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Calmodulin Mutations Associated With Recurrent Cardiac Arrest in Infants

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Background—Life-threatening disorders of heart rhythm may arise during infancy and can result in the sudden and tragic death of a child. We performed exome sequencing on 2 unrelated infants presenting with recurrent cardiac arrest to discover a genetic cause.

Methods and Results—We ascertained 2 unrelated infants (proband) with recurrent cardiac arrest and dramatically prolonged QTc interval who were both born to healthy parents. The 2 parent-child trios were investigated with the use of exome sequencing to search for de novo genetic variants. We then performed follow-up candidate gene screening on an independent cohort of 82 subjects with congenital long-QT syndrome without an identified genetic cause. Biochemical studies were performed to determine the functional consequences of mutations discovered in 2 genes encoding calmodulin. We discovered 3 heterozygous de novo mutations in either *CALM1* or *CALM2*, 2 of the 3 human genes encoding calmodulin, in the 2 probands and in 2 additional subjects with recurrent cardiac arrest. All mutation carriers were infants who exhibited life-threatening ventricular arrhythmias combined variably with epilepsy and delayed neurodevelopment. Mutations altered residues in or adjacent to critical calcium binding loops in the calmodulin carboxyl-terminal domain. Recombinant mutant calmodulins exhibited several-fold reductions in calcium binding affinity.

Conclusions—Human calmodulin mutations disrupt calcium ion binding to the protein and are associated with a life-threatening condition in early infancy. Defects in calmodulin function will disrupt important calcium signaling events in heart, affecting membrane ion channels, a plausible molecular mechanism for potentially deadly disturbances in heart rhythm during infancy. (*Circulation*. 2013;127:1009-1017.)

Key Words: arrhythmia ■ calcium signaling ■ death, sudden, cardiac ■ exome

Sudden unexplained death during early development (prenatal period through infancy) may be caused by inborn errors, including severe chromosomal abnormalities and monogenic conditions that predispose to life-threatening

cardiac arrhythmias. Highly malignant arrhythmias may predispose to intrauterine fetal morbidity and mortality^{1,2} and sudden death of a neonate or infant as in the sudden infant death syndrome.³⁻⁶ When a genetic cause has been identified

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in these early-onset and highly malignant conditions, mutations are often de novo,^{3,7–9} but many cases do not have a clear genetic or molecular explanation.

Editorial see p 968 Clinical Perspective on p 1017

In conditions such as the congenital long-QT syndrome (LQTS) and other inherited arrhythmia syndromes, the predisposition to sudden cardiac death is due to dysfunctional cardiac ion channels caused by mutations in genes encoding either pore-forming subunits or channel-interacting proteins.¹⁰ The identification of novel arrhythmia susceptibility genes, particularly in clinically extreme cases, has great value for understanding the molecular basis of sudden cardiac death, including unexplained infant mortality, and has the potential to inspire new therapeutic approaches.

Here, we report that mutations in 2 genes encoding calmodulin, a ubiquitous and essential calcium signaling protein involved critically in myriad physiological events, are associated with life-threatening cardiac arrhythmias accompanied variably by neurological complications. We made this discovery by performing exome sequencing on 2 unrelated infants with recurrent cardiac arrest and subsequently by examining calmodulin genes for mutations in a cohort of LQTS patients without a defined genetic origin. These findings suggest phenotypic and biochemical consequences of human calmodulin mutations and offer a molecular basis for a novel life-threatening condition occurring in infancy.

Methods

Study Subjects

Study subjects were ascertained following informed consent procedures approved by the Ethics Review Board of the Fondazione IRCCS Policlinico San Matteo (Pavia, Italy), the Ethics Review Board of Klinikum Grosshadern (Munich, Germany), or the Institutional Review boards of the Advocate Lutheran General Hospital and Cincinnati Children's Hospital. Peripheral blood leukocytes were collected and used for isolation of genomic DNA. Study subjects included 2 probands (1 white, 1 Hispanic) with highly malignant ventricular arrhythmia syndromes, their respective unaffected parents, and 82 additional unrelated patients with LQTS without an identified genetic cause. Control subjects were from 2 sources. A panel of Hispanic Americans (n=92) was obtained from the Coriell Institute for Medical Research, and a panel of white Europeans (n=1800) for whom exome data were available was obtained through the Institute of Human Genetics (Helmholtz Zentrum München).

