QT intervals were shorter than normal in the BrS/SQT, ERS, and IVF groups but were in the normal range in the BrS group (Table 1).

Of the 23 mutations uncovered, 21 were missense and 2 were deletion/duplication (Table 3). Four of the mutations (p.A39V, p.G490R, p.T11I, p.S481L) were previously reported by our group. Nine mutations were localized in the α subunit, 10 in the β subunit, and 4 in the α2δ subunit.

Four of the 9 mutations in CACNA1C were identified in BrS probands, 4 in BrS/SQT, and 1 in the ERS group (Table 3). Six of the 10 mutations in CACNB2 were identified in BrS, 1 each in BrS/SQT and IVF, and 2 in ERS probands (Table 3). The mutation p.S709N was found in two unrelated patients. Two mutations (p.D550Y and p.Q917H) were identified in the same individual. The mutation p.S143F was found in three BrS patients. Two mutations (p.L399F in exon 13 and p.K170N in alternative exon 7b) were genotyped in the same BrS proband (Table 3 and Figure 2H). Three of the 4 mutations identified in the CACNA2D1 gene were found in BrS patients and 1 in an ERS patient (Table 3). Six rare SNPs were identified in screened probands in two subunits of the calcium channel listed in Table 3. Four of the 6 (p.P817S, p.A1717G, p.T1787M, p.R1973Q) are novel, and two are present in NCBI’s dbSNP (p.G37R, rs34534613 in CACNA1C and p.R552G, rs61733968 in CACNB2b). Variation p.G37R has a reported heterozygous frequency of 0.028. The estimated frequencies of other identified rare polymorphisms varied from 0.5% to 1.6%.

Each mutation was tested for degree of conservation among multiple species (Rhesus monkey, dog, horse, mouse, rat, chicken; Table 3). Fourteen (61%) of the 23 mutations were in residues highly conserved among species, 6 (26%) were conserved among large mammals, and 3 (13%) were not conserved. In the case of rare polymorphisms, 2 of the 6 were highly conserved, 20 were conserved among large mammals, and 2 were not conserved.

Figure 3 shows the predicted topology of the three subunits of LTCC and the location of the mutations. Interestingly, 6 of the 9 mutations in the Ca_{1.2} α1 subunit were in either the N-terminus or the C-terminus, with no mutations detected in any of the transmembrane regions. Larger symbols with numbers denote the frequency of appearance the mutation among probands.

Pedigrees of the available families of probands with mutations are shown in Figure 2. Penetrance was complete in five families (A, C, D, G, I). Families B and F showed incomplete penetrance for BrS, which could be explained on the basis of female gender and young age. Family J with a diagnosis of ERS represents a rare case in which both the proband and his wife carried the same mutation (p.S160T in CACNB2), resulting in a homozygous appearance of the mutation in one son and heterozygous in the other. Both sons experienced ventricular tachycardia/ventricular fibrillation. Family H, with a diagnosis of BrS, presented with a double mutations in CACNB2 on the same allele inherited from the mother. The first child (female) died suddenly at age 16 months. The proband, a 9-year-old boy, was diagnosed at age 10 months with a ventricular conduction defect and BrS (procainamide challenge). The same genotype was found in his asymptomatic brother. Such diversity between siblings may be due to protective or deleterious effects of some additional genetic variation, which may be revealed with further genetic testing.
Loss-of-function mutations involving LTCC are known to predispose to a phenotype consisting of BrS with an abbreviated QTc. Yet the majority of BrS probands in this study presented with normal QTc intervals. It is noteworthy that a QT-prolonging variation could be identified in 12 (86%) of the 14 BrS cases (Table 3). The most common modulating variation involved the co-presence of a p.D601E polymorphism in CACNB2b that augments late I_{Ca} (Figures 5C and 5G). Another common variant modulating the manifestation of the QT interval is a common polymorphism in KCNH2, p.K897T. Although this SNP has been reported to exert a modifying effect on QTc, whether it confers risk or a protective effect remains controversial. Some studies have shown that it reduces I_{Kr} and aggravates LQTS,\textsuperscript{25,26} whereas others have shown it increases I_{Kr} and confers a protective effect.\textsuperscript{27,28} Other additional variations typically associated with LQTS include p.T10M-KCNE2, p.R1047L-KCNH2, p.D76N-KCNQ1, and p.G643S-KCNQ1.\textsuperscript{29–34} In contrast, these QT-prolonging variants are only present in 1 (20%) of the 5 BrS/SQT probands (Table 3).

