Clinical Phenotype and Functional Characterization of 
CASQ2 Mutations Associated With Catecholaminergic Polymeric Ventricular Tachycardia

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Background—Four distinct mutations in the human cardiac calsequestrin gene (CASQ2) have been linked to catecholaminergic polymorphic ventricular tachycardia (CPVT). The mechanisms leading to the clinical phenotype are still poorly understood because only 1 CASQ2 mutation has been characterized in vitro.

Methods and Results—We identified a homozygous 16-bp deletion at position 339 to 354 leading to a frame shift and a stop codon after 5aa (CASQ2G112stop) in a child with stress-induced ventricular tachycardia and cardiac arrest. The same deletion was also identified in association with a novel point mutation (CASQ2L167H) in a highly symptomatic CPVT child who is the first CPVT patient carrier of compound heterozygous CASQ2 mutations. We characterized in vitro the properties of CASQ2 mutants: CASQ2G112stop did not bind Ca2+, whereas CASQ2L167H had normal calcium-binding properties. When expressed in rat myocytes, both mutants decreased the sarcoplasmic reticulum Ca2+ storing capacity and reduced the amplitude of ICa-induced Ca2+ transients and of spontaneous Ca2+ sparks in permeabilized myocytes. Exposure of myocytes to isoproterenol caused the development of delayed afterdepolarizations in CASQ2G112stop.

Conclusions—CASQ2L167H and CASQ2G112stop alter CASQ2 function in cardiac myocytes, which leads to reduction of active sarcoplasmic reticulum Ca2+ release and calcium content. In addition, CASQ2G112stop displays altered calcium-binding properties and leads to delayed afterdepolarizations. We conclude that the 2 CASQ2 mutations identified in CPVT create distinct abnormalities that lead to abnormal intracellular calcium regulation, thus facilitating the development of tachyarrhythmias. (Circulation. 2006;114:1012-1019.)

Key Words: genetics ■ tachyarrhythmias ■ electrophysiology ■ calcium

Catecholaminergic polymorphic ventricular tachycardia (CPVT; Online Mendelian Inheritance in Man [OMIM], Johns Hopkins University, Baltimore, Md; MIM No. 604772) is a familial arrhythmic disorder characterized by adrenergically mediated polymorphic ventricular tachyarrhythmias that lead to syncope and sudden cardiac death. Physical or emotional stress triggers arrhythmias in young individuals, in whom no structural abnormalities of the heart can be observed. Two genetic variants of the disease have been identified, a recessive form caused by homozygous mutations in the calsequestrin-2 (CASQ2) gene, located on chromosome 1, site p13.3-p11 and encoding for the cardiac isoform of calsequestrin, and an autosomal dominant form caused by mutations in the RyR2 gene encoding for the cardiac isoform of the ryanodine receptor on chromosome 1q42.1-q43.

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The cardiac ryanodine receptor (RyR2) is an intracellular Ca2+ release channel located in the membrane of the sarcoplasmic reticulum (SR). Calsequestrin is a high-capacity, low-affinity Ca2+-binding protein that represents a major Ca2+-reservoir element within the SR lumen. Both of these Ca2+-handling proteins are critically involved in cardiac excitation-contraction (EC) coupling through the mechanism of calcium-induced calcium release, thus suggesting that abnormalities in the control of intracellular calcium may represent the central pathogenic pathway in CPVT. Accordingly, it has been speculated that the electrophysiological mechanism for arrhythmias in CPVT is triggered activity initiated by delayed afterdepolarizations (DADs). We recently reported the first functional characterization of the CASQ2 mutant identified by Lahat et al, and we were able to...
demonstrate that DADs develop as a consequence of a loss of function in calsequestrin, which leads to triggered activity.