Exome Sequencing

Exome enrichment was performed with the Agilent SureSelect Human All Exon 50 Mb capture reagent used according to the supplier's instructions. Paired-end (2×100 bp) sequencing was performed on the Illumina HiSeq2000 platform. Proband 1 was sequenced at the Institute of Human Genetics, Helmholtz Zentrum München; proband 2 was sequenced by the Genome Sciences Resource, Vanderbilt University.

Exome Sequence Data Analysis

After removal of low-quality reads, alignments to a reference human genome (UCSC assembly hg19) were performed with BWA (version 0.5.8),¹¹ and then sequences were processed with the Genome Analysis Toolkit¹² to remove duplicate reads and to call variants. Default settings were used in the BWA alignments, including a

maximum of 2 mismatches in the seed portion of reads (first 32 bp) and no more than 3 mismatches for the entire read. The threshold for detecting variants was set at a genotype quality score of 40.

Variants identified in the probands that were also found in dbSNP (version 130), 1000Genomes, Exome Variant Server, and Helmholtz exome databases were excluded from further analyses. Synonymous and intronic (other than canonical splice sites) variants were also excluded. Variant annotation was performed with custom scripts. Furthermore, on the basis of the hypothesis that disease-causing mutations in the probands are de novo, we excluded all inherited variants (ie, that were observed in either parent). Variants that remained after filtering were validated in proband and parental DNA with conventional Sanger sequencing and then further annotated on the basis of evolutionary nucleotide conservation,¹³ and predicted impact on protein function (Polyphen2, SIFT).^{14,15}

Additional Mutation Detection

Mutation screening was performed in 82 unrelated LQTS subjects (Schwartz Score ≥ 3.5 ¹⁶ and/or resting QTc ≥ 480 milliseconds) without a previously identified genetic cause to search for additional variants in the candidate disease-causing genes identified in the 2 probands. Specifically, the coding exons of *CALM1*, *CALM2*, and *CALM3*, 3 genes encoding identical calmodulin proteins, were amplified by polymerase chain reaction (Table 1 in the online-only Data Supplement provides the primer sequences) and then sequenced through the use of an automated capillary electrophoresis DNA sequencing platform (Applied Biosystems, Foster City, CA).

Calmodulin Gene Expression and Functional Analyses

The methods for determining calmodulin gene expression in human heart and biochemical studies of recombinant calmodulin proteins are described in detail in the online-only Data Supplement.

Results

Study Subjects

We ascertained 2 unrelated infants (probands) with recurrent cardiac arrest. Proband 1 was a white girl from Italy who suffered cardiac arrest caused by ventricular fibrillation (VF) at 6 months of age. Her birth and prenatal history were unremarkable; her family history was negative for sudden cardiac death; and both parents were asymptomatic with normal ECGs. There had been no evidence of fetal bradycardia. After successful external defibrillation, an ECG demonstrated a markedly prolonged QTc interval (630 milliseconds), frequent episodes of T-wave alternans, and intermittent 2:1 atrioventricular block (Figure 1). Echocardiogram revealed normal cardiac anatomy and contractile function. The patient was treated with propranolol, and an internal cardioverter-defibrillator was placed. Genetic testing for mutations in *KCNQ1*, *KCNH2*, *SCN5A*, *KCNE1*, and *KCNE2* (Molecular Cardiology Laboratory, Fondazione IRCCS Policlinico S. Matteo, Pavia, Italy), the most frequently mutated genes in LQTS, was negative.

In the following months, the child had multiple episodes of VF that were terminated by internal cardioverter-defibrillator shocks. Propranolol dosage was progressively increased to 10 mg·kg⁻¹·d⁻¹ without arrhythmia suppression. Left cardiac sympathetic denervation was performed at 12 months of age, but QTc remained prolonged (550–630 milliseconds), and episodes of T-wave alternans continued to occur. A loading dose of mexiletine did not shorten the QT interval. Verapamil

Table. Summary of Clinical Features of Calmodulin Mutation Carriers

Subject	Sex	Age at Diagnosis	VF	QTc, ms	TWA	2:1 AVB	Seizures	Developmental Delay	Treatments	Mutation*
Proband 1	F	6 mo	+	630	+	+	–	+/-	BB, MEX, VER, FLEC, ICD, LCSD, RCSD	CALM1-D130G
Proband 2	F	Prenatal	+	690	+	+	+	+	BB, MEX, ICD	CALM2-D96V
Patient 3	M	1 mo	+	610	+	+	+	+	BB, MEX, ICD	CALM1-D130G
Patient 4	M	? Neonatal	+	>600	+	-	+	+	BB, MEX, LCSD, ICD	CALM1-F142L

AVB indicates atrioventricular block; BB, β -blocker (propranolol); FLEC, flecainide; ICD, implantable cardioverter-defibrillator; LCSD, left cardiac sympathetic denervation; MEX, mexiletine; QTc, rate-corrected QT interval; RCSD, right cardiac sympathetic denervation; TWA, T-wave alternans; VER, verapamil; and VF, ventricular fibrillation.