The total yields of probands with mutations and rare polymorphisms in each of the diagnostic groups is listed in Table 2. A total of 12.3% of BrS and BrS/SQT probands displayed mutations in the \( \alpha 1 \) (5.5%), \( \beta 2 \) (4.9%),

Table 1  Demographic and clinical characteristics of screened probands and probands with mutations

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>BrS</th>
<th>BrS/SQT</th>
<th>IVF</th>
<th>ERS</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of probands</td>
<td>152</td>
<td>10</td>
<td>19</td>
<td>24</td>
</tr>
<tr>
<td>Age at diagnosis (years)</td>
<td>43 ± 16</td>
<td>41 ± 14</td>
<td>37 ± 11</td>
<td>30 ± 11</td>
</tr>
<tr>
<td>Gender (% male)</td>
<td>74%</td>
<td>90%</td>
<td>68%</td>
<td>81%</td>
</tr>
</tbody>
</table>

Clinical characteristics and demographics of probands with mutations

| No. of patients with mutations in all Ca, subunits | 15 | 5 | 1 | 4 |
| Age at diagnosis (years) | 34 ± 19 | 42 ± 15 | 50 | 40 ± 4 |
| Range (years) | (1–72) | (25–65) | — | (32-51) |
| Gender (% male) | 80% | 100% | 0 | 75% |
| Symptomatic patients (%) | 93% | 100% | 100% | 75% |
| Patients with syncope (%) | 46% | 40% | 100% | 75% |
| Ventricular tachycardia/ventricular fibrillation (%) | 60% | 60% | 100% | 75% |
| Family history of unexplained sudden death (%) | 42% | 60% | 100% | 100% |
| Type I ST-segment elevation at baseline or with sodium blockers (%) | 100% | 100% | 0 | 0 |
| Early repolarization pattern (%) | 26% | 0 | 0 | 100% |
| Average QTc (ms) | 432 ± 38 | 350 ± 15 | 376 | 375 ± 13 |

Age at time of diagnosis and average QTc values are given as mean ± SD.

BrS = Brugada syndrome; BrS/SQT = Brugada syndrome with shorter than normal QT; ERS = early repolarization syndrome; IVF = idiopathic ventricular fibrillation.

Loss-of-function mutations involving LTCC are known to predispose to a phenotype consisting of BrS with an abbreviated QTc. Yet the majority of BrS probands in this study presented with normal QTc intervals. It is noteworthy that a QT-prolonging variation could be identified in 12 (86%) of the 14 BrS cases (Table 3). The most common modulating variation involved the co-presence of a p.D601E polymorphism in CACNB2b that augments late I_{Ca} (Figures 5C and 5G). Another common variant modulating the manifestation of the QT interval is a common polymorphism in KCNH2, p.K897T. Although this SNP has been reported to exert a modifying effect on QTc, whether it confers risk or a protective effect remains controversial. Some studies have shown that it reduces I_{Kr} and aggravates LQTS,\textsuperscript{25,26} whereas others have shown it increases I_{Kr} and confers a protective effect.\textsuperscript{27,28} Other additional variations typically associated with LQTS include p.T10M-KCNE2, p.R1047L-KCNH2, p.D76N-KCNQ1, and p.G643S-KCNQ1.\textsuperscript{29–34} In contrast, these QT-prolonging variants are only present in 1 (20%) of the 5 BrS/SQT probands (Table 3).

