Identification and Functional Characterization of a Novel CACNA1C-Mediated Cardiac Disorder Characterized by Prolonged QT Intervals With Hypertrophic Cardiomyopathy, Congenital Heart Defects, and Sudden Cardiac Death

Nicole J. Boczek, PhD; Dan Ye, MD; Fang Jin, MD; David J. Tester, BS; April Huseby, BS; J. Martijn Bos, MD, PhD; Aaron J. Johnson, PhD; Ronald Kanter, MD; Michael J. Ackerman, MD, PhD

Background—A portion of sudden cardiac deaths can be attributed to structural heart diseases, such as hypertrophic cardiomyopathy (HCM) or cardiac channelopathies such as long-QT syndrome (LQTS); however, the underlying molecular mechanisms are distinct. Here, we identify a novel CACNA1C missense mutation with mixed loss-of-function/gain-of-function responsible for a complex phenotype of LQTS, HCM, sudden cardiac death, and congenital heart defects.

Methods and Results—Whole exome sequencing in combination with Ingenuity variant analysis was completed on 3 affected individuals and 1 unaffected individual from a large pedigree with concomitant LQTS, HCM, and congenital heart defects and identified a novel CACNA1C mutation, p.Arg518Cys, as the most likely candidate mutation. Mutational analysis of exon 12 of CACNA1C was completed on 5 additional patients with a similar phenotype of LQTS plus a personal or family history of HCM-like phenotypes and identified 2 additional pedigrees with mutations at the same position, p.Arg518Cys/His. Whole cell patch clamp technique was used to assess the electrophysiological effects of the identified mutations in Ca$_{\alpha_{1.2}}$ and revealed a complex phenotype, including loss of current density and inactivation in combination with increased window and late current.

Conclusions—Through whole exome sequencing and expanded cohort screening, we identified a novel genetic substrate p.Arg518Cys/His-CACNA1C, in patients with a complex phenotype including LQTS, HCM, and congenital heart defects annotated as cardiac-only Timothy syndrome. Our electrophysiological studies, identification of mutations at the same amino acid position in multiple pedigrees, and cosegregation with disease in these pedigrees provide evidence that p.Arg518Cys/His is the pathogenic substrate for the observed phenotype. (Circ Arrhythm Electrophysiol. 2015;8:1122-1132. DOI: 10.1161/CIRCEP.115.002745.)

Key Words: calcium channels, L-type ■ cardiomyopathy, hypertrophic ■ death, sudden, cardiac ■ genetics ■ long QT syndrome ■ Timothy syndrome

Received January 16, 2015; accepted July 23, 2015.
From the Department of Molecular Pharmacology and Experimental Therapeutics, Windland Smith Rice Sudden Death Genomics Laboratory (N.J.B., D.Y., D.J.T., J.M.B., M.J.A.), Division of Immunology and Neurology (F.J., A.H., A.J.J.), and Departments of Medicine (Division of Cardiovascular Diseases) and Pediatrics (Division of Pediatric Cardiology) (M.J.A.), Mayo Clinic, Rochester, MN; Division of Cardiology, Nicklaus Children's Hospital, Miami, FL (R.K.).

Correspondence to Michael J. Ackerman, MD, PhD, Windland Smith Rice Sudden Death Genomics Laboratory, Mayo Clinic, Guggenheim 501, Rochester, MN 55905. E-mail ackerman.michael@mayo.edu

Circ Arrhythm Electrophysiol is available at http://circep.ahajournals.org DOI: 10.1161/CIRCEP.115.002745

1122
Boczek et al  Novel CACNA1C Mutation in New Disorder COTS

WHAT IS KNOWN

- LQTS is a disorder of delayed ventricular myocardial repolarization that often manifests as prolonged QT intervals on a resting ECG in the setting of a structurally normal heart, and to date, 17 LQTS-susceptibility genes, encoding ion channel or ion channel interacting proteins, have been described.
- Hypertrophic cardiomyopathy (HCM) is defined by cardiac hypertrophy and to date, 25 HCM-susceptibility genes, primarily encoding for structural proteins, have been described; and although some cases of HCM have concurrent QT prolongation, a genetic etiology has never been identified for patients presenting with LQTS and HCM-like phenotypes.

WHAT THE STUDY ADDS

- This study identifies a novel disorder, cardiac-only Timothy syndrome (COTS), characterized by QT prolongation, hypertrophic cardiomyopathy, congenital heart defects, and sudden cardiac death.
- Using whole exome sequencing and expanded cohort screening, novel genetic substrates p.Arg518Cys/His, within the CACNA1C-encoded L-type calcium channel, were attributed to the COTS phenotype.
- The p.Arg518Cys/His variants were characterized functionally and found to lead to a complex electrophysiological phenotype including loss of current density, increased window and late current, and decelerating voltage-dependent inactivation.

Methods

Study Subjects

An 11-member (5 affected) multigenerational pedigree presented with a multitude of cardiovascular signs and symptoms, including marked QT prolongation, HCM, SCD, and CHDs. However, none of the family members had syndactyly, cognitive impairments, facial dysmorphisms, or any other noncardiac clinical characteristics suggestive of Timothy syndrome (TS). The proband, who was genotype negative by commercially available LQTS genetic testing, was referred to the Mayo Clinic Windland Smith Rice Sudden Death Genomics Laboratory for further genetic testing. After written consent was obtained for this institutional review board–approved study, peripheral blood lymphocytes were obtained from 6 family members. Genomic DNA was obtained using the Puregene DNA Isolation Kit (Qiagen Inc, Valencia, CA). The symptomatic index case, unaffected mother, affected sister, and affected nephew were selected for WES (Figure 1A, cases III.1, II.2, III.4, and IV.1).

Whole Exome Sequencing

Expanded Methods on the whole exome sequencing and subsequent bioinformatic analysis are available in the Data Supplement.