*Mutation position is based on RefSeq NP_005175 and counting the predicted translational start codon (Met) as position 1.

reduced the frequency of T-wave alternans, but persistent cardiac electric instability prompted right cardiac sympathetic denervation. Despite all treatments, there were 16 episodes of VF during the first 2 years of life, mostly induced by adrenergic stimulation and beginning either abruptly or preceded by a brief episode of torsades de pointes that was not pause dependent. Verapamil was replaced with flecainide, and in the following year, she had only 1 VF episode triggered by strong emotional stress. A mild delay in language development was noted.

Proband 2 was a Hispanic female infant living in the United States who presented with sinus bradycardia, T-wave

alternans, markedly prolonged QTc (690 milliseconds), and 2:1 atrioventricular block occurring 2 hours after a normal delivery (Figure 1). Fetal bradycardia (98–110 bpm) was first noted at 21 weeks' gestation and lasted throughout the pregnancy. Fetal echocardiogram performed at 27 weeks' gestation revealed normal cardiac anatomy and function except for a heart rate of 90 to 95 bpm. An episode of 2:1 atrioventricular block was noted at 28 weeks' gestation. There was no family history of arrhythmia, miscarriages, sudden death, seizures, or drowning, and ECGs from both parents and an older sister were normal. Postnatally, esmolol and

