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

Marina Raffaele di Barletta, PhD; Serge Viatchenko-Karpinski, PhD; Alessandra Nori, PhD; Mirella Memmi, PhD; Dmitry Terentyev, PhD; Federica Turcato, PhD; Giorgia Valle, PhD; Nicoletta Rizzi, PhD; Carlo Napolitano, MD, PhD; Sandor Gyorke, PhD; Pompeo Volpe, MD; Silvia G. Priori, MD, PhD

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 ($CASQ2^{G112+5X}$) in a child with stress-induced ventricular tachycardia and cardiac arrest. The same deletion was also identified in association with a novel point mutation ($CASQ2^{L167H}$) 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: $CASQ2^{G112+5X}$ did not bind Ca^{2+}, whereas $CASQ2^{L167H}$ had normal calcium-binding properties. When expressed in rat myocytes, both mutants decreased the sarcoplasmic reticulum Ca^{2+}-storing capacity and reduced the amplitude of I_{Ca}-induced Ca^{2+} transients and of spontaneous Ca^{2+} sparks in permeabilized myocytes. Exposure of myocytes to isoproterenol caused the development of delayed afterdepolarizations in $CASQ2^{G112+5X}$.

Conclusions—$CASQ2^{L167H}$ and $CASQ2^{G112+5X}$ alter CASQ2 function in cardiac myocytes, which leads to reduction of active sarcoplasmic reticulum Ca^{2+} release and calcium content. In addition, $CASQ2^{G112+5X}$ 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 arrhythmogenic disorder characterized by adrenergically mediated polymorphic ventricular tachyarrhythmias that lead to syncope and sudden cardiac death.[1,2] 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,[3] 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[4] on chromosome 1q42.1-q43.

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The cardiac ryanodine receptor (RyR2) is an intracellular Ca^{2+} release channel located in the membrane of the sarcoplasmic reticulum (SR).[5] Calsequestrin is a high-capacity, low-affinity Ca^{2+}-binding protein that represents a major Ca^{2+}-reservoir element within the SR lumen.[6] Both of these Ca^{2+}-handling proteins are critically involved in cardiac excitation-contraction (EC) coupling through the mechanism of calcium-induced calcium release,[7] 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[8] the first functional characterization of the *CASQ2* mutant identified by Lahat et al,[3] and we were able to

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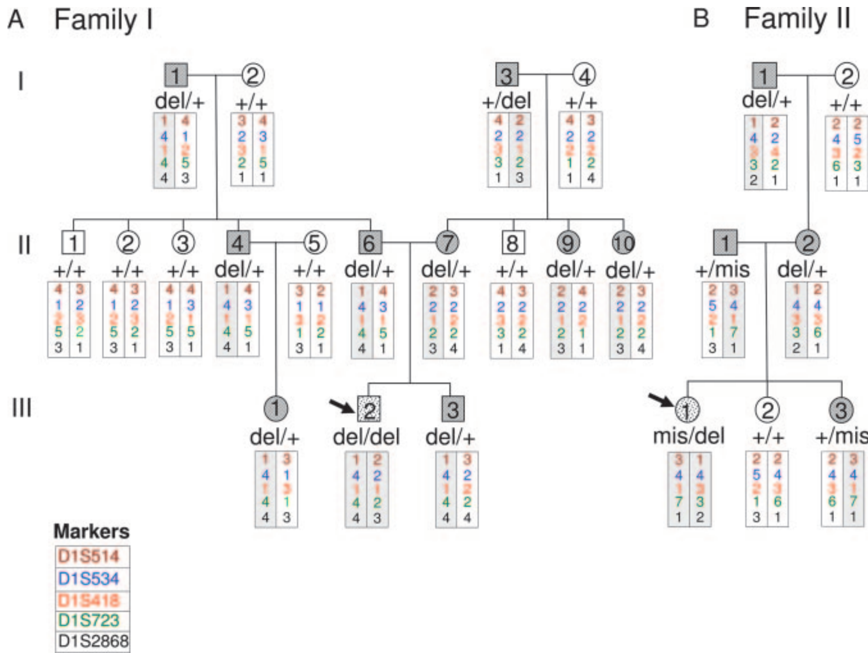

Figure 1. A and B, Pedigrees of the 2 *CASQ2* families. Squares indicate males; circles, females; dotted symbols, carriers of 2 *CASQ2* mutations who were symptomatic for CPVT; shaded symbols, asymptomatic heterozygous carriers of 1 *CASQ2* mutation; white symbols, individuals without *CASQ2* mutations; del, G112+5X mutation; and mis, L167H mutation. Typing for the 5 markers used for haplotype analysis is shown below symbols.