CACNA1C Mutational Analysis

Next, we examined our cohort of 37 unrelated patients with clinically robust but genetically elusive LQTS for coexisting echocardiographic evidence of HCM or family history of HCM-like phenotypes; we found 5 cases with this phenotype (Table 1). These cases were mutation negative after LQTS mutational analysis (by denaturing high-performance liquid chromatography and DNA sequencing) of the 3 major LQTS genes, KCNQ1, KCNH2, and SCN5A, and 8 minor LQTS genes, AKAP9, ANKB, CAV3, KCNE1, KCNE2, KCNJ2, SCN4B, and SNTA1.

For these 5 unrelated cases with LQTS and concomitant personal or familial HCM, genetic analysis was performed on exon 12 of CACNA1C (NM_000719) using polymerase chain reaction and DNA sequencing (ABI Prism 377; Applied Biosystems Inc, Foster City, CA). Primer sequences and polymerase chain reaction conditions are available on request.

WHAT IS KNOWN

- LQTS is a disorder of delayed ventricular myocardial repolarization that often manifests as prolonged QT intervals on a resting ECG in the setting of a structurally normal heart, and to date, 17 LQTS-susceptibility genes, encoding ion channel or ion channel interacting proteins, have been discovered, a significant number of patients expressing these potentially lethal phenotypes remains genetically elusive.
- Hypertrophic cardiomyopathy (HCM) is defined by cardiac hypertrophy and to date, 25 HCM-susceptibility genes, primarily encoding for structural proteins, have been described; and although some cases of HCM have concurrent QT prolongation, a genetic etiology has never been identified for patients presenting with LQTS and HCM-like phenotypes.

WHAT THE STUDY ADDS

- This study identifies a novel disorder, cardiac-only Timothy syndrome (COTS), characterized by QT prolongation, hypertrophic cardiomyopathy, congenital heart defects, and sudden cardiac death.
- Using whole exome sequencing and expanded cohort screening, novel genetic substrates p.Arg518Cys/His, within the CACNA1C-encoded L-type calcium channel, were attributed to the COTS phenotype.
- The p.Arg518Cys/His variants were characterized functionally and found to lead to a complex electrophysiological phenotype including loss of current density, increased window and late current, and decelerating voltage-dependent inactivation.
**Table 1. Patients With LQTS With Personal or Family History of HCM-Like Phenotype**

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Sex</th>
<th>Age at Dx, y</th>
<th>QTc, ms</th>
<th>Symptomatic</th>
<th>HCM-Like Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>1*</td>
<td>M</td>
<td>10</td>
<td>480</td>
<td>No</td>
<td>Father with HCM</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>13</td>
<td>493</td>
<td>No</td>
<td>SCD in 2 cousins with reported increased left ventricular wall thickness on autopsy</td>
</tr>
<tr>
<td>3*</td>
<td>F</td>
<td>25</td>
<td>558</td>
<td>Yes: torsades de pointes during surgery</td>
<td>Sister, aunt, and cousin with HCM</td>
</tr>
<tr>
<td>4</td>
<td>F</td>
<td>28</td>
<td>513</td>
<td>Yes: syncope</td>
<td>Cardiomyopathy with septal wall motion abnormalities</td>
</tr>
<tr>
<td>5</td>
<td>F</td>
<td>46</td>
<td>493</td>
<td>Yes: cardiac arrest</td>
<td>Postpartum cardiomyopathy</td>
</tr>
</tbody>
</table>

Dx indicates diagnosis; F, female; HCM, hypertrophic cardiomyopathy; LQTS, long-QT syndrome; M, male; and SCD, sudden cardiac death.

*p.Arg518Cys/His mutation positive.

We expanded the pedigrees to include additional blood samples from family members of 2 of 5 of these patients to examine exon 12 of CACNA1C. All patients and family members signed written consent for this institutional review board–approved study.

**CACNA1C Mammalian Expression Vectors and Site-Directed Mutagenesis**

The human wild-type (WT) CACNA1C cDNA with an N-terminal enhanced yellow fluorescence protein (EYFP) tag ([EYFP] N-terminal) in the pcDNA vector and the cDNA of the CACNA2D1 gene cloned in pcDNA3.1 vector, and the CACNB2 cDNA were gifts from Dr. Charles Antzelevitch, Masonic Medical Research Laboratory, Utica, NY. The cDNA of CACNB2 was subcloned into the bicistronic pIRE2-dsRED2 vector (Clontech, Mountain–Whitney, CA). The p.Arg518Cys-CACNA1C and p.Arg518His-CACNA1C missense mutations were engineered into pcDNA3-CACNA1C-WT-EYFP vector using primers containing the missense mutations (available on request) in combination with the Quikchange II XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). The integrity of all constructs was verified by DNA sequencing.

**HEK293 Cell Culture and Transfection**

Expanded Methods on the HEK293 cell culture and transfection, as well as subsequent electrophysiological measurements, data analysis, and confirmatory examination of CACNA1C, are available in the Data Supplement.

**Statistical Analysis**

All data points are shown as the mean value, and error bars represent the standard error of the mean (SEM). Statistics were completed using SigmaPlot 12.0 (San Jose, CA) and GraphPad Prism (La Jolla, CA). Normality was tested by Shapiro–Wilk, and constant variance was assessed for each comparison, and if normality failed, nonparametric methods were used. Student t test or Mann–Whitney rank sum test was performed to determine statistical significance between two groups, and a P<0.05 was considered significant. One-way ANOVA or Kruskal–Wallis in combination with Dunn method for multiple comparisons was performed to determine statistical significance among our variants versus controls. After correction for multiple comparisons, a P<0.05 was considered to be significant.

**Results**

**Multigenerational Pedigree With LQTS, HCM, SCD, and CHDs**

The index case is a 33-year-old woman (Figure 1A, III.1; Table 2, III.1) who presented at 25 years of age with QT prolongation during pregnancy. Subsequent medical history revealed a ventricular septal defect and a family history of SCD. Her ECG exhibited a QTc of 500 ms, first-degree AV block, and her medical therapy included the placement of an implantable cardioverter defibrillator. She later experienced peripartum cardiomyopathy after giving birth to triplets. Several years later, she had an abnormal echocardiogram with ventricular septal hypertrophy with a maximum septal wall thickness of 15 mm.