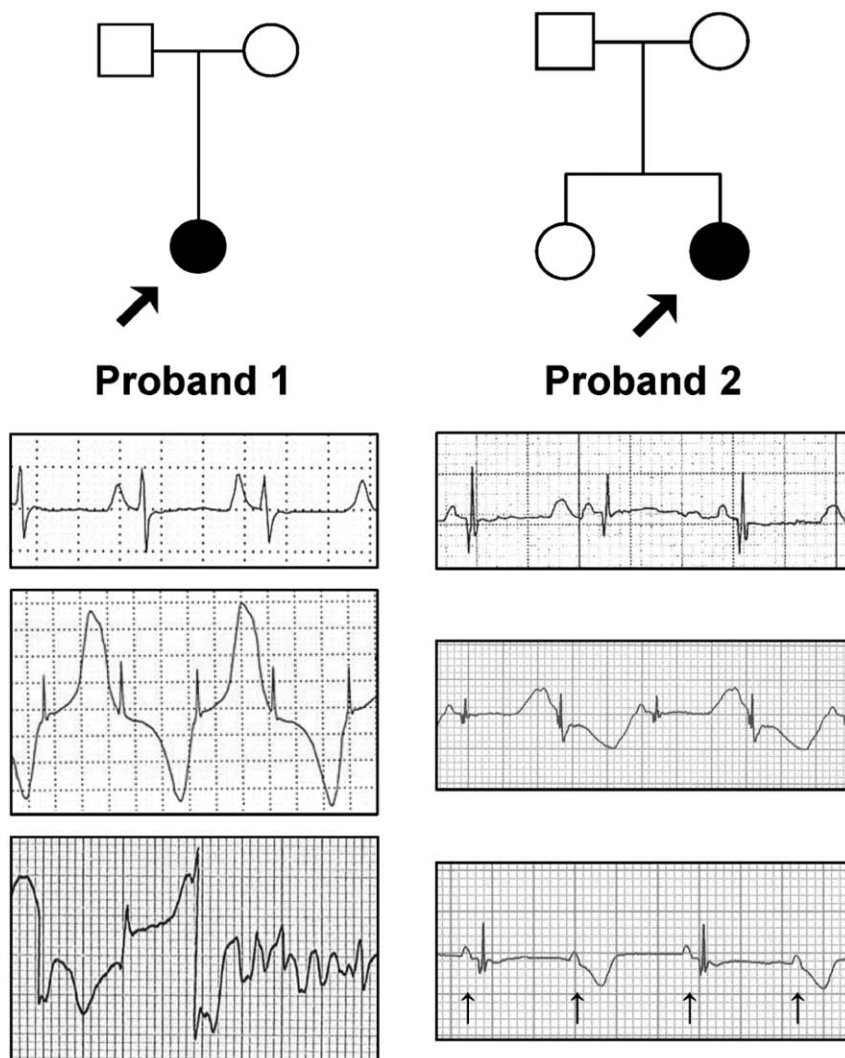


Figure 1. Clinical phenotypes and pedigrees. Representative ECG recordings from 2 probands with early-onset, life-threatening cardiac arrhythmias. **Top trace** represents baseline ECG for both probands. **Middle trace** illustrates T-wave alternans. **Bottom trace** for proband 1 illustrates the onset of ventricular fibrillation after a period of T-wave alternans. **Bottom trace** for proband 2 illustrates 2:1 atrioventricular block (arrows mark p waves coincident with atrial depolarization).

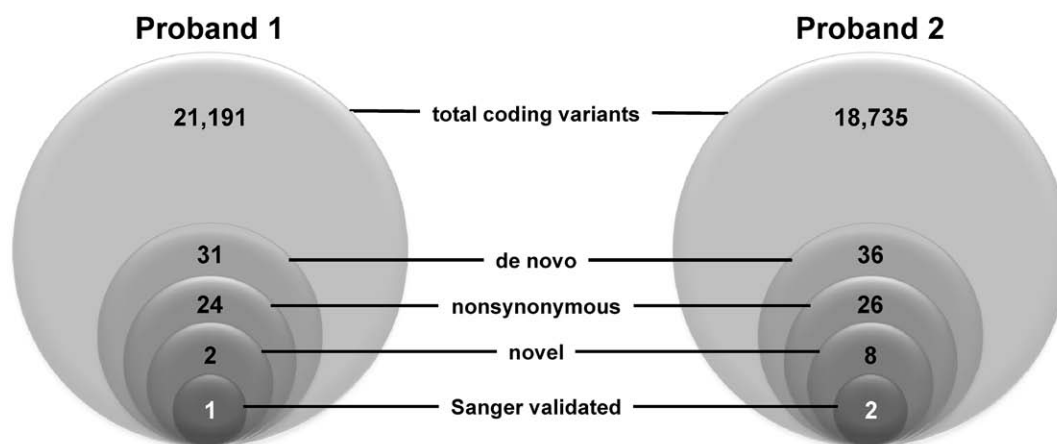


Figure 2. Exome variant filtering strategy. Sequential filters were applied to the pool of variants discovered by exome sequencing in the 2 probands. Total coding variants include all synonymous, nonsynonymous, nonsense, and frameshift-inducing insertions or deletions and canonical splice-site variants within captured exons, but they exclude variants in 5' and 3' untranslated regions and introns. De novo variants are those found in the proband but not in either parent. Novel variants were defined as those absent in public databases (dbSNP, 1000genomes). Novel variants in proband 1 were also absent in 1800 exomes generated in the Institute of Human Genetics (Helmholtz Zentrum München).

propranolol treatment restored 1:1 atrioventricular conduction, but lidocaine did not shorten the QTc interval. Genetic testing was negative for mutations in *KCNQ1*, *KCNH2*, *SCN5A*, *KCNE1*, and *KCNE2* (Familion, Transgenomic Labs).

She was discharged from the hospital on postnatal day 8 but returned at 3 weeks of age after cardiac arrest and multiple episodes of VF. She was successfully defibrillated but suffered a right parietal lobe cerebral infarction that was documented by brain magnetic resonance imaging. Treatment at that time included propranolol, mexiletine, and an internal cardioverter-defibrillator. In the first 2 years of life, she was hospitalized numerous times for episodes of VF that were successfully terminated by internal cardioverter-defibrillator shocks. At 2 years of age, she developed seizures that were attributed to the prior brain injury. At 3 years of age, seizures were well controlled with levetiracetam, and her QTc was 500 to 510 milliseconds on a combination of mexiletine and propranolol, but she exhibited developmental delays (10% to 33%) in all categories of the Hawaii Early Learning Profile.