The total yields of probands with mutations and rare polymorphisms in each of the diagnostic groups is listed in Table 2. A total of 12.3% of BrS and BrS/SQT probands displayed mutations in the \( \alpha 1 \) (5.5%), \( \beta 2 \) (4.9%),

Figure 2  Pedigree of the available families for CACNA1C and CACNB2 mutations. BrS = Brugada syndrome; BrS/SQT = Brugada syndrome with shorter than normal QT; ER = early repolarization pattern; ERS = early repolarization syndrome; IVF = idiopathic ventricular fibrillation. Arrows indicates proband. Numbers represent the Masonic Medical Research Laboratory ID number. Asterisk denotes previously published mutations.\textsuperscript{10,11}
and α2δ (1.8%) subunits of the LTCC; a total of 5.2% of IVF patients had mutations in the β2 subunit; and 16.0% of ERS patients had mutations in the α1 (4.1%), β2 (8.3%), and α2δ (4.1%) subunits. The total yield of probands with mutations and rare polymorphisms together was 17.9% for BrS and BrS/SQT, 21% for IVF, and 29.1% for the ERS group.

Functional expression studies
Expression studies probing the functional consequences of mutations in LTCC are limited. Previous studies have shown a loss of function of I, as the basis for BrS associated with mutations in CACNA1C and CACNB2b.10,11 As a further test of the hypothesis that loss-of-function mutations in LTCC underlie BrS as well as ERS and some forms of IVF, we are in the process of performing functional studies of the variants uncovered. We present two cases here.

The first case is a 41-year-old woman of Panamanian descent who presented with palpitations, incomplete right bundle branch block, and a history of presyncope (MMRL219). A diagnosis of BrS was confirmed following a positive procainamide challenge (Figure 4A). Family history was negative for SCD but positive for stroke and coronary disease. Genetic testing identified a heterozygous mutation in SCN5A. This proband with mutations and rare polymorphisms together was 17.9% for BrS and BrS/SQT, 21% for IVF, and 29.1% for the ERS group.

Interestingly, this proband also had a p.D601E polymorphism in CACNB2. To examine the functional effect of this variant, we expressed it in human embryonic kidney (TSA201) cells. Figures 5C and 5G show the effect of p.D601E in CACNB2 in significantly increasing late I, which is known to prolong QT. The modulatory effect of
this SNP likely accounts for the fact that QTC (449 ms) in this proband is not accompanied by SQT, as is the case with loss-of-function mutations involving LTCC.

The second case is a 33-year-old man who presented with presyncope incomplete right bundle branch block (MMRL300, Figure 6A). An ajmaline challenge performed was positive, confirming a diagnosis of BrS (data not shown). QTc interval was 346 ms. Genetic analysis showed duplication of five amino acids in exon 43 of CACNA1C p.E1829_Q1833dup (Table 3), with no other variations. Family members were not available for genetic screening.

To determine the functional consequences of the mutation, we expressed WT and p.E1829_Q1833dup CACNA1C in TSA201 cells. Figure 6C shows I_{Ca} traces recorded during application of 500-ms pulses from –90 mV to potentials ranging between –50 and +50 mV. The p.E1829_Q1833dup mutation reduced peak current density at potentials between –20 and +50 mV, resulting in nearly complete suppression of I_{Ca} (n = 8 in each group, P < .01; Figure 6D).