After reviewing her family history (Table 2), it was identified that her father (II.1) died at the age of 36 years because of a cardiac arrhythmia secondary to HCM with cardiac hypertrophy and fibrosis noted at autopsy. Her brother (III.2) died at the age of 24 years, when first responders found him in ventricular fibrillation and were unable to resuscitate him. His autopsy also revealed underlying HCM with cardiomegaly and interstitial fibrosis. An ECG performed 2 years before his death revealed QT prolongation with a QTc of 490 ms. Her deceased brother’s son (IV.1) has marked QT prolongation (QTc, 522 ms) and has been treated with an implantable cardioverter defibrillator. Her living brother (III.3) has normal QTc values. Her sister’s (III.4) ECGs showed QT prolongation with a QTc of 491 ms.

Overall, the family history is unique and atypical, with overt QT prolongation presenting in some cases and cardiomyopathies including HCM, with or without CHDs, in an autosomal dominant inheritance pattern. However, none of the patients have extracardiac phenotypes, such as those observed in TS. Taken altogether, the phenotypic sequelae is representative of an autosomal dominant condition, with features consistent with cardiac-only TS (COTS).

**WES for Novel Pathogenic Substrate Identification in a Large Multigenerational Pedigree**

Using WES, we genetically interrogated our index case (III.1), her affected sister (III.4), affected nephew (IV.1), and unaffected mother (II.2). Overall, 146,020 variants were identified (Figure 1B). Using Ingenuity variant analysis, we were able to filter our total number of plausible candidate variants to 29 (Figure 1B). Using genes that have been associated previously with LQTS, cardiomyopathy, SCD, and diseases consistent with these phenotypes, we identified 1 novel, ultrarare variant, c.1552C>T in exon 12 of the CACNA1C-encoded Ca,1.2 L-type calcium channel (LTCC), which leads to an arginine to cysteine change at amino acid position 518 (p.Arg518Cys) in the I–II cytoplasmic linker of Ca,1.2 (Figure 2).

**Follow-Up Cohort Analysis**

On the basis of the unique and novel phenotype identified within this pedigree, we examined our cohort of 37 unrelated
patients with clinically robust but genetically elusive LQTS for cases with a personal or family history of phenotypes suggestive of HCM, and a total of 5 patients met those criteria (Table 1). Using polymerase chain reaction amplification and Sanger-based DNA sequencing, we interrogated exon 12 of \textit{CACNA1C}. Interestingly, 2 of 5 (40\%) had a variant in exon 12, both at the 518th amino acid, first encoding for arginine to cysteine (p.Arg518Cys), like our original pedigree, and the second encoding for arginine to histidine (p.Arg518His).

The index case (II.1) in pedigree 2 was diagnosed with LQTS with a QTc of 480 ms after a screening ECG was performed because of his father's (I.2) diagnosis of HCM. (Table 2, pedigree 2; Figure 3, pedigree 2). The index case (III.1) in pedigree 3 was diagnosed with LQTS, cleft mitral valve, ASD, torsades de pointes during surgery, and premature atrial complexes (Table 2, pedigree 3; Figure 3, pedigree 3). Genetic interrogation using polymerase chain reaction and Sanger-based DNA sequencing of exon 12 of \textit{CACNA1C} in family members in all 3 pedigrees revealed that p.Arg518Cys/His cosegregated with the abnormal cardiac phenotypes (Figure 3).

**Table 2. Summary of Pedigrees 1, 2, and 3**

<table>
<thead>
<tr>
<th>Pedigree</th>
<th>Patient</th>
<th>Symptoms</th>
<th>QTc, ms</th>
<th>R518 Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>I.1</td>
<td>Died unexpectedly at the age of 67 y: heart attack</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>1</td>
<td>I.2</td>
<td>Died unexpectedly at the age of 66 y: heart attack</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>1</td>
<td>I.3</td>
<td>Died at the age of 52 y during heart surgery</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>1</td>
<td>II.1</td>
<td>SCD at 36 y caused by cardiac arrhythmia secondary to primary cardiomyopathy and hypertrophy</td>
<td>NA</td>
<td>Obligate positive</td>
</tr>
<tr>
<td>1*</td>
<td>II.2</td>
<td>Asymptomatic</td>
<td>NA</td>
<td>Negative</td>
</tr>
<tr>
<td>1*†</td>
<td>III.1</td>
<td>LQTS, VSD, peripartum cardiomyopathy, HCM, first-degree AV block</td>
<td>500</td>
<td>Positive</td>
</tr>
<tr>
<td>1</td>
<td>III.2</td>
<td>SCD at 24 y, prolonged QT, autopsy-revealed HCM-like phenotype of cardiomegaly and interstitial fibrosis</td>
<td>490</td>
<td>Obligate positive</td>
</tr>
<tr>
<td>1</td>
<td>III.3</td>
<td>Asymptomatic</td>
<td>405</td>
<td>Negative</td>
</tr>
<tr>
<td>1*</td>
<td>III.4</td>
<td>LQTS</td>
<td>491</td>
<td>Positive</td>
</tr>
<tr>
<td>1*</td>
<td>IV.1</td>
<td>LQTS: syncope</td>
<td>522</td>
<td>Positive</td>
</tr>
<tr>
<td>1</td>
<td>IV.2</td>
<td>Asymptomatic</td>
<td>NA</td>
<td>Negative</td>
</tr>
<tr>
<td>2</td>
<td>I.1</td>
<td>Asymptomatic</td>
<td>NA</td>
<td>Negative</td>
</tr>
<tr>
<td>2</td>
<td>I.2</td>
<td>HCM</td>
<td>NA</td>
<td>Positive</td>
</tr>
<tr>
<td>2</td>
<td>I.3</td>
<td>Died at the age of 23 y during single motor vehicle accident</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>2†</td>
<td>II.1</td>
<td>LQTS, sinoatrial node dysfunction</td>
<td>480</td>
<td>Positive</td>
</tr>
<tr>
<td>2</td>
<td>II.2</td>
<td>Asymptomatic</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>3</td>
<td>I.1</td>
<td>SCD at the age of 64 y while in the hospital</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>3</td>
<td>I.2</td>
<td>Asymptomatic</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>3</td>
<td>II.1</td>
<td>Septal hypertrophy and QT prolongation</td>
<td>NA</td>
<td>Obligate positive</td>
</tr>
<tr>
<td>3</td>
<td>II.2</td>
<td>HCM</td>
<td>NA</td>
<td>Obligate positive</td>
</tr>
<tr>
<td>3†</td>
<td>III.1</td>
<td>LQTS, cleft mitral valve, ASD, torsades de pointes during surgery, and premature atrial complexes</td>
<td>500</td>
<td>Positive</td>
</tr>
<tr>
<td>3</td>
<td>III.2</td>
<td>SCD at the age of 30 y, previous history of HCM and QT prolongation</td>
<td>NA</td>
<td>Positive</td>
</tr>
<tr>
<td>3</td>
<td>III.3</td>
<td>HCM, subaortic stenosis, and first-degree AV block</td>
<td>444</td>
<td>Positive</td>
</tr>
</tbody>
</table>