Discovery of Calmodulin Mutations by Exome Sequencing

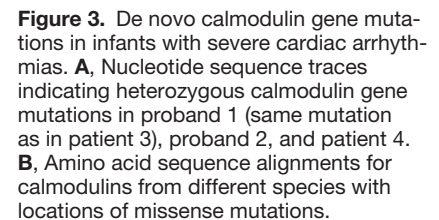
Because both sets of parents were healthy with normal ECGs and there was no overt family history of sudden death or related symptoms suggesting an inherited cardiac arrhythmia syndrome, we hypothesized that de novo mutations were most likely. Furthermore, because genetic testing for the major LQTS susceptibility genes was unrevealing in the probands, we predicted that a novel genetic basis for the clinical disease was plausible. Therefore, we performed exome sequencing on the 2 probands and their parents (parent-child trios) and then searched for novel variants that were not inherited and predicted to have deleterious effects on protein structure or function. Exome sequencing of parent-child trios has emerged as a powerful approach for discovering de novo mutations in novel genes.^{17,18}

For proband 1 and her parents, the mean coverage depth across 50 Mb of captured sequence was 104-fold for the 3

samples, with 20-fold coverage depth for 90.5% of targeted bases and 88% of high-quality reads mapped uniquely to the reference human genome (hg19 build). We detected 31 heterozygous de novo "coding" variants (within exons or canonical splice sites), of which 24 were nonsynonymous (Figure 2). However, only two of these variants were novel, and only one was validated by Sanger DNA sequencing (see below).

For proband 2, >98% of high-quality reads mapped uniquely to hg19, and the mean coverage depth was 58-fold with at least 20-fold coverage for 83% of targeted bases. Initially, we identified 36 de novo coding sequence variants, of which 10 were synonymous. Of the remaining 26 nonsynonymous de novo variants, 8 were novel (absent in reference databases), but only 2 were validated by Sanger sequencing (Figure 2). One of the 2 validated variants occurred in a non-cardiac expressed gene with no known function (*C6orf108*) at a nucleotide position with poor evolutionary conservation (E17K; evolutionary nucleotide conservation score, -6.1) and therefore was deemed unlikely to be pathogenic. The remaining novel, nonsynonymous de novo variant is discussed further below.

Among the validated, de novo variants discovered in the probands, 2 variants were in genes encoding the ubiquitous calcium signaling protein calmodulin (Figure 3A). In proband 1, a missense mutation in *CALM1* (chr 14q31) predicted replacement of a highly conserved aspartic acid residue at position 130 (first methionine residue assigned position 1) with glycine (D130G). In proband 2, a missense mutation in *CALM2* (chr 2p21) predicted replacement of another highly conserved aspartic acid residue at position 96 with valine (D96V). Both mutations are predicted to be damaging by SIFT and PolyPhen2 analyses and to replace acidic residues within the carboxyl terminal domain (C domain; Figure 3B). Neither mutation was found by direct screening of DNA from ethnically matched control subjects or was found in publicly accessible databases of genetic variants (see Methods). We also did not observe either variant in 1800 exomes sequenced at the Institute of Human Genetics (Helmholtz Zentrum München) in which



The correlation between the symptoms exhibited by these infants and the known involvement of calmodulin in modulating the activity of ion channels and other critical proteins in heart motivated the investigation of the effect of