As expected for a recessive disease, CASQ2 mutations are less commonly identified in CPVT patients than RyR2 mutations. To date, only 3 homozygous disease-associated sequence variations have been identified in the CASQ2 gene (http://www.fsm.it/cardmoc): a 1-bp deletion, 1 splicing junction mutation,9 and 1 missense mutation.3 However, functional characterization is available only for the D307H mutation.8 Here, we report the identification of 2 new mutations in the CASQ2 gene, and we used the model previously developed8 to characterize in vitro the mutant peptides and functional derangements of the 2 CASQ2 mutations. Our data have implications for molecular diagnosis of CPVT and for genetic counseling in CPVT families.

Methods

Clinical Evaluation

Two unrelated probands with unexplained syncopal episodes or idiopathic ventricular tachyarrhythmia and 20 family members were referred to our center for clinical and molecular evaluation. Cardiac evaluation included ECG, echocardiogram, exercise stress testing, and Holter recording. Genetic counseling was performed and DNA obtained for genetic analysis. Patients or their guardians provided written informed consent for clinical and genetic evaluation. Protocols were approved by the institutional review board of the Fondazione Salvatore Maugeri.

Genetic Analysis

DNA was extracted from peripheral blood lymphocytes. The coding region of the RyR2 gene was amplified by polymerase chain reaction (PCR) with intronic primers10 and analyzed by the single-strand conformation polymorphism method. The 11 exons of CASQ2 (GenBank: NM_001232) were amplified with intronic primers and analyzed by denaturing high-performance liquid chromatography (WAVE, Transgenomic, Inc, Omaha, Neb). Abnormal elution profiles were sequenced with a BigDye terminator sequencing kit (Applied Biosystems, Foster City, Calif) and an ABI Prism 310 genetic analyzer (Applied Biosystems) and compared with 600 alleles from healthy subjects with normal ECGs. Haplotype analysis was performed with markers as described11 previously (Figure 1).

Cloning of Human Cardiac Calsequestrin cDNA and Mutagenesis

The full-length coding sequence of human CASQ2 gene was amplified with the Gene Amp XL-PCR kit (Roche, Basel, Switzerland) from DNA pools of a Human Heart cDNA Library Lambda ZAP II Vector (Stratagene, La Jolla, Calif) with Cas1F and Cas11R primers, annealing to the 5′-UTR and 3′-UTR regions, respectively. The 1.2-kb PCR product was cloned into the pGEM-dT Easy Vector (Promega, Madison, Wis), and the correct sequence was verified by sequencing. Mutations were introduced by site-directed mutagenesis with the QuickChange Mutagenesis Kit (Stratagene) and verified by sequencing.

Recombinant Adenovirus and Gene Transfer

The wild-type (WT) or mutant human CASQ2 cDNA were subcloned in pENTR-4 Vector (Invitrogen Corp, Carlsbad, Calif) and transferred into the Adenoviral Expression pAD/DEST Vector (Invitrogen). Ventricular myocytes were enzymatically dissociated from adult rat hearts, infected with adenoviruses (multiplicity of infection of 100), and maintained in a 5% CO2 incubator at 37°C.8 Experiments were performed 48 to 56 hours after infection.

Electrophysiological Recordings

Whole-cell patch-clamp recording of transmembrane ionic currents was performed with an Axopatch 200B amplifier (Axon Instruments, Foster City, Calif).8 The external solution contained (in mmol/L): 140 NaCl, 5.4 KCl, 1.0 CaCl2, 0.5 MgCl2, 10 HEPES, and 5.6 glucose, pH 7.3. Micropipettes (borosilicate glass, Sutter Instruments, Novato, Calif; 1- to 3-MΩ resistance) were filled with the following solution (in mmol/L): 90 cesium aspartate, 50 CsCl, 3 Na2ATP, 3.5 MgCl2, 10 HEPES, and 0.05 Fluo-3 potassium salt, pH 7.3. Holding potential was −50 mV; 400-ms voltage pulses were applied at 1-minute intervals from the holding potential to specified membrane potentials.