ASD indicates atrial septal defect; HCM, hypertrophic cardiomyopathy; LQTS, long-QT syndrome; NA, not available; SCD, sudden cardiac death; and VSD, ventricular septal defect.

*Whole exome sequenced.
†Index cases.

had QT prolongation which at the time was attributed to her HCM. She died suddenly at 30 years of age, and her autopsy reported symmetrical hypertrophy and fibrosis; cause of death was likely because of arrhythmia. Her mother (II.1) has an enlarged septum and QT prolongation, and she has a pacemaker. Her maternal aunt (II.2) and maternal cousin (III.3) have been diagnosed with HCM. Family member III.3 had subaortic stenosis, had a left ventricular septal myectomy, has a history of supraventricular tachycardia and first-degree AV block, and has been treated with verapamil. Her maternal grandfather (I.1) died suddenly in the hospital at the age of 64 years (Table 2, pedigree 3; Figure 3, pedigree 3). Genetic interrogation using polymerase chain reaction and Sanger-based DNA sequencing of exon 12 of \textit{CACNA1C} in family members in all 3 pedigrees revealed that p.Arg518Cys/His cosegregated with the abnormal cardiac phenotypes (Figure 3).

**In Vitro Functional Analysis of p.Arg518Cys/His-CACNA1C**

To better understand the effect that p.Arg518Cys/His has on the Ca\textsubscript{1.2} channel, we used whole cell patch clamp...
technique to determine whether there are electrophysiological differences between these mutants and WT-CaV1.2 channels in HEK293 cells. Typical $I_{Ca}$ tracings of voltage-dependent activation from WT and p.Arg518Cys/His are shown in Figure 4A. Analysis of the current–voltage relationship shows that p.Arg518Cys/His mutants dramatically decrease $I_{Ca}$ current density from −10 to +70 mV ($P<0.05$ versus WT; Figure 4B). The peak current density was reduced by 55.6% and 63.2%, respectively, from −69.3±7.0 pA/pF (WT, $n=15$) to −30.8±5.2 pA/pF (p.Arg518Cys, $n=14$; $P<0.0001$) and to −25.5±5.8 pA/pF (p.Arg518His, $n=15$; $P<0.0001$; Figure 4C).

Because of the reduction of current density observed with the p.Arg518Cys/His variants, we used the EYFP-tag on CACNA1C to examine the localization of mutant and WT-CaV1.2 proteins. Visual comparison highlights a centralized punctate pattern of fluorescence with p.Arg518Cys, which is not observed in WT cells (Figure 4D). Using the confocal microscopy images, we compared the peripheral (membrane):central (cytosol) ratio between WT and p.Arg518Cys. We found that WT had a ratio of 0.97±0.05 ($n=10$), whereas p.Arg518Cys had a ratio of 0.81±0.04 ($n=10$; $P=0.02$; Figure 4E), suggesting that less mutant channels are localized to the membrane and that p.Arg518Cys may affect trafficking.

Typical tracings of steady-state inactivation for WT and p.Arg518Cys/His are shown in Figure 5A. Steady-state inactivation was assessed by a standard 2-pulse voltage-clamp

Figure 2. Topology of CACNA1C. Shown is the topology of CACNA1C-encoded CaV1.2 in the membrane. The location of the p.Arg518Cys/His mutations, associated with cardiac-only Timothy syndrome (COTS), as identified in the pedigrees, are highlighted in red circles. In addition, other published CACNA1C mutations in Brugada syndrome (BrS)/Brugada syndrome+short-QT syndrome (BrS-SQTS; black diamonds),9,10 long-QT syndrome (LQTS; black circles),11–15 and Timothy syndrome (TS; white circles),13,14,16–19 are highlighted. Shown in green is the α-interaction domain (AID; amino acids 428–445), the domain in which the β-subunit is known to bind.

*Variants represent those that have been functionally characterized.

Figure 3. Pedigrees harboring p.Arg518Cys/His. The original whole exome sequencing (WES) pedigree (pedigree 1), in addition to the pedigrees identified through the cohort analysis (pedigrees 2 and 3), shows the phenotypic status of each patient (key on right). In addition, each individual with a red circle is p.Arg518Cys/His positive, dotted red circles represent obligate positive p.Arg518Cys/His individuals, those who have been tested and are negative for p.Arg518Cys/His are demarcated with neg. LQTS indicates long-QT syndrome; NA, samples that were not available.
protocol (Figure 5A, inset). A plot of the inactivation curves shows that p.Arg518Cys and p.Arg518His shifted $V_{1/2}$ of inactivation to more depolarized potentials by +6.8 and +7.0 mV, respectively, from −20.9±0.4 mV (WT, n=15) to −14.1±0.5 mV (p.Arg518Cys, n=14; $P<0.001$) and to −13.9±0.7 mV (p.Arg518His, n=15; $P<0.001$). The respective $k$ (slope factor) showed a significant difference between the mutants and the WT from 5.6±0.2 (WT, n=15) to 8.2±0.4 (p.Arg518Cys, n=14; $P<0.001$) and to 8.6±0.6 (p.Arg518His, n=15; $P<0.001$). A plot of the activation curves shows that the p.Arg518His mutation shifted $V_{1/2}$ of activation to a more depolarized potential by +4.5 mV from +13.4±1.1 mV (WT, n=15) to +17.9±0.8 mV (p.Arg518His, n=15; $P=0.004$; Figure 5B). However, p.Arg518Cys did not significantly shift activation. In addition, the respective $k$ (slope factor) showed no significant difference between the groups: 6.9±0.4 (WT, n=15), 7.7±0.4 (p.Arg518Cys, n=14), 8.0±0.4 (p.Arg518His, n=15). When voltage-dependent activation and steady-state inactivation are plotted together, large increases in the window current can be observed (Figure 5B).