**Discussion**

This study is the first comprehensive attempt to associate inherited cardiac arrhythmia syndromes with genetic variations in the cardiac LTCC. We identified 23 mutations in three genes encoding the three subunits of the LTCC in 25 unrelated probands and six rare polymorphisms in 17 additional probands diagnosed with BrS, BrS/SQT, IVF, or ERS. A total of 12.3%, 5.2%, and 16% of BrS/BrS+SQT, IVF, and ERS probands displayed mutations in α1, β2, and α2δ subunits of LTCC, respectively. The total yield of probands with mutations and rare polymorphisms is 17.9% for BrS and BrS/SQT, 21% for IVF, and 29.1% for ERS diagnostic groups (Table 2). The yield of probands with LTCC mutations associated with BrS (12.3%) is second only to SCN5A mutations, which have been reported to range between 11% and 28% at different international centers.5 In the case of ERS, CACNA1C, CACNB2, and CACNA2D1 represent the second, third, and fourth genes proposed to underlie this phenotype, the first one being KCNJ8.6

Topologically, it is interesting that no mutations were detected in any of the transmembrane regions of Ca_{1.2} (Figure 3). Six of the nine mutations were located in the N- or C-terminus of the α1 subunit. Relevant to this finding is the demonstration by Soldatov’s group of voltage-gated mobility of the C- and N-cytoplasmic tails of Ca_{1.2} and their important regulatory role in voltage- and Ca^{2+}-dependent inactivation.35,36 In addition, cleavage of the C-terminus of native Ca_{1.2} channels has been shown to result in a proteolytic fragment that is able to act as a represor of Ca_{1.2} promoter activity.37,38 Thus, mutations in the C-terminus could have significant effects on the regulation of expression level and function of the Ca_{1.2} channel. Another mutation of great interest is p.E1115K because it is proposed to underlie this phenotype, the first one being KCNJ8.6
uncovered likely are disease-causing. Twelve of the 14 probands with rare polymorphisms had variations in residues that were either highly conserved or conserved among large mammals (Table 3). Moreover, excellent genotype–phenotype correlation was seen among available families, with pathogenic phenotypes co-segregating with a positive genotype (Figure 2). Failure to do so in all but one case could be attributed to female gender and/or young age, both of which are known to diminish expression of the disease phenotype.

In previous studies we demonstrated a loss of function of \( I_{\text{Ca}} \) for four of these mutations (marked with an asterisk in Figures 2 and Figure 3 and Table 3).\(^{10,11}\) In the present study, we demonstrated a loss of function of \( I_{\text{Ca}} \) in BrS and BrS/SQT probands carrying a p.V2014I or p.E1829_Q1833dup mutation in \( \text{CACNA1C} \). The BrS proband, unlike the BrS/SQT proband, was also found to carry a rare polymorphism, p.D601E, in \( \text{CACNB2b} \), which when expressed was found to augment late \( I_{\text{Ca}} \), thereby explaining the absence of an abbreviated QTc. QT-prolonging variants (p.D601E-\( \text{CACNB2b} \), p.K897T-\( \text{KNCH2} \), p.T10M-\( \text{KCNE2} \), p.R1047L-\( \text{KCNH2} \), p.D76N-\( \text{KCNE1} \), p.G643S-\( \text{KCNQ1} \)) were found in 12 of the 14 BrS probands presenting with a normal QTc (Table 3).\(^{25,26,33}\)

Our study results suggest that mutations in all three subunits of the LTCCs are detected in a relatively high percentage of probands with inherited cardiac arrhythmia syndromes, including BrS, ERS, and some forms of IVF. These findings suggest that genetic screening of \( \text{Ca} \) genes may prove to be a valuable diagnostic tool for identifying individuals who might be at risk. \( \text{CACNA1C} \), \( \text{CACNB2} \), and \( \text{CACNA2D1} \) should be included in the genotyping of patients who have diseases with a high occurrence of sudden death, particularly in cases where J-wave syndromes are suspected.\(^3\)

### Study limitations

The LTCC subunit genes, especially \( \text{CACNB} \), have multiple isoforms. Our focus on \( \text{CACNB2} \) in this study may have resulted in an underestimation of linkage of LTCC mutations to inherited cardiac arrhythmia disease. Thus far, a total of seven genes have been identified as associated with BrS.\(^{30}\) Our findings of three BrS probands associated with mutations in highly conserved residues of \( \text{CACNA2D1} \) suggest that it may be a new gene for BrS. In support of this hypothesis, our preliminary functional expression studies indicate that the double mutation in \( \text{CACNA2D1} \) [p.D550Y and p.Q917H (MMRL194)] reduces \( I_{\text{Ca}} \) to 25% of normal (Barajas et al, unpublished observation).