Confocal Ca2+ Measurements

Myocytes were imaged with a Bio-Rad Laser Scanning Confocal System (Bio-Rad MRC-1024ES interfaced to an Olympus IX-70 inverted microscope; Bio-Rad Laboratories, Hercules, Calif) as
described previously. Fluo-3 was excited by light at 488 nm, and the fluorescence was acquired at wavelengths >515 nm in the line-scan mode of the confocal system at a rate of 2 or 6 ms per scan.

**Production and Purification of Recombinant CASQ2**

CASQ2 constructs were created by nested PCR. CASQ2 signal sequence was eliminated or substituted with the T7-Tag coding sequence using the following oligonucleotides: internal forward T7-EE F, external forward NdeI-ATG-pT7 F coupled either with reverse EcoRI-XX R (WT and L167H) or EcoRI-XX-Trunc R (G112+5X). PCR products were digested with NdeI and EcoRI ligated in pET-5a (Novagen, Madison, Wis), and transformed in BL21 (DE3) Escherichia coli (Novagen). Expression of recombinant proteins was induced according to the manufacturer's instructions. Cells were sonicated either in 50 mmol/L Tris-Cl (pH 7.5), 5 mmol/L DTT, 1 mmol/L EDTA, and 0.1 mg/mL lysozyme (phenyl-sepharose purification) or 1XT7-Tag bind/wash buffer (Novagen; T7-Tag affinity purification) and incubated with the suitable resin. Recombinant proteins were eluted from phenyl-sepharose in the presence of 10 mmol/L CaCl2, 20 mmol/L MOPS (pH 7.2), 1 mmol/L DTT, and 500 mmol/L NaCl or according to Novagen’s procedure. Proteins were quantified according to Bradford and Lowry et al.

**Western Blot and Stains: All Staining**

SDS-PAGE was performed on either 7.5% or 15% gels. Slab gels were stained with the cationic carbocyanine dye “Stains-all” (Sigma-Aldrich, St Louis, Mo) for identification of Ca2+-binding proteins. Western blot with anti-CASQ2 polyclonal antibodies (ABR–Affinity Bioreagents, PAI-913) and for detection of rat CASQ2 (ABR–Affinity Bioreagents, Golden, Colo) was performed as described previously. Levels of CASQ2 in cultured myocytes were determined as described previously. Cell lysate proteins (10 μg) were subjected to SDS-PAGE (4% to 20% linear gradient gel), blotted onto nitrocellulose membranes (Bio-Rad Laboratories). Anti-CASQ2 antibodies were used for detection of both rat and human CASQ2 (ABR–Affinity Bioreagents, PAI-913) and for detection of rat CASQ2 (06-382, Upstate, Charlottesville, Va). Blots were quantified with a Visage 2000 Blot Scanning and Analysis system (BioImage Systems Corp).

**Calcium Binding**

45Ca ligand overlay was performed on purified CASQ2 (2 to 3 μg of protein) electroblotted on nitrocellulose membranes, in a medium containing 5 mmol/L MgSO4, 60 mmol/L KCl, 5 mmol/L imidazole, pH 7.0, and 0.6 to 6 mmol/L Ca2+ (specific activity 5 to 50 mCi/mg Ca). Single lanes were incubated at room temperature for 20 minutes at different total Ca2+ concentrations ranging from 10 μmol/L to 6 mmol/L. After ethanol wash, CASQ2 bands were counted for radioactivity. Background subtraction was obtained by counting an aliquot of Lane 1. Background subtraction was obtained by counting an aliquot of Lane 1.

**Results**

**Clinical Phenotype**

**Family I**

A 6-year-old boy (III-2, Figure 1A) was referred to our center for the evaluation of effort-induced syncopal episodes since age 3. Physical examination, resting ECG, and echocardiogram were unremarkable, but exercise stress testing demonstrated rapid polymorphic ventricular tachycardia (Figure 2A). Holter monitoring showed severe runs of asymptomatic polymorphic and bidirectional sustained ventricular tachycardia at rates of 170 to 180 bpm during outdoor playing. The diagnosis of CPVT was established and β-blocker treatment initiated. No family history for sudden cardiac death, syncopal events, or effort- or emotion-related arrhythmias was reported. His parents had normal ECGs, and exercise stress testing and Holter monitoring showed no ventricular arrhythmias. They denied consanguinity, as confirmed by haplotype analysis (Figure 1A).