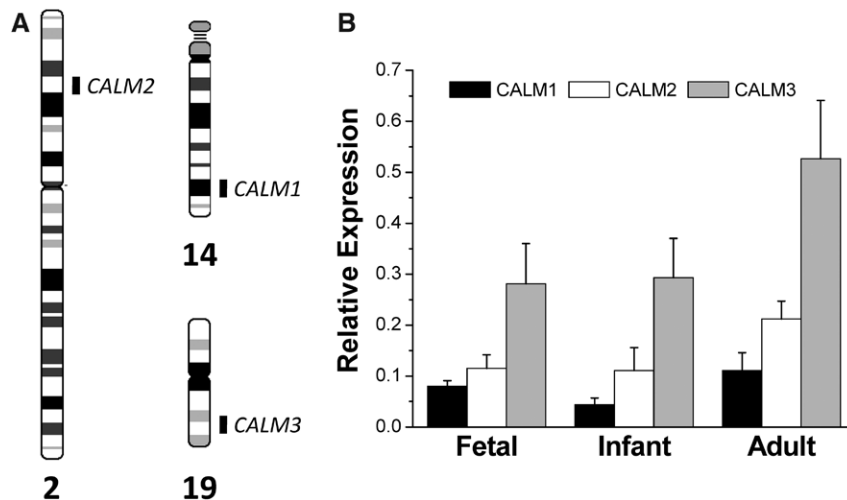


Figure 4. Expression of calmodulin genes in human heart. **A**, Chromosomal locations of the 3 human calmodulin genes indicated on ideograms representing G-banded chromosomes. **B**, Relative expression of mRNA for *CALM1*, *CALM2*, and *CALM3* in normal human heart (left ventricle) normalized to expression of β -actin as determined by real-time quantitative reverse transcriptase-polymerase chain reaction using gene-specific Taqman probes. Human heart samples include fetal ($n=4$), infant ($n=4$), and adult ($n=8$) developmental stages. Data plotted are mean $\pm$ SEM. Differences in expression among the 3 genes were significant ($P<0.05$, 1-way ANOVA) in fetal, infant, and adult hearts.

the mutations on protein function. Two of the mutations we discovered (*CALM1*-D130G, *CALM2*-D96V) alter highly conserved aspartic acid residues that directly chelate Ca^{2+} ions in EF-hand domains IV and III, respectively (positions X and Y, respectively, in the pentagonal bipyramidal coordination sphere²⁰; Figure 5A) and were predicted to reduce Ca^{2+} affinity. The predicted effect of the *CALM1*-F142L mutation

was an alteration of the energetic coupling of Ca^{2+} binding and the conformational change associated with calmodulin activation.^{21,22} To determine the functional consequences of calmodulin mutations, we generated wild-type and mutant recombinant calmodulin proteins in bacteria and performed in vitro Ca^{2+} binding studies by monitoring intrinsic tyrosine and phenylalanine fluorescence.²³ The data revealed that