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 developed[8] 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 primers[10] 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 described[11] 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% CO_2 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 $CaCl_2$, 0.5 $MgCl_2$, 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 Na_2ATP, 3.5 $MgCl_2$, 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 Ca^{2+} 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

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described previously.[8] 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 *Nde*I-ATG-pT7 F coupled either with reverse *Eco*RI-XX R (WT and L167H) or *Eco*RI-XX-Trunc R (G112+5X). PCR products were digested with *Nde*I and *Eco*RI, 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)[12] 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 $CaCl_2$ in 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[13] and Lowry et al.[14]

Western Blot and Stains: All Staining

SDS-PAGE was performed on either 7.5% or 15% gels.[15] Slab gels were stained with the cationic carbocyanine dye "Stains-all" (Sigma-Aldrich, St Louis, Mo) for identification of Ca^{2+}-binding proteins.[16] Western blot with anti-CASQ2 polyclonal antibodies (ABR–Affinity Bioreagents, Golden, Colo) was performed as described previously.[17] Levels of CASQ2 in cultured myocytes were determined as described previously.[18] 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

^{45}Ca ligand overlay was performed on purified CASQ2 (2 to 3 μg of protein) electroblotted on nitrocellulose membranes,[19] in a medium containing 5 mmol/L $MgSO_4$, 60 mmol/L KCl, 5 mmol/L imidazole, pH 7.4, and 0.6 to 6 μmol/L ^{45}Ca (specific activity 5 to 50 mCi/mg Ca). Single lanes were incubated at room temperature for 20 minutes at different total Ca^{2+} 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 area of nitrocellulose equivalent to that of CASQ2.

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

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 several 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).

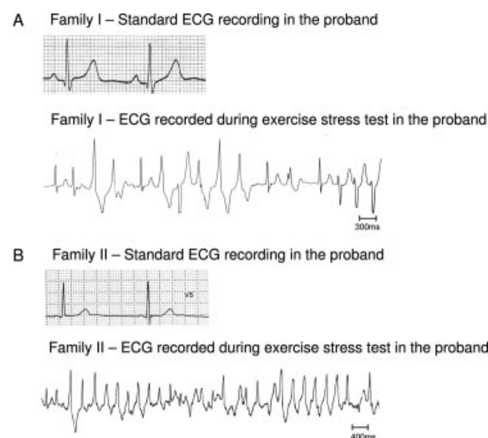

Figure 2. A, Resting ECG (upper trace) and example of ECG recorded during exercise stress test (lower trace) recorded in proband of family 1. B, Resting ECG (upper trace) and example of ventricular tachycardia recorded during exercise stress test in proband of family II.

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 I (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-

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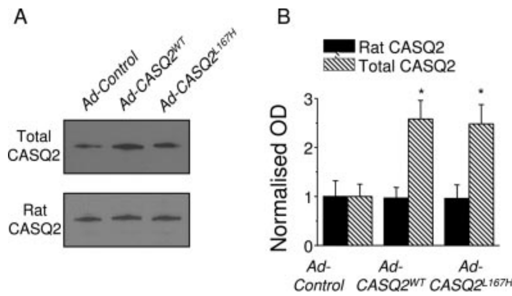

Figure 3. Immunoblot analysis of CASQ2 levels in myocytes infected with *Ad-Control*, *Ad-CASQ2^{WT}*, and *Ad-CASQ2^{L167H}* vectors. A, Representative Western blot of total CASQ2 (rat and human; top) and rat CASQ2 alone (bottom). B, Normalized optical density (OD) for rat and total CASQ2. Comparisons were performed by 1-way ANOVA. *$P<0.05$ (n=6 and n=5 for total and rat CASQ2, respectively). Measurements were performed 48 hours after infection of myocytes with the adenoviral constructs.