**Family II**

A 17-year-old girl (III-1, Figure 1B) was referred for evaluation of “idiopathic ventricular arrhythmias.” Family history was negative for sudden death, unexplained ventricular tachyarrhythmias, and syncopal events. Parents denied consanguinity. The first manifestation of the disease occurred at age 4, when the child collapsed while playing; the first recorded rhythm at the emergency room was polymorphic ventricular tachycardia at a rate of 200 bpm. Between age 4 and 17 years, the patient experienced multiple syncopal events, and runs of polymorphic ventricular tachycardia were often recorded at Holter monitoring; antiarrhythmic therapy with class I and class III drugs failed to modify the clinical manifestations. When we first saw the patient at age 17 years, both the ECG (Figure 2B) and echocardiogram were unremarkable; the diagnosis of CPVT was established, and therapy with β-blockers was initiated. After several years of apparent reduction of arrhythmias, sustained runs of ventricular tachycardia were again documented despite compliance with therapy. Electrophysiological studies failed to demonstrate inducibility of ventricular arrhythmias. The patient underwent left cardiac sympathetic denervation that was unable to control cardiac symptoms, and eventually, she received an implantable cardioverter defibrillator.

**Genetic Analysis**

A homozygous 16-bp deletion in exon 3 (deletion 339 to 354) of CASQ2 was identified in the proband (III-2) of family 1 (Figure 1A). This deletion generates a frame shift that leads to a stop codon 5 amino acids downstream from the deletion site (G112+5X). Evaluation of family members revealed that asymptomatic family members were heterozygous carriers of the deletion, and none of them developed ventricular arrhyth-
mias during Holter or exercise stress test. No other homozygous carrier was identified.

The proband (III-1) of family II (Figure 1B) presented with the same 16-bp deletion in exon 3, previously identified in family I (the 2 families were unrelated, as shown by haplotype analysis in Figure 1), on 1 allele and a new missense mutation on the other (L167H). As a consequence, she has no WT cardiac calsequestrin in her cardiac cells. Family screening showed that the proband’s mother and the maternal grandfather are asymptomatic heterozygous carriers of the 16-bp deletion, whereas the father and 1 sister (III-3) of the proband are asymptomatic heterozygous carriers of the missense mutation. The other sister (III-2) inherited 2 WT alleles.

Electrophysiological Recordings and Confocal Ca2+ Measurements

To characterize the effects of the new CASQ2 mutations on SR Ca2+ handling in vivo, we performed experiments in adult rat ventricular myocytes infected with adenoviral constructs. 

- Virus-mediated expression of human WT and mutant L167H CASQ2 results in an ~2.5-fold increase in the total amount of CASQ2 protein in rat ventricular myocytes. 
- Importantly, the level of endogenous rat CASQ2 remained unchanged in cells expressing exogenous human CASQ2, as revealed with rat CASQ2-specific antibody.
- Caffeine (10 mmol/L) was applied to myocytes expressing either WT or mutant CASQ2 to evaluate the SR Ca2+ content by measurements of the global Ca2+ content and of sparks. In agreement with measurements of the global Ca2+ content, the effects observed with CASQ2L167H were much more prominent than those observed with CASQ2G112S.

The effects of the new CASQ2 mutants on active Ca2+ release during EC coupling were studied in myocytes undergoing voltage-clamp stimulation (Figures 4B and 4C; Table 2). Expression of CASQ2G112S and expression of CASQ2L167H caused a significant decrease in the amplitude of Ca2+ transients triggered by Ica at membrane potentials ranging from –40 to 60 mV as compared with WT expression. In addition, both the time-to-peak and the rate of decay of Ca2+ transients were shortened. The effects observed with CASQ2G112S were much more prominent than those observed with CASQ2L167H.