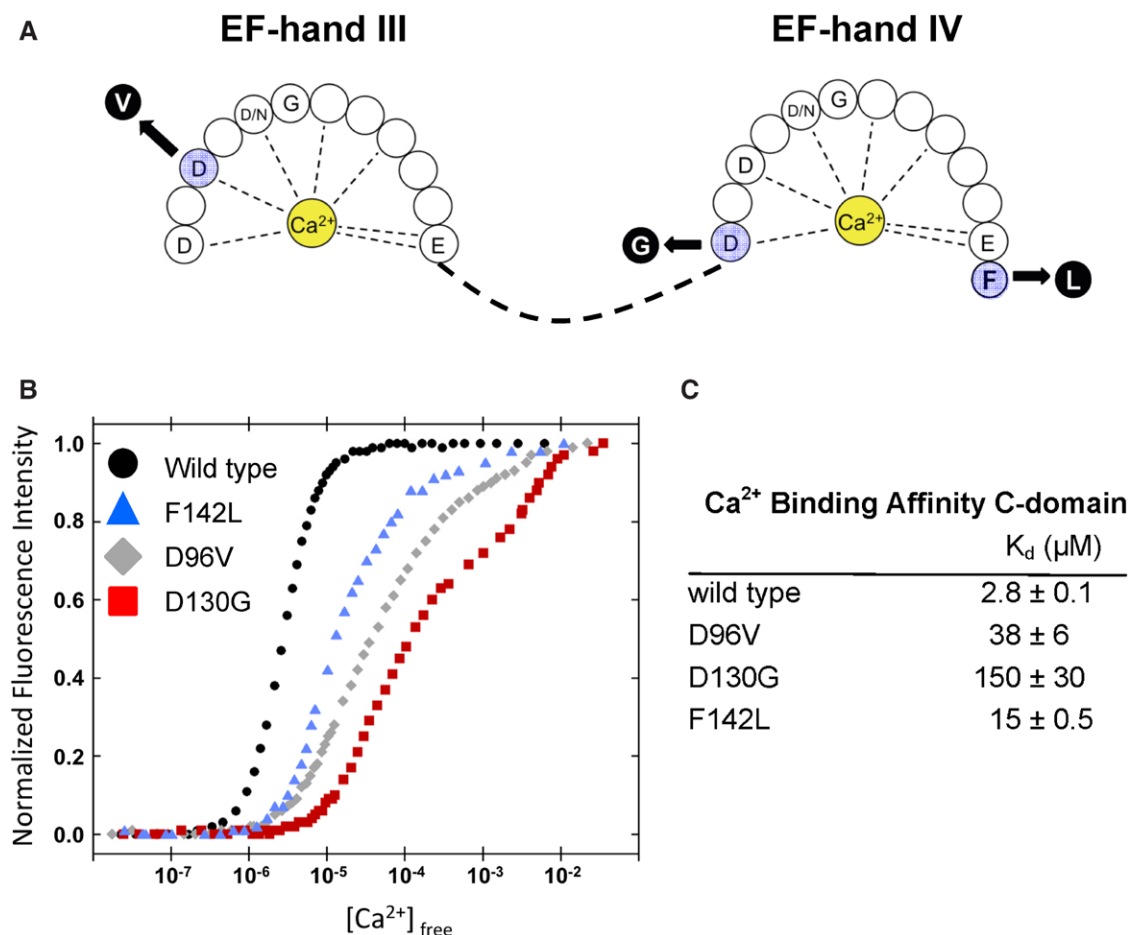


Figure 5. Impaired Ca^{2+} binding by mutant calmodulin C domains. **A**, Schematic model of the Ca^{2+} binding loops in the C-terminal EF-hand domains of calmodulin showing the locations of the mutations. **B**, Titration curves for Ca^{2+} binding to recombinant wild-type (WT; black circles) and mutant (blue triangles, F142L; grey diamonds, D96V; red squares, D130G) calmodulins. **C**, Calcium ion binding affinities for WT and mutant calmodulins.

all 3 mutations have reduced Ca^{2+} affinity in the C domain (5- to 53-fold; Figure 5B and 5C) and no significant effect on N-domain Ca^{2+} affinity (data not shown). The structural integrity of the mutant proteins was then validated with heteronuclear nuclear magnetic resonance spectroscopy (see Figure I in the online-only Data Supplement). These biochemical data predict a significant alteration in the ability of mutant calmodulins to transduce Ca^{2+} signals and to perform essential physiological functions.

Discussion

We report the discovery of de novo calmodulin mutations in a severe, early-onset cardiac arrhythmia syndrome with features of LQTS. Calmodulin is a ubiquitous, multifunctional Ca^{2+} binding protein essential for myriad intracellular signaling processes in eukaryotic cells.²⁴ In electrically excitable tissues such as heart and brain, calmodulin transduces Ca^{2+} signals to influence the activity of ion channels, kinases, and other target proteins that contribute importantly to physiological functions of these organs.^{25–27} Calcium ion binding by 4 highly conserved EF-hand domains promotes conformational changes that are integral to calmodulin function.²⁸ Given that calmodulin is essential to fundamental cell processes and that its protein sequence is perfectly conserved among vertebrates, complete absence of calmodulin is not expected to be compatible with survival.

The main clinical features of the conditions associated with calmodulin mutations are summarized in the Table. The common cardiac features of this syndrome include life-threatening ventricular arrhythmias occurring very early in life, frequent episodes of T-wave alternans, markedly prolonged QTc interval (>600 milliseconds), and intermittent 2:1 atrioventricular block. VF was typically triggered by adrenergic activation and either occurred spontaneously or was preceded by a short period of torsades de pointes that was not pause dependent. Treatment with β -adrenergic antagonists was of some value but was not sufficient to prevent all arrhythmic events. All mutation carriers were treated with a second antiarrhythmic agent (most often mexiletine or flecainide) and an implantable defibrillator, cardiac sympathetic denervation, or both of these interventions.

The clinical syndrome we describe bears many similarities to congenital LQTS, but the intensity and frequency of arrhythmic events and the very early age of onset are not entirely typical of LQTS. Furthermore, all patients exhibited some degree of neurodevelopmental delay, ranging from mild delay in language development (proband 1) to moderate and severe cognitive or motor impairment (proband 2, patients 3 and 4). Seizures were present in 3 of the 4 patients, and 1 subject (proband 2) suffered a cerebrovascular accident after her first cardiac arrest. Neurodevelopmental phenotypes and epilepsy can be attributed to brain injury secondary to cardiac arrest during early life, as seen in young children with congenital heart disease.^{29,30} However, given that calmodulin is highly expressed in brain, we cannot completely exclude the possibility that calmodulin mutations confer increased susceptibility to neuronal injury in the setting of circulatory insufficiency, thereby contributing to the high prevalence of neurological and neurodevelopmental deficits.