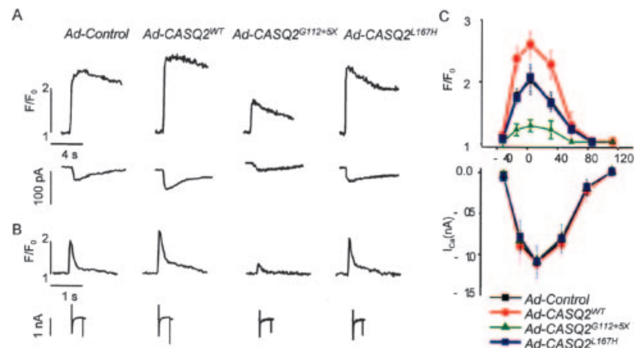

Figure 4. Effects of *CASQ2* mutations on SR Ca^{2+} content in caffeine- and I_{Ca}-induced Ca^{2+} transients. A, Caffeine-induced intracellular Ca^{2+} transients (upper traces) and Na^+/Ca^{2+} exchange current (lower traces) in myocytes infected with *Ad-Control*, *Ad-CASQ2^{WT}*, *Ad- CASQ2$^{G112+5X}$*, and *Ad-CASQ2^{L167H}*. B, Recordings of Ca^{2+} transients (top traces) and I_{Ca} (bottom traces) in cardiomyocytes infected with *Ad-Control*, *Ad-CASQ2^{WT}*, *Ad-CASQ2$^{G112+5X}$*, and *Ad-CASQ2^{L167H}*. C, Voltage dependence of Ca^{2+} transients (upper profile) and I_{Ca} (lower profile) in myocytes infected with *Ad-Control*, *Ad-CASQ2^{WT}*, *Ad- CASQ2$^{G112+5X}$*, and *Ad-CASQ2^{L167H}*.

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 Ca^{2+} Measurements

To characterize the effects of the new *CASQ2* mutations on SR Ca^{2+} 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[8,20] (Figure 3). Importantly, the level of endogenous rat CASQ2 remained unchanged in cells expressing exogenous human CASQ2, as revealed with rat CASQ2-specific antibody (Figure 3). Caffeine (10 mmol/L) was applied to myocytes expressing either WT or mutant CASQ2 to evaluate the SR Ca^{2+}-storing capacity by measurement of fluo-3 fluorescence and of Na^+/Ca^{2+} exchange current (I_{NCX}; Figure 4A; Table 1).[21] Expression of WT increased the SR Ca^{2+} content by 50%, whereas CASQ2 $^{G112+5X}$ reduced the SR content, not only as compared with WT expression but also as compared with native cells (Ad-control). Expression of the CASQ2^{L167H} mutant significantly reduced the SR Ca^{2+} content compared with WT expression, albeit to a lesser extent than observed in myocytes expressing CASQ2$^{G112+5X}$.

The effects of the new *CASQ2* mutants on active Ca^{2+} release during EC coupling were studied in myocytes undergoing voltage-clamp stimulation (Figures 4B and 4C; Table 2). Expression of CASQ2$^{G112+5X}$ and expression of CASQ2^{L167H} caused a significant decrease in the amplitude of Ca^{2+} transient triggered by I_{Ca} 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 Ca^{2+} transients were shortened. The effects observed with CASQ2$^{G112+5X}$ were much more prominent than those observed with CASQ2^{L167H}.

The effects of the new *CASQ2* mutants on the properties of focal fluorescence signals, ie, Ca^{2+} sparks, were studied in saponin-permeabilized myocytes kept at a constant cytosolic $[Ca^{2+}]$ (≈100 nmol/L; Figure 5; Table 3). When CASQ2^{WT} was overexpressed, Ca^{2+} sparks were greater and longer than in native cells, in agreement with our previous results.[20] Compared with WT, expression of CASQ2$^{G112+5X}$ and of CASQ2^{L167H} 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 Ca^{2+} transients, the effects observed with CASQ2$^{G112+5X}$ were more prominent than those observed with CASQ2^{L167H}.

Finally, we examined the effects of expressing the *CASQ2* mutants on Ca^{2+} transients and action potentials in paced

TABLE 1. Effects of Overexpression of *CASQ2* Mutants on SR Ca^{2+} Content in Caffeine- and I_{Ca}-Induced Ca^{2+} Transients in Rat Ventricular Myocytes

	Ca^{2+} Transients by Fluo-3 Fluorescence, F/F_0	I_{NCX}, (pC/pF)	n
Ad-Control	2.6±0.2	0.57±0.11	10
Ad-CASQ2^{WT}	3.4±0.3*	0.88±0.15*	4
Ad-CASQ2$^{G112+5X}$	1.64±0.4*†	0.28±0.11*†	3
Ad-CASQ2^{L167H}	2.8±0.3†	0.55±0.12†	3

I_{NCX} indicates Na^+/Ca^{2+} exchange current.