The effects of the new CASQ2 mutants on the properties of focal fluorescence signals, ie, Ca2+ sparks, were studied in saponin-permeabilized myocytes kept at a constant cytosolic [Ca2+]i (~100 nmol/L; Figure 5; Table 3). When CASQ2WT was overexpressed, Ca2+ sparks were greater and longer than in native cells, in agreement with our previous results. Compared with WT, expression of CASQ2G112S and of CASQ2L167H decreased the magnitude of sparks, the spatiotemporal spread of sparks, and the duration of the rising phase of sparks. In agreement with measurements of the global Ca2+ transients, the effects observed with CASQ2G112S were more prominent than those observed with CASQ2L167H.

Finally, we examined the effects of expressing the CASQ2 mutants on Ca2+ transients and action potentials in paced rat ventricular myocytes.
myocytes exposed to isoproterenol (1 μmol/L). Myocytes expressing CASQ2G112+5X exhibited spontaneous extrasystolic Ca2+ elevations and DADs in 4 of 4 myocytes tested (Figure 6). Myocytes expressing either the WT or the L167H mutant CASQ2 showed no spontaneous Ca2+ transients or DADs (n=4 and n=3, respectively; not shown).

**Molecular Properties of Mutant CASQ2**

To investigate the molecular mechanisms causing aberrant Ca2+ handling in ventricular myocytes, recombinant CASQ2 was expressed in vitro and thereafter purified by standard procedures. All recombinant WT and mutant CASQ2 (CASQ2WT, T7-CASQ2WT, CASQ2L167H, and T7-CASQ2G112+5X) were found in the soluble fraction after high-speed centrifugation, which indicated that either addition of the T7-Tag, the point mutation (L167H), or the long 260 aa deletion, induced by G112+5X mutation, did not drastically affect solubility and folding of the relative polypeptides. Western blot experiments showed that all recombinant CASQ2s were recognized by specific anti-CASQ2 polyclonal antibodies. Metachromatically blue staining by Stains-all was preserved for all recombinant CASQ2s except for the deleted form. Recombinant CASQ2WT without the T7-Tag migrated with a molecular weight of 55 kDa; slower migration was detected for the T7-bearing recombinant protein (T7-CASQ2WT), whereas the deleted T7-CASQ2G112+5X had an estimated molecular weight of ~14 kDa.

Some biochemical properties of native CASQ2 were likewise shared by all recombinant CASQ2s except for T7-CASQ2G112+5X. In the absence of Ca2+, successful hydrophobic binding to phenyl-sepharose was obtained; subsequently, quantitative Ca2+-dependent elution of CASQ2WT, T7-CASQ2WT, and CASQ2L167H showed that the hydrophobic site involved in interaction with phenyl-sepharose (aa 214 to 222) is exposed in the absence of Ca2+ and that 2 to 10 mM/moL Ca2+ induced the expected conformational change that led to internalization of the region, as in native CASQ2.

**Calcium Binding to CASQ2**

To investigate whether a decrease of SR Ca2+ content in ventricular myocytes is related to modifications of Ca2+-binding properties of mutant CASQ2, Ca2+ overlay experiments were performed to compare Ca2+-binding properties of recombinant WT and mutant CASQ2. Ca2+ affinity (Kd) and capacity (Bmax) values are shown in Table 4. Human CASQ2WT displayed Kd and Bmax values comparable to those reported for native CASQ2 from different animal species.12,23 CASQ2L167H did not display any significant change in Kd and Bmax compared with CASQ2WT. On the contrary, CASQ2G112+5X did not bind Ca2+ at all according to pink staining with Stains-all. This effect was not due to the presence of T7-Tag, because Kd and Bmax of Ca2+ binding for CASQ2WT and T7-CASQ2WT were not significantly different (Table 4).