Mutations in only one gene, \( \text{KCNJ8} \), have thus far been associated with ERS.\(^6,7\) The present study identifies four probands in whom mutations in highly conserved residues...
### Table 3
Summary of L-type calcium channel mutations and rare polymorphisms in CACNA1C, CACNB2, and CACNA2D1

<table>
<thead>
<tr>
<th>No.</th>
<th>Amino acid change</th>
<th>Nucleotide change</th>
<th>Mutation type</th>
<th>Conserv.</th>
<th>Exon</th>
<th>Location</th>
<th>Probands (n)</th>
<th>Diagnosis</th>
<th>Additional variations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mutations in CACNA1C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>p.E1829_Q1833dup</td>
<td>c.5485_5499 dup15</td>
<td>Duplication</td>
<td>NC</td>
<td>43</td>
<td>C-terminus</td>
<td>300</td>
<td>BrS</td>
<td>SCN5A p.S1103Y-SNP</td>
</tr>
<tr>
<td>9</td>
<td>p.E850 del</td>
<td>c.2548-550del GAG</td>
<td>Deletion</td>
<td>HC</td>
<td>19</td>
<td>DIII/DIII</td>
<td>445</td>
<td>ERS</td>
<td></td>
</tr>
<tr>
<td>Mutations in CACNB2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>p.T11I*</td>
<td>c.32 C&gt;T</td>
<td>Missense</td>
<td>CM</td>
<td>2</td>
<td>N-terminus</td>
<td>284</td>
<td>BrS</td>
<td>CACNB2 p.D601E-SNP</td>
</tr>
<tr>
<td>Mutations in CACNA2D1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>No.</th>
<th>Gene</th>
<th>Amino acid change</th>
<th>Nucleotide change</th>
<th>Mutation type</th>
<th>Conserv.</th>
<th>Exon</th>
<th>Location</th>
<th>Probands (n)</th>
<th>Diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rare SNP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>CACNA1C</td>
<td>p.G37R</td>
<td>c.109 G&gt;A</td>
<td>Missense</td>
<td>HC</td>
<td>2</td>
<td>N-terminus</td>
<td>2</td>
<td>BrS</td>
</tr>
<tr>
<td>2</td>
<td>CACNA1C</td>
<td>p.P817S</td>
<td>c.2449 C&gt;T</td>
<td>Missense</td>
<td>NC</td>
<td>17</td>
<td>DII/DIII</td>
<td>2</td>
<td>BrS</td>
</tr>
<tr>
<td>3</td>
<td>CACNA1C</td>
<td>p.A1717G</td>
<td>c.5150 C&gt;G</td>
<td>Missense</td>
<td>CM</td>
<td>42</td>
<td>C-terminus</td>
<td>2</td>
<td>BrS</td>
</tr>
<tr>
<td>4</td>
<td>CACNA1C</td>
<td>p.A1717G</td>
<td>c.5150 C&gt;G</td>
<td>Missense</td>
<td>CM</td>
<td>42</td>
<td>C-terminus</td>
<td>2</td>
<td>BrS</td>
</tr>
<tr>
<td>5</td>
<td>CACNA1C</td>
<td>p.R1973Q</td>
<td>c.5918 G&gt;A</td>
<td>Missense</td>
<td>HC</td>
<td>46</td>
<td>C-terminus</td>
<td>2</td>
<td>BrS</td>
</tr>
<tr>
<td>6</td>
<td>CACNB2</td>
<td>p.R552G</td>
<td>c.1654 C&gt;G</td>
<td>Missense</td>
<td>CM</td>
<td>14</td>
<td>C-terminus</td>
<td>2</td>
<td>BrS</td>
</tr>
</tbody>
</table>