The average amplitude (F/F_0) of caffeine-induced Ca^{2+} transients and integrals of I_{NCX} density for *Ad-Control*, *Ad-CASQ2^{WT}*, *Ad-CASQ2$^{G112+5X}$*, and *Ad-CASQ2^{L167H} are shown.*

*$P<0.001$ compared with *Ad-Control*.

†$P<0.001$ compared with *Ad-CASQ2^{WT}*.

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TABLE 2. I_{Ca} and Ca^{2+} Transients in Control Condition and With Overexpression of *CASQ2* Mutants

	I_{Ca}			Ca^{2+} Transients			
	Peak Amplitude, nA	τ_{fast}, ms	τ_{slow}, ms	F/F_0	Rise Time, ms	τ_{decay}, ms	No. of Cells
Ad-Control	−1.10±0.14	16.5±4.3	80±27	2.0±0.2	30±4	320±24	10
Ad-CASQ2^{WT}	−1.12±0.20	16.8±4.7	86±26	2.6±0.2*	43±4*	407±35*	6
Ad- CASQ2$^{G112+5X}$	−1.09±0.16	16.3±4.1	82±19	1.5±0.1*†	20±3*†	261±21*†	7
Ad-CASQ2^{L167H}	−1.10±0.20	16.4±4.9	88±28	2.1±0.2†	33±6	324±31†	3

*$P<0.001$ compared with *Ad-Control*.
†$P<0.001$ compared with *Ad-CASQ2*^{WT}.

myocytes exposed to isoproterenol (1 μmol/L). Myocytes expressing CASQ2$^{G112+5X}$ exhibited spontaneous extrasystolic Ca^{2+} elevations and DADs (in 4 of 4 myocytes tested; Figure 6). Myocytes expressing either the WT or the L167H mutant CASQ2 showed no spontaneous Ca^{2+} transients or DADs (n=4 and n=3, respectively; not shown).

Molecular Properties of Mutant CASQ2

To investigate the molecular mechanisms causing aberrant Ca^{2+} handling in ventricular myocytes, recombinant CASQ2 was expressed in vitro and thereafter purified by standard procedures. All recombinant WT and mutant CASQ2 (CASQ2^{WT}, T7-CASQ2^{WT}, CASQ2^{L167H}, and T7-CASQ2$^{G112+5X}$) were found in the soluble fraction after high-speed centrifugation, which indicates that either addition of the T7-Tag, the point mutation (L167H), or the long 260 aa deletion, introduced 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 CASQ2^{WT} without the T7-Tag migrated with a molecular weight of 55 kDa; slower migration was detected for the T7-bearing recombinant protein (T7-CASQ2^{WT}), whereas the deleted T7-CASQ2$^{G112+5X}$ had an estimated molecular weight of ≈14 kDa.

Some biochemical properties of native CASQ2 were likewise shared by all recombinant CASQ2s except for T7-CASQ2$^{G112+5X}$. In the absence of Ca^{2+}, successful hydrophobic binding to phenyl-sepharose was obtained; subsequently, quantitative Ca^{2+}-dependent elution of CASQ2^{WT}, T7-CASQ2^{WT}, and CASQ2^{L167H} showed that the hydrophobic site involved in interaction with phenyl-sepharose (aa 214 to 222)[22] is exposed in the absence of Ca^{2+} and that 2 to 10 mmol/L Ca^{2+} induced the expected conformational change that led to internalization of the region, as in native CASQ2. T7-CASQ2$^{G112+5X}$ lacked the Ca^{2+}-regulated hydrophobic site and could not be eluted from phenyl-sepharose. This observation indicates that the hydrophobic/hydrophilic distribution of the residues at the surface of the molecule is sufficient for binding to phenyl-sepharose but does not allow any Ca^{2+}-dependent conformational change. These results demonstrate that the L167H mutation does not modify the chemical properties of CASQ2, whereas the long deletion drastically affects both the Stains-all–CASQ complex and Ca^{2+}-induced conformational changes, which indicates that different molecular mechanisms could be involved in the aberrant SR Ca^{2+}-handling phenotypes.