$I_{\text{CaL}}$ decay after 90% of peak was best fit by 2 exponentials with 2 $\tau$ values representing fast and slow inactivation.
From +40 to +50, p.Arg518Cys/His revealed slower inactivation $\tau$ in the fast component of decay time (corrected $P<0.05$; Figure 5C). From +20 to +50 mV, p.Arg518Cys/His revealed a slower inactivation $\tau$ in the slow component of the decay time (corrected $P<0.05$; Figure 5D).

Finally, we examined late current of $I_{CaL}$: the typical traces can be seen in Figure 5E. In addition, we normalized late current to peak current shown as percentages in a bar graph for WT, p.Arg518Cys, and p.Arg518His. All values represent means±SEM. *$P<0.05$ vs CACNA1C-WT for Student t tests; **$P<0.05$ after ANOVA/Kruskal–Wallis with Dunn correction for multiple comparisons.

**In Vitro Functional Analysis of p.Arg518Cys-CACNA1C With or Without BaCl$_2$ Perfusion**

$I_{CaL}$ CACNA1C-WT and p.Arg518Cys reached peak at +30 mV, whereas $I_{BaL}$ CACNA1C-WT and p.Arg518Cys reached peak at +10 mV. Both WT and p.Arg518Cys mutant displayed robust $I_{BaL}$ currents at +10 mV when compared with $I_{CaL}$ currents. The p.Arg518Cys mutation exhibited slower inactivation kinetics than WT under both CaCl$_2$ and BaCl$_2$ perfusion. Typical $C_a$,1.2 CACNA1C-WT and p.Arg518Cys $I_{CaL}$ and $I_{BaL}$ tracings of voltage-dependent activation at +10 and +30 mV are shown in Figure 6A. Voltage-dependent inactivation (VDI) was presented as fraction of current remaining after 500-ms depolarization normalized to peak current ($r_{500}$) across various voltages. The extent of Ca$^{2+}$-dependent inactivation was calculated as $f_{500}=r_{500(Ba)}-r_{500(Ca)}$. The p.Arg518Cys mutation significantly decelerated VDI from +10 to +30 mV under both BaCl$_2$ and CaCl$_2$ perfusion (Figure 6B). However, p.Arg518Cys did not change Ca$^{2+}$-dependent inactivation of Ca,1.2 channel at +30 mV significantly with $f_{500}=0.62±0.07$ (p.Arg518Cys, n = 5) when compared with $f_{500}=0.55±0.09$ (WT, n=5).

**Discussion**

Familial WES is a powerful tool for identifying the underlying genetic substrate in novel disorders. In this study, we identified a pedigree with multiple cardiac abnormalities, including LQTS,
HCM, CHDs, and SCD. WES of 3 affected and 1 unaffected family member in combination with Ingenuity variant analysis suggested a novel mutation p.Arg518Cys-CACNA1C as the probable pathogenic substrate for the COTS phenotype observed in this pedigree. Follow-up cohort analysis revealed 2 additional pedigrees, with similar phenotypes, having mutations at the exact same amino acid position (p.Arg518Cys and p.Arg518His).

CACNA1C encodes for the α-subunit of the CaV1.2 LTCC, which is critical for the plateau phase of the cardiac action potential, cellular excitability, excitation–contraction coupling, and regulation of gene expression.20,21 Perturbations of CACNA1C have been associated with several different cardiac arrhythmia disorders, which can be differentiated into 2 groups: loss-of-function CACNA1C-mediated disease leading to Brugada syndrome9 and gain-of-function CACNA1C-mediated disease leading to either TS16 or LQTS.11

Of the gain-of-function CACNA1C-mediated diseases, TS, is an extremely rare, sporadic disorder, characterized by a myriad of multisystem abnormalities, including a cardiac phenotype of QT prolongation, HCM, CHDs, premature SCD and an extracardiac phenotype of syndactyly, facial dysmorphisms, and neurological symptoms including autism and intellectual disability.22 Although it was speculated initially that gain-of-function mutations within CACNA1C would lead to this complex multisystem phenotype of TS, CACNA1C mutations were described recently in cases of pure congenital LQTS devoid of other cardiac or other organ system abnormalities.11 Conversely, concomitant QT prolongation is common in patients with HCM although the exact pathophysiology of this electrocardiographic finding remains unclear.23 Interestingly, the pedigrees identified within this study seem to have a phenotype that lies between TS and LQTS, representing a COTS phenotype of LQTS, HCM, CHDs, and SCD.