**Discussion**

Homozgyous mutations in the CASQ2 gene cause the recessive form of CPVT, a malignant disease that predisposes young individuals to sudden arrhythmic death. We report the first CPVT patient carrier of 2 heterozygous CASQ2 mutations and a CPVT proband carrier of a new homozygous CASQ2 mutation, and we demonstrate that these mutations impair CASQ2 function. The evidence that carriers of 2

**TABLE 2. Ica and Ca2+ Transients in Control Condition and With Overexpression of CASQ2 Mutants**

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<td></td>
<td>Peak Amplitude, nA</td>
<td>t½, ms</td>
<td>t½, ms</td>
<td>F/F0</td>
<td>Rise Time, ms</td>
<td>t½, ms</td>
<td>No. of Cells</td>
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<td>Ad-Control</td>
<td>-1.10±0.14</td>
<td>16.5±4.3</td>
<td>80±27</td>
<td>2.0±0.2</td>
<td>30±4</td>
<td>320±24</td>
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<td>Ad-CASQ2WT</td>
<td>-1.12±0.20</td>
<td>16.8±4.7</td>
<td>86±26</td>
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<td>43±4*</td>
<td>407±35*</td>
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<td>Ad-CASQ2L167H</td>
<td>-1.09±0.16</td>
<td>16.3±4.1</td>
<td>82±19</td>
<td>1.5±0.1†</td>
<td>20±3†</td>
<td>261±21†</td>
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<tr>
<td>Ad-CASQ2G112+5X</td>
<td>-1.10±0.20</td>
<td>16.4±4.9</td>
<td>88±28</td>
<td>2.1±0.2†</td>
<td>33±6</td>
<td>324±31†</td>
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*P<0.001 compared with Ad-Control.  †P<0.001 compared with Ad-CASQ2WT.

**TABLE 3. Effects of Overexpression of CASQ2 Mutants on Ca2+ Sparks in Saponin-Permeabilized Myocytes**

<table>
<thead>
<tr>
<th></th>
<th>∆F/F0</th>
<th>Rise Time, ms</th>
<th>Duration HA, ms</th>
<th>Width HM, μm</th>
<th>Frequency, s-1</th>
<th>100 μm-1</th>
<th>No. of Sparks</th>
<th>No. of Cells</th>
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<td>Ad-Control</td>
<td>1.32±0.02</td>
<td>8.0±0.2</td>
<td>15.6±0.2</td>
<td>2.44±0.03</td>
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<td>Ad-CASQ2WT</td>
<td>1.73±0.04*</td>
<td>13.6±0.3*</td>
<td>25.9±0.5*</td>
<td>3.03±0.05*</td>
<td>2.9±0.4</td>
<td>885</td>
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<td>Ad-CASQ2L167H</td>
<td>1.08±0.02†</td>
<td>6.8±0.2†</td>
<td>13.7±0.2†</td>
<td>2.14±0.03†</td>
<td>3.7±0.7</td>
<td>684</td>
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<tr>
<td>Ad-CASQ2G112+5X</td>
<td>1.29±0.02†</td>
<td>7.8±0.1†</td>
<td>15.3±0.2†</td>
<td>2.39±0.03†</td>
<td>4.3±0.5</td>
<td>774</td>
<td>18</td>
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</table>

*P<0.001 compared with Ad-Control.  †P<0.001 compared with Ad-CASQ2WT.
distinct CASQ2 mutations develop CPVT supports the screening of the entire coding region of the CASQ2 gene even in the absence of consanguineous marriage.

Biochemical Properties of the CASQ2 Mutants

Our first objective was to investigate the biochemical properties of CASQ2G112S and of CASQ2L167H. The CPVT phenotype may be due either to a lowered affinity of CASQ2 for Ca2+ or the inability of mutant CASQ2 to undergo Ca2+-dependent conformational changes.