Conserv. = degree of conservation for the mutated site among multiple species: CM = conserved among large mammals; HC = highly conserved; NC = not conserved. BrS = Brugada syndrome; BrS/SQT = Brugada syndrome with shorter than normal QT; ERS = early repolarization syndrome; IVF = idiopathic ventricular fibrillation; MMRL ID no. = three-digit Masonic Medical Research Laboratory identification number; SNP = single nucleotide polymorphism.

*Previously published mutations.\(^{10,11}\)

Heart Rhythm, Vol 7, No 12, December 2010
**Figure 5** The p.V2014I-CACNA1C mutation causing a loss of function of $I_{Ca}$ together with a p.D601E-CACNB2b single nucleotide polymorphism causing a gain of function of late $I_{Ca}$ result in Brugada syndrome (BrS) with normal QTc (MMRL219). A: Representative calcium current traces recorded in Chinese hamster ovary (CHO) cells transfected with wild-type (WT; left) or p.V2014I (right) CACNA1C subunits in response to the voltage clamp protocol shown at the top. B: $I_{Ca}$ recorded in response to the inactivation protocol shown. C: Overlapping calcium traces recorded from human embryonic kidney (TSA201) cells expressing WT and p.D601E-CACNB2b rare polymorphism. D: Current–voltage relationship. E: Activation curve showing conductance–voltage. F: Normalized inactivation curves in WT or p.V2014I CACNA1C. G: Bar graph showing $I_{Ca}$ current density recorded with WT versus p.D601E CACNB2b at different times (100, 200, and 300 ms) into the depolarized testing pulse at 0 mV (protocol inset). *$P < .05$, **$P < .01$ vs WT data. Each datapoint/bar represents mean ± SEM of 6–8 experiments.

**Figure 6** A: Duplication of five amino acids in Cav1.2 leads to loss of function of $I_{Ca}$ resulting in Brugada syndrome (BrS) with shorter than normal QTc interval (QTc = 346 ms). ECG recorded in leads V1–V3 of patient #300 at baseline. B: Electropherogram of wild-type (WT) and mutant CACNA1C showing duplication of five amino acids EETSQ. C: Representative calcium current traces recorded in human embryonic kidney (TSA201) cells transfected with WT (left) and p.E1829_Q1833-dup mutant (right) CACNA1C subunits by applying the protocol shown at the top. D: Current–voltage relationship (I–V curve) p.E1829_Q1833-dup mutant effect in Cav1.2 channels. Data are given as mean ± SEM of at least eight cells. *$P < .05$. 
of CACNA1C, CACNB2, and CACNA2D1 are associated with ERS, suggesting linkage of these genes with ERS. Although many of the mutations in these genes occur in highly conserved residues and genotype-phenotype correlation among male members of available families is excellent, confirmation of these hypotheses must await the availability of functional expression studies. The requirement for such studies is underscored by the study of Kapa et al suggesting that, in the case of LQTS, mutations of highly conserved residues may not always be disease-causing. It is possible that the same may be true in the case of calcium channel mutations associated with BrS and ERS.

We present functional expression data for two of the phenotypes evaluated. Although functional studies for the other mutations are in process, the data likely will not be available for many months, and it would be unreasonable to delay reporting of these results until that time.

Although in most cases of IVF we made a diligent effort to exclude the diagnosis of known channelopathies, we recognize that these tests are not always definitive and that patients whom we categorize as IVF may properly belong to another category.

Acknowledgments
We thank Judy Hefferon for creative work on the figures, Susan Bartkowiak for maintaining the genetics database, and Gabriel Caceres for DNA isolation. We also thank Drs. Nikolai Soldatov and Michael C. Sanguinetti for expression constructs.

References