To confirm the disease-causing nature of the p.Arg518Cys/His mutations and to attempt to better understand the resultant

Figure 6. Deceleration of voltage-dependent inactivation by p.Arg518Cys-CACNA1C. A, Whole cell I_{Ca} and I_{Ba} current representative tracings from HEK293 cells expressing wild-type (WT) or mutant p.Arg518Cys determined from a holding potential of −90 mV to testing potential of +10 mV and +30 mV with 500-ms duration. B, Voltage-dependent inactivation of I_{Ca} and I_{Ba} currents for WT and p.Arg518Cys channels (n=5 for each group, red *P<0.05 vs WT I_{Ba}, black *P<0.05 vs WT I_{Ca}; statistics completed for each of the 10 voltages). r_{500}, represented fraction of current remaining after 500-ms depolarization normalized to peak current (I_{peak}).
COTS phenotype, heterologous expression of the Ca$_{1.2}$ was used to elucidate the functional perturbation of p.Arg518Cys/His. Interestingly, unlike the previously reported LQTS-associated mutations, which produced an overall gain of current density,11,12 p.Arg518Cys/His led to an overall loss of current density by $\approx$59%. Confocal imaging provided evidence that there was a higher proportion of CACNA1C mutant channels in the center versus the periphery of the cell, highlighting the possibility of a trafficking defect, which may explain the overall reduction in current density. The p.Arg518Cys/His variant lies within the I-II linker of CACNA1C, and within this linker is the $\alpha$-interaction domain, in which the $\beta$-subunit binds (Figure 2). Although p.Arg518Cys/His does not fall within the $\alpha$-interaction domain, the $\beta$-subunit is known to aid in $\alpha$-subunit trafficking and regulation of channel kinetics; therefore one could hypothesize that the p.Arg518Cys/His may disrupt folding of the I–II linker, altering the normal interaction between the $\alpha$-interaction domain and the $\beta$-subunit, leading to significant changes in trafficking and channel kinetics.

In addition, p.Arg518Cys/His also caused a gain-of-function shift in the inactivation curves to more depolarized potentials, leading to a significant increase in the window current. Increases in window current, because of gain-of-function shifts in activation that were identified recently in p.Ile1166Thr-CACNA1C, and within this linker is the $\alpha$-interaction domain, in which the $\beta$-subunit binds (Figure 2). Although p.Arg518Cys/His does not fall within the $\alpha$-interaction domain, the $\beta$-subunit is known to aid in $\alpha$-subunit trafficking and regulation of channel kinetics; therefore one could hypothesize that the p.Arg518Cys/His may disrupt folding of the I–II linker, altering the normal interaction between the $\alpha$-interaction domain and the $\beta$-subunit, leading to significant changes in trafficking and channel kinetics.

The exact pathophysiology of HCM and CHDs in TS is yet to be established. However, it has been suggested previously that protein expression in pathways regulating Ca$^{2+}$ within cardiac tissue may be perturbed in patients with CHDs and may result in hypertrophy.28 Those studies, in combination with what has been observed in our pedigrees and TS, suggest that proper regulation of Ca$^{2+}$ and potentially the LTCC are crucial for cardiac development, and perturbation of Ca$^{2+}$ handling could lead to developmental abnormalities leading to CHDs.

Abnormal calcium handling has also been described in various models of cardiac hypertrophy. What has been unclear in the past is whether this Ca$^{2+}$ irregularity is secondary to hypertrophy or related to the primary pathogenesis of disease.29 Recently, Lan et al30 examined Ca$^{2+}$ handling in an induced pluripotent stem cell model of HCM and found irregular Ca$^{2+}$ transients and elevated diastolic [Ca$^{2+}$], before overt phenotypic expression of cardiac hypertrophy as seen in HCM. This finding, in combination with their finding that LTCC blockers, such as verapamil, mitigated cellular hypertrophy, suggest that dysregulation of Ca$^{2+}$ may be a central mechanism for disease development.30 The phenotypes observed within our pedigrees also support the findings of Lan et al,30 suggesting that Ca$^{2+}$ handling could be a primary cause for hypertrophy. As shown in our electrophysiological studies, the p.Arg518Cys/His leads to Ca$^{2+}$ mishandling, through constitutively active LTCCs, and over time, many of the patients within the pedigrees who are mutation positive acquire left ventricular hypertrophy.

**CACNA1C-Mediated Clinical Phenotypes**

A large paradox emerges in the context of CACNA1C mutations in the human heart as to why some gain-of-function mutations cause a multiple organ system phenotype like TS, some an LQTS-only phenotype, and some a COTS phenotype such as the one described within this study. Initially, analyzing electrophysiological phenotypes of each of these diseases as described above, it was easy to distinguish CACNA1C-mediated LQTS. On the basis of our original findings, we found that the major electrophysiological phenotype of CACNA1C-associated LQTS was a marked gain in Ca$_{1.2}$ current density,11 whereas TS16,20 and p.Arg518Cys/His altered the kinetic properties of the channel. It also seemed as if TS-associated mutations were localized to specific channel regions, at the S6/interdomain linker boundaries,17 whereas the COTS- and LQTS-associated mutations were primarily localized to intracellular linker loops and the N and C terminus (Figure 2). Therefore, it could have been hypothesized that the mechanism for pure LQTS is largely because of the location and the electrophysiological perturbation caused by the mutation.

However, since the initial discovery of CACNA1C-mediated LQTS, the spectrum and prevalence of mutations within CACNA1C have expanded greatly. Two novel case reports identified the previously TS-associated p.Gly402Ser mutation in patients without the neurological phenotypes associated with TS, rather these patients presented primarily with QT prolongation and ventricular fibrillation. In addition, these case reports suggest that the neurological phenotype observed with p.Gly402Ser may stem from the cardiac insults that had taken place early in development, rather than observed with the p.Gly402Ser mutation itself.11,14 In a similar
fashion, Wemhöner et al\textsuperscript{15} found an individual with phenotypes consistent with TS with a p.Ile1166Thr mutation, matching previous reports.\textsuperscript{17} However, they found a second mutation, p.Ile1166Val, in a patient exhibiting a LQTS-only phenotype. Although the electrophysiological characteristics between p.Ile1166Thr and p.Ile1166Val were distinct, this finding, along with the novel case reports surrounding p.Gly402Ser, begins to highlight that mutation location may not be the final determinant of disease manifestation.