CASQ2G112S lacks the second, the third, and part of the first domain and is devoid of most of the acidic residues at the COOH-terminal tail, responsible for ion binding24; as predicted, we show that CASQ2G112S has lost its ability to bind Ca2+. CASQ2G112S also lacks the amino acids involved in either front-to-front or back-to-back interactions, so that its dimerization capability should be compromised.25,26 On the other hand, the dibasic hydrophobic site, known to be involved in back-to-back interaction25,26 in native CASQ2, is preserved in CASQ2G112S; thus, we predict that anomalous, stable, Ca2+-insensitive, pseudohomologous interactions may take place between endogenous CASQ2, CASQ2WT or CASQ2L167H, and CASQ2G112S.

The functional consequences of the mutant CASQ2L167H are less predictable. Alignment of the amino acid sequence of CASQ2 from different vertebrates shows that the sequence variation L167H occurs in a highly conserved residue located in a region supposedly involved in heterologous interactions between junctional SR proteins and CASQ2.27 The hypothesis that the new histidine residue could modify the charge balance of the protein, thus disrupting Ca2+-binding properties, was not supported by our experiments, ie, no change in Ca2+-binding properties of CASQ2L167H was detected. We next asked whether the transition from a hydrophobic amino acid to a basic one could have an impact on Ca2+-sensitive structural changes of CASQ2, as described for the D307H mutation by Houle et al.28 However, we showed that CASQ2L167H had normal solubility and folding properties and displayed the standard Ca2+-sensitive interaction with phenyl-Sepharose. Thus, CASQ2L167H responds to Ca2+ with the expected conformational changes, and it does not affect the dimerization capability of CASQ2. We conclude that CASQ2G112S is completely functionally impaired, whereas major biochemical and biophysical properties of CASQ2L167H are preserved, thus prompting the need for further characterization of electrophysiological properties of myocytes expressing this mutant.

Functional Properties of CASQ2 Mutants in Isolated Adult Cardiac Myocytes

We compared EC coupling and SR Ca2+ handling of the new CASQ2 mutants expressed in ventricular myocytes with those of CASQ2WT, and we showed that both mutations alter CASQ2 function. Overexpression of the deletion mutant showed dominant-negative effects, as judged by (1) reduction of active SR Ca2+ release and SR Ca2+ content compared with myocytes that overexpress CASQ2WT and control myocytes that express the endogenous CASQ2 and (2) generation of spontaneous, extrasyctolic Ca2+ transients and DADs in paced myocytes. Thus, abnormalities observed with CASQ2G112S were similar to those we previously identified in the CPVT mutant CASQ2D307H,3,8 and they were even more severe given the degree of inhibition of the Ca2+ signals and SR Ca2+ content. Overexpression of CASQ2G112S showed reduction of active SR Ca2+ release and SR Ca2+ content with respect to myocytes that overexpressed CASQ2WT, yet no generation of
spontaneous extrasystolic Ca\(^{2+}\) transients and DADs. Despite normal biochemical and biophysical properties, CASQ2\(^{L167H}\) is not a fully functional CASQ2, which suggests that the mutation either influences regulative properties of CASQ2 or retains partial activity and simultaneously acts as a partial dominant negative; these speculations deserve further investigation.

In conclusion, the 2 mutations lead to CASQ2 dysfunction through distinct molecular mechanisms. The inhibitory effects on cellular Ca\(^{2+}\) handling by CASQ2\(^{G112+5X}\) are almost certainly caused by disruption of the CASQ2 polymerization required for high-capacity Ca\(^{2+}\) binding that compromises the Ca\(^{2+}\) sequestration ability of the SR. This is in agreement with the observation that mutants lacking either the N- or C-terminal domains disrupt the “head-to-tail” polymerization of CASQ2.\(^{29,30}\) Additionally, in vitro Ca\(^{2+}\)-binding measurements showed a complete lack of Ca\(^{2+}\) binding by CASQ2\(^{G112+5X}\). Because Ca\(^{2+}\) binding is required for polymerization, these results are also consistent with the notion that mutant monomers cannot form polymers.