Calcium Binding to CASQ2

To investigate whether a decrease of SR Ca^{2+} content in ventricular myocytes is related to modifications of Ca^{2+}-binding properties of mutant CASQ2, Ca^{2+} overlay experiments were performed to compare Ca^{2+}-binding properties of recombinant WT and mutant CASQ2. Ca^{2+} affinity (K_d) and capacity (B_{max}) values are shown in Table 4. Human CASQ2^{WT} displayed K_d and B_{max} values comparable to those reported for native CASQ2 from different animal species.[12,23] CASQ2^{L167H} did not display any significant change in K_d and B_{max} compared with CASQ2^{WT}. On the contrary, CASQ2$^{G112+5X}$ did not bind Ca^{2+} at all according to pink staining with Stains-all. This effect was not due to the presence of T7-Tag, because K_d and B_{max} of Ca^{2+} binding for CASQ2^{WT} and T7-CASQ2^{WT} were not significantly different (Table 4).

Discussion

Homozygous 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 3. Effects of Overexpression of *CASQ2* Mutants on Ca^{2+} Sparks in Saponin-Permeabilized Myocytes

	ΔF/F_0	Rise Time, ms	Duration HA, ms	Width HM, μm	Frequency, $s^{-1}\cdot 100\ \mu m^{-1}$	No. of Sparks	No. of Cells
Ad-Control	1.32±0.02	8.0±0.2	15.6±0.2	2.44±0.03	4.1±0.4	821	22
Ad-CASQ2^{WT}	1.73±0.04*	13.6±0.3*	25.9±0.5*	3.03±0.05*	2.9±0.4	885	17
Ad- CASQ2$^{G112+5X}$	1.08±0.02*†	6.8±0.2*†	13.7±0.2*†	2.14±0.03*†	3.7±0.7	684	15
Ad-CASQ2^{L167H}	1.29±0.02†	7.8±0.1†	15.3±0.2†	2.39±0.03†	4.3±0.5	774	18

*$P<0.001$ compared with *Ad-Control*.
†$P<0.001$ compared with *Ad-CASQ2*^{WT}.

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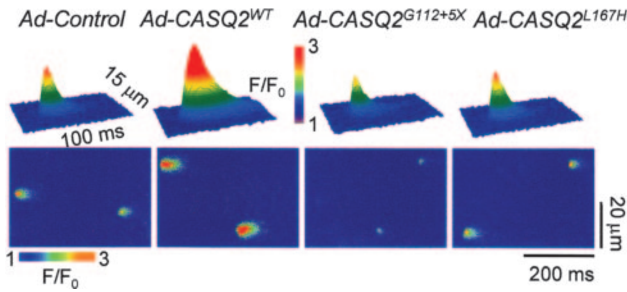

Figure 5. Effect of the *CASQ2* mutants on Ca^{2+} sparks in saponin-permeabilized myocytes. Surface plots of averaged Ca^{2+} sparks (20% of the largest events) in myocytes infected with *Ad-Control* (n=36), *Ad-CASQ2*^{WT} (n=16), *Ad- CASQ2*$^{G112+5X}$ (n=24), and *Ad-CASQ2*^{L167H} (n=25). Representative line-scan images of Ca^{2+} sparks acquired in myocytes infected with *Ad-Control*, *Ad-CASQ2*^{WT}, *Ad- CASQ2*$^{G112+5X}$, and *Ad-CASQ2*^{L167H}.

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 CASQ2$^{G112+5X}$ and of CASQ2^{L167H}. The CPVT phenotype may be due either to a lowered affinity of CASQ2 for Ca^{2+} or the inability of mutant *CASQ2* to undergo Ca^{2+}-dependent conformational changes.

CASQ2$^{G112+5X}$ 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 binding[24]; as predicted, we show that CASQ2$^{G112+5X}$ has lost its ability to bind Ca^{2+}. CASQ2$^{G112+5X}$ 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 interaction[25,26] in native CASQ2, is preserved in CASQ2$^{G112+5X}$; thus, we predict that anomalous, stable, Ca^{2+}-insensitive, pseudohomologous interactions may take place between endogenous CASQ2, CASQ2^{WT} or CASQ2^{L167H}, and CASQ2$^{G112+5X}$.