After the identification of more CACNA1C mutations in LQTS and TS, a more comprehensive comparison of the electrophysiological characteristics could be made. Taken altogether, there are LQTS-associated mutations that present with only increases in peak current density (p.Pro857Arg and p.Ile66Val), there are mutations that affect only channel kinetics (p.Arg860Gly, p.Ile1475Met, and p.Glu1496Lys), and there are mutations that affect both (p.Ala238Thr, p.Ala582Asp, and p.Asp585His). In addition to our CACNA1C-mediated LQTS, we have TS mutations (p.Gly406Arg exon 8/8A, p.Gly402Ser exon 8, and p.Ile1166Thr) that affect kinetics and in some cases current density. And finally, we have the COTS-associated mutations (p.Arg518Cys/His) that affect both current density and channel kinetics. Therefore, these variants cannot easily be separated by the electrophysiological characteristics.

Collectively, it seems as if the disease phenotypes observed with mutations in the calcium channel are more complex than the location of the mutation within the channel and the underlying electrophysiological perturbations that the particular mutation causes. There are several different unexplored explanations that may determine the disease pathogenesis. It may be possible that the amino acid change itself, for instance p.Ile1166Thr, causes TS, whereas p.Ile1166Val causes LQTS and confers disease phenotype. It is also possible that the binding of partner proteins leading to complex signaling cascades may also be important to disease manifestation. For example, Krey et al\textsuperscript{18} found that the location of the p.Gly406Arg mutation may be critical for the neurogenic phenotypes seen in TS. The p.Gly406Arg mutation leads to more dendritic retraction than WT cells regardless of the presence of pore mutations eliminating Ca\textsuperscript{2+} influx, highlighting the independence of this function from proper Ca\textsubscript{v}1.2 electrophysiology. They identified that p.Gly406Arg mutation disrupted binding between Gem and Ca\textsubscript{v}1.2, altering signaling cascades regulated by Gem and RhoA, providing evidence that this interaction is essential to prevent dendritic retraction, and highlighting that binding partners and signaling cascades may be critical for disease presentation.\textsuperscript{19} Finally, it is also may be possible that there are other genetic or epigenetic factors may play a role in the disease pathogenesis causing different CACNA1C mutations to lead to different phenotypic manifestations in each patient.

Only 1 conclusion can be made for certain; there definitely are regions within CACNA1C that seem to resemble genetic hotspots, which lead to cardiac disease. The S6/interdomain linker loop regions are capable of leading to severe LQTS or TS phenotypes, p.Arg518Cys/His lead to a COTS phenotype, the II-III linker has a multitude of LQTS-associated CACNA1C mutations. Future studies will be necessary to unravel the differences between these mutations, and why, despite their location and electrophysiological similarities, these regions are capable of producing distinct clinical phenotypes ranging the spectrum of TS, COTS, and LQTS.

Conclusions
Through WES and expanded cohort screening, we identified a novel genetic substrate, p.Arg518Cys/His-CACNA1C, in patients with a complex phenotype including LQTS, HCM, CHDs, and SCD here referred to as COTS. Our electrophysiological studies identified a complex phenotype, including loss of current density in combination with increased window current and late current, and decelerating VDI. On the basis of functional studies, the identification of mutations at the same amino acid position, and cosegregation with disease in 3 different pedigrees, these 2 novel, ultrarare missense mutations—p.Arg518Cys and p.Arg518His—cause COTS.

Sources of Funding
This work was supported by the Windland Smith Rice Comprehensive Sudden Cardiac Death Program, the Dr. Scholl Foundation, Hannah Wernike Memorial Foundation, the Sheikh Zayed Saif Mohammed Al Nahyan Fund in Pediatric Cardiology Research (Dr Ackerman). Dr Boczek was supported by CTSA Grant (UL1 TR000135) from the National Center for Advancing Translational Science, a component of the National Institutes of Health, as well as was a member of the Clinical and Translational Science program and was supported by the Mayo Clinic Graduate School.

Disclosures
Mayo Clinic and Dr Ackerman received sales-based royalties from Transgenomic’s FAMILION-long-QT syndrome and FAMILION-CPVT (catecholaminergic polymorphic ventricular tachycardia) genetic tests.

References


Identification and Functional Characterization of a Novel CACNA1C-Mediated Cardiac Disorder Characterized by Prolonged QT Intervals With Hypertrophic Cardiomyopathy, Congenital Heart Defects, and Sudden Cardiac Death

Nicole J. Boczek, Dan Ye, Fang Jin, David J. Tester, April Huseby, J. Martijn Bos, Aaron J. Johnson, Ronald Kanter and Michael J. Ackerman

Circ Arrhythm Electrophysiol. 2015;8:1122-1132; originally published online August 7, 2015; doi: 10.1161/CIRCEP.115.002745

Circulation: Arrhythmia and Electrophysiology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2015 American Heart Association, Inc. All rights reserved.
Print ISSN: 1941-3149. Online ISSN: 1941-3084

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circep.ahajournals.org/content/8/5/1122

Data Supplement (unedited) at:
http://circep.ahajournals.org/content/suppl/2015/08/07/CIRCEP.115.002745.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation: Arrhythmia and Electrophysiology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation: Arrhythmia and Electrophysiology is online at:
http://circep.ahajournals.org//subscriptions/
SUPPLEMENTAL MATERIAL

Supplemental Methods

**Whole Exome Sequencing**

Paired-end libraries were prepared following the manufacturer’s protocol (Illumina, San Diego, CA and Agilent, Santa Clara, CA) using the Bravo liquid handler from Agilent. Whole exon capture was carried out using the protocol for Agilent’s SureSelect Human All Exon v5 + UTRs 75 MB kit. Exome libraries were loaded onto TruSeq Rapid run paired end flow cells at concentrations of 9 pM to generate cluster densities of 600,000-800,000/mm² following Illumina’s standard protocol using the Illumina cBot and TruSeq Rapid Paired end cluster kit version 1. The flow cells were sequenced as 100 X 2 paired end reads on an Illumina HiSeq 2500 using TruSeq Rapid SBS kit version 1 and HiSeq data collection version 2.0.12.0 software. Base-calling was performed using Illumina’s RTA version 1.17.21.3.