As to CASQ2\(^{L167H}\), its overexpression failed to increase SR Ca\(^{2+}\) content and Ca\(^{2+}\) release compared with CASQ2\(^{WT}\), thus implying altered regulation of RyR2 Ca\(^{2+}\) release channel function rather than changes in SR Ca\(^{2+}\) capacity. The L167H mutation is localized in the domain that might be involved in heterologous interactions between CASQ2 and the junctional proteins triadin and junctin,\(^{27}\) which are considered transducers of the CASQ2 effects on RyR2; because CASQ2 modulates RyR2 activity by inhibiting its open probability, defective interactions of CASQ2 with the RyR channel complex could lead to hyperactive RyRs and leaky SR Ca\(^{2+}\) stores. This in turn could contribute to the reduced total SR Ca\(^{2+}\) content in myocytes expressing CASQ2\(^{L167H}\) compared with myocytes overexpressing CASQ2\(^{WT}\). Future experiments with direct monitoring of intra-SR [Ca\(^{2+}\)] and measurements of CASQ2-triadin interactions may help to address this possibility.

The cellular mechanisms of CPVT caused by the homozygous G112+5X mutation and by the compound heterozygous G112+5X and L167H mutations appear to be similar to those we previously described for the D307H mutation.\(^{8}\) Specifically, abnormal intrastore Ca\(^{2+}\) handling (ie, impaired Ca\(^{2+}\) buffering or disrupted interactions of CASQ2 with the RyR2 complex) results in premature functional restitution of the release mechanism from a luminal Ca\(^{2+}\)-dependent refractoriness state, which in turn leads to spontaneous Ca\(^{2+}\) release and arrhythmogenic DADs.

**Implications of In Vitro Characterization of CASQ2\(^{G112+5X}\) and CASQ2\(^{L167H}\)**

The experimental investigations reported here raise several interesting points relevant to the understanding of the relationship between CASQ2 mutations and the clinical phenotype. First, we observe that CASQ2\(^{G112+5X}\) is not only unable to perform its physiological task but, when overexpressed in rat myocytes, it also interferes in a “dominant negative” fashion with the endogenous, native CASQ2. The proband of family I is a homozygous carrier of this mutation, and therefore he can be regarded as a CASQ2 “knockout” individual: his clinical phenotype consists of severe ventricular tachyarrhythmias, but he presents no macroscopic (because no biopsy was performed) structural alterations of the myocardium, which suggests that although CASQ2 serves an important role in cardiac cells, its absence is not lethal and does not affect cardiac development in humans. Second, CPVT does not develop in the heterozygous carriers who express either CASQ2\(^{WT}\) or CASQ2\(^{G112+5X}\), in fact, none of the 11 heterozygous carriers present in the 2 families developed ventricular arrhythmias. To account for the lack of clinical phenotype in the heterozygous carriers, we may speculate that the CASQ2\(^{G112+5X}\) allele may be translated at a lower level than the WT one, by a nonsense-mediated decay mechanism.\(^{31}\) Alternatively, long-term adaptation might have occurred, such as increased expression of another SR luminal Ca\(^{2+}\) binding protein (eg, calreticulin) or isoform transition (to skeletal muscle CASQ1). Lack of clinical phenotype in heterozygous carriers of CASQ2\(^{L167H}\) is consistent with the contention that this CASQ2 mutant may perform all regulative functions on RYR2. In fact, CASQ2\(^{L167H}\) does not affect active SR Ca\(^{2+}\) release, SR Ca\(^{2+}\) content, or the Ca\(^{2+}\) binding capacity, suggesting that endogenous (wild-type) CASQ2 can vicariate all the regulative functions regardless of the presence of CASQ2\(^{L167H}\).

Nonetheless, the combination of CASQ2\(^{L167H}\) with CASQ2\(^{G112+5X}\) brings about a critical reduction in calsequestrin functions, ie, it evokes a severe clinical phenotype, as indeed was observed in the proband of family II. Plausible interpretations are that either CASQ2\(^{L167H}\) is more susceptible to interference by the truncated CASQ2\(^{G112+5X}\) than the WT protein or that the regulative function on RYR2 cannot be complemented by CASQ2\(^{G112+5X}\).

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**Disclosures**

None.

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