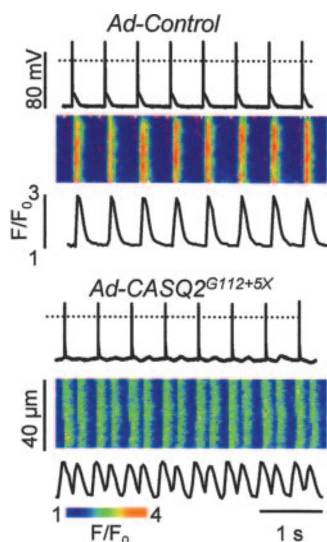

Figure 6. Abnormal Ca^{2+} cycling in paced myocytes. Recordings of membrane potential (upper traces), line-scan images (middle traces), and time-dependent profiles (lower traces) of $[Ca^{2+}]$ in control (A) and *CASQ2*$^{G112+5X}$ (B) myocytes exposed to 1 μmol/L isoproterenol and stimulated at 2 Hz.

TABLE 4. Ca^{2+} Binding Properties of WT and Mutant *CASQ2*

Mutant	K_d, mmol/L	B_{max}, pmol/μg	n
CASQ2^{WT}	2.15±0.197	789±71.8	7
CASQ2^{L167H}	2.02±0.240	723±67.9	7
T7-CASQ2^{WT}	2.29±0.277	714±87.2	7
T7-CASQ2$^{G112+5X}$	Not determined	Not detectable	2

K_d and B_{max} values are expressed as mean of n experiments±SD. No significant differences were observed between *CASQ2*^{WT} *and CASQ2*^{L167H} *either with or without T7 tag.*

The functional consequences of the mutant CASQ2^{L167H} 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 Ca^{2+}-binding properties, was not supported by our experiments, ie, no change in Ca^{2+}-binding properties of CASQ2^{L167H} was detected. We next asked whether the transition from a hydrophobic amino acid to a basic one could have an impact on Ca^{2+}-sensitive structural changes of CASQ2, as described for the D307H mutation by Houle et al.[28] However, we showed that CASQ2^{L167H} had normal solubility and folding properties and displayed the standard Ca^{2+}-sensitive interaction with phenyl-sepharose. Thus, CASQ2^{L167H} responds to Ca^{2+} with the expected conformational changes, and it does not affect the dimerization capability of CASQ2. We conclude that CASQ2$^{G112+5X}$ is completely functionally impaired, whereas major biochemical and biophysical properties of CASQ2^{L167H} 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 Ca^{2+} handling of the new *CASQ2* mutants expressed in ventricular myocytes with those of CASQ2^{WT}, 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 Ca^{2+} release and SR Ca^{2+} content compared with myocytes that overexpress CASQ2^{WT} and control myocytes that express the endogenous CASQ2 and (2) generation of spontaneous, extrasystolic Ca^{2+} transients and DADs in paced myocytes. Thus, abnormalities observed with CASQ2$^{G112+5X}$ were similar to those we previously identified in the CPVT mutant CASQ2^{D307H},[3,8] and they were even more severe given the degree of inhibition of the Ca^{2+} signals and SR Ca^{2+} content. Overexpression of CASQ2^{L167H} showed reduction of active SR Ca^{2+} release and SR Ca^{2+} content with respect to myocytes that overexpressed CASQ2^{WT}, yet no generation of

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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 *$CASQ2^{G112+5X}$* with *$CASQ2^{L167H}$* 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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CLINICAL PERSPECTIVE

Homozygous mutations in the cardiac calsequestrin gene (*CASQ2*) cause the autosomal recessive form of catecholaminergic polymorphic ventricular tachycardia (CPVT), a disease characterized by adrenergically mediated polymorphic and bidirectional ventricular tachycardia and sudden death. We identified a novel homozygous deletion in the calsequestrin gene that led to a stop codon and to a truncated calsequestrin protein in a child with stress-induced ventricular tachycardia and cardiac arrest. The same deletion was also identified in association with a novel point mutation (L167H) in a highly symptomatic CPVT child who was the first CPVT patient carrier of compound heterozygous *CASQ2* mutations. In vitro studies demonstrated that both *CASQ2* mutants created distinct abnormalities that led to abnormal intracellular calcium regulation, thus facilitating the development of tachyarrhythmias. The first proband, who was homozygous for the deletion that is functionally completely impaired, survived without functional calsequestrin (as if he were a "functional" knockout for the CASQ2) and with no cardiac structural abnormalities. This study shows that carriers of 2 distinct *CASQ2* mutations develop CPVT, which indicates that when genetic screening is performed for this disease, the entire coding region of the *CASQ2* gene should be screened, even in the absence of consanguinity of the parents of the affected individual.

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