**Bioinformatics**

The Illumina paired end reads were aligned to the hg19 reference genome using Novoalign 2.08 (http://novocraft.com) followed by the sorting and marking of duplicate reads using Picard (http://picard.sourceforge.net). Local realignment of INDELs and base quality score recalibration were then performed using the Genome Analysis Toolkit 2.7-4 (GATK).¹ Single nucleotide variants (SNVs) and insertions/deletions (INDELs) were called across all of the samples simultaneously using GATK's Unified Genotyper with variant quality score recalibration.²

Ingenuity Variant Analysis (Qiagen, Valencia, CA) was utilized to complete our filtering strategy. Variants with a call quality <20, read depth <10, found in the top 1% of genes with high variability; with a frequency of >0.01% in National Heart Lung and Blood Institute Exome Sequencing Project (NHLBI ESP), 1000 Genomes Project, or public Complete Genomics Genomes; and synonymous and noncoding variants with the exception of potential splice site
variants were excluded. We then excluded any variants present in the unaffected mother (II.2), and kept only variants shared by the index case (III.1), affected sister (III.4), and affected nephew (IV.1; Figure 1A). Finally, we looked for mutations in genes related to LQTS, cardiomyopathy, SCD, and other diseases consistent with these phenotypes (Figure 1B).

**HEK293 Cell Culture and Transfection**

HEK293 cells were cultured in minimum essential medium supplemented with 1% nonessential amino acid solution, 10% horse serum, 1% sodium pyruvate solution, and 1.4% penicillin/streptomycin solution in a 5% CO₂ incubator at 37°C. Heterologous expression of Ca,1.2 was accomplished by co-transfecting 1 μg CACNA1C wild type (WT) or mutant cDNA with 1 μg CACNB2b, 1 μg CACNA2D1 and 0.25 μg Green Fluorescence Protein (GFP) cDNA (kindly provided by Dr. Gianrico Farrugia, Mayo Clinic, Rochester, MN) with the use of 9 μl Lipofectamine 2000 (Invitrogen, Carlsbad, CA). The media was replaced with OPTI-MEM (Gibco, Carlsbad, CA) after 4-6 hours. Transfected HEK293 cells were cultured in OPTI-MEM and incubated for 48 hours. Cells exhibiting yellow (CACNA1C), green (transfection control), and red (CACNB2b) fluorescence were selected for electrophysiological experiments.

**Electrophysiological Measurements and Data Analysis**

Standard whole-cell patch clamp technique was used to measure CACNA1C wild type and mutant calcium currents at room temperature (22-24°C) with the use of an Axopatch 200B amplifier, Digidata 1440A and pclamp version 10.4 software (Axon Instruments, Sunnyvale, CA). The extracellular (bath) solution contained (mmol/L): 130 NMDG, 5 KCl, 15 CaCl₂, 1 MgCl₂, 5 mM TEA-Cl and 10 HEPES, pH adjusted to 7.35 with HCl. The pipette solution contained (mmol/L): 120 CsCl, 2 MgCl₂, 10 EGTA, 2 MgATP, 5 CaCl₂ and 10 HEPES, pH adjusted to 7.25 with CsOH.³ Microelectrodes were pulled on a P-97 puller (Sutter Instruments,
Novato, CA) and fire polished to a final resistance of 2-3 MΩ. Series resistance was compensated by 80-85%. Currents were filtered at 1 kHz and digitized at 5 kHz with an eight-pole Bessel filter. The voltage dependence of activation and inactivation was determined using voltage-clamp protocols described in the relevant figures. Data were analyzed using Clampfit (Axon Instruments, Sunnyvale, CA), Excel (Microsoft, Redmond, WA), and fitted with Origin 8 (OriginLab Corporation, Northampton, MA) software.

The voltage-dependence of activation curve was fitted with a Boltzmann function:
\[ \frac{G_{Ca}}{G_{Ca \ max}} = \frac{1}{1 + \exp \left( \frac{V-V_{1/2}}{k} \right)} \]
where \( V_{1/2} \) and \( k \) are the half-maximal voltage of activation and the slope factor respectively. The steady-state inactivation curve was fitted with a Boltzmann function:
\[ \frac{I_{Ca}}{I_{Ca \ max}} = \frac{1}{1 + \exp \left( \frac{(V-V_{1/2})}{k} \right)} \]
where \( V_{1/2} \) and \( k \) (slope factor) are the half-maximal voltage of inactivation and the slope factor respectively. \( I_{Ca} \) decay was fitted with a two-exponential function:
\[ y = y_0 + \left( 1 - [A_f \exp(-t/\tau_f)] + [A_s \exp(-t/\tau_s)] \right) \]
where \( A_f \) and \( A_s \) represent the amplitudes of the fast and the slow inactivating components respectively and \( \tau_f \) and \( \tau_s \) represent the fast and slow time constants of inactivation respectively. Late \( I_{Ca} \) current was measured at the end of 500 ms long depolarization of +30 mV.

In order to explore the BaCl₂ effects on CaV1.2 wild type (WT) and mutant channels and explain the mechanism by which mutants caused slower inactivation, the standard whole-cell patch clamp technique was used to measure CaV1.2-WT or p.Arg518Cys calcium currents at room temperature (22-24°C) under both 15 mM CaCl₂ and 15 mM BaCl₂ extracellular (bath) solution in the same cells. Fraction of current remaining after 500 ms depolarization normalized to peak current (\( r_{500} \)) across various voltages was measured. The extent of Ca²⁺-dependent inactivation (CDI) was calculated as:
\[ f_{500} = \frac{(r_{500}/Ba-r_{500}/Ca)}{r_{500}/Ba} \]
Confocal Examination of CACNA1C

48-hours after transfection, cells grown on 10-micro well slides were examined using Olympus Leica DM 2500 confocal microscope and images were acquired using LAS AF 6000 acquisition software. The Tiff images (WT n=10 vs p.Arg518Cys n=10) were analyzed with Olympus Fluoview Ver3.1a Software. After software conversion, we scaled a region of interest measurement confined to within 1.163 μm of the membrane. The average intensity within this region was defined as peripheral staining. The average intensity of the inside of the cells was defined as central staining. We calculated the ratio of the peripheral to central fluorescence.7

Supplemental References