The impaired Ca^{2+} binding exhibited by calmodulin mutants suggests a molecular basis for the life-threatening condition experienced by the 4 mutation carriers we report here. Calmodulin serves as the Ca^{2+} sensor for Ca^{2+} -dependent inactivation of L-type voltage-gated Ca^{2+} channels in cardiac myocytes.^{31,32} Overexpression of calmodulin mutants with defective Ca^{2+} binding causes dramatic prolongation of ventricular action potentials in guinea pig myocytes,³³ a plausible mechanistic link to prolonged QT interval, and predisposition to ventricular arrhythmia. Other essential heart proteins require functional calmodulin for normal cardiac repolarization. A voltage-gated potassium channel (KCNQ1 or $\text{K}_{\text{v}}7.1$) responsible for the slow component of the delayed rectifier current (I_{Ks}) is needed for myocardial repolarization and requires calmodulin for activity.^{34,35} Inhibition of calmodulin or chelation of intracellular Ca^{2+} inhibits I_{Ks} , which would delay repolarization, setting up conditions favoring early afterdepolarizations and triggered arrhythmias. Inactivation of cardiac sodium channels involves calmodulin, and disrupting this interaction might evoke arrhythmogenic sodium channel dysfunction.^{36–39} Finally, disturbances in calmodulin-dependent kinase II activity can promote ventricular arrhythmogenesis by several mechanisms.⁴⁰

Functional studies in native cardiac myocytes should be informative as to the major electrophysiological events disrupted by the calmodulin mutations we discovered and help explain the pathogenesis of the associated arrhythmia syndrome. We predict that expression of mutant calmodulins will adversely affect repolarization because of ion channel dysfunction such as impaired calcium channel inactivation. Understanding the manner in which these effects are generated will also help clarify how a single mutant allele can have such severe consequences despite the redundancy of calmodulins in heart. However, a thorough understanding of the molecular mechanisms responsible for the complex heart rhythm disturbances we report in association with calmodulin mutations will require extensive experimental work, including studies of genetically engineered animals, and is beyond the scope of this report.

A recent study demonstrated genetic linkage of autosomal-dominant catecholaminergic polymorphic ventricular tachycardia in an *RYR2* and *CASQ2* mutation-negative Swedish family to chromosome 14q31–32, a locus that includes *CALM1* among nearly 70 genes.⁴¹ Screening *CALM1* revealed a missense mutation affecting a conserved asparagine residue within EF-hand domain II (reported as N53I). A subsequent search for *CALM1* mutations in a cohort of 62 patients with other mutation-negative catecholaminergic polymorphic ventricular tachycardia identified a single de novo missense mutation (reported as N97S; EF-hand domain III) in an Iraqi child. Both mutations impair Ca^{2+} binding to recombinant calmodulin and disrupt binding to a peptide encompassing the calmodulin binding site on *RYR2*. There was no evidence of prolonged QT intervals in any of these mutation carriers. Combined with our observations, these findings suggest an intriguing genotype-phenotype correlation among calmodulin mutations and further suggest different pathophysiological mechanisms.

The amino acid sequence of calmodulin is perfectly conserved among vertebrates. Furthermore, vertebrates have 3 genes encoding calmodulin, but the transcriptional regulation, tissue-specific expression, and intracellular distribution of the 3 gene products are incompletely understood. We demonstrated the expression of all 3 calmodulin genes in human fetal, infant, and adult left ventricle, indicating that heterozygous mutations in *CALM1* or *CALM2* will be present in mutation carriers among all other wild-type protein alleles encoded by the 3 calmodulin genes. This suggests that haploinsufficiency is unlikely to account for the severe phenotype observed in heterozygous carriers of either *CALM1* or *CALM2* mutations and raises the possibility of a dominant-negative mechanism. Furthermore, calmodulin mutations with such severe phenotypic consequences as we report here are less likely to be inherited and will therefore appear as sporadic cases, but only if lifesaving measures are successful. Finally, the reason why mutations in ubiquitously expressed calmodulin genes present predominantly with a cardiac phenotype, albeit with notable neurological deficits after cardiac arrest, is not clear. We speculate that the heart may simply have less physiological reserve with respect to Ca^{2+} signaling than other tissues.

Conclusions

We discovered calmodulin mutations that offer an explanation for recurrent cardiac arrest during early infancy with features of severe LQTS. The high degree of conservation and the absence of inherited mutations attest to the importance of calmodulin in transducing Ca^{2+} signals into essential cellular responses. Additional investigations to determine the contribution of mutant calmodulins to unexplained sudden death in early development are warranted.

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Disclosures

None.

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CLINICAL PERSPECTIVE

Calmodulin is a ubiquitous calcium binding protein essential for myriad intracellular signaling processes in eukaryotic cells. In heart, calmodulin transduces Ca^{2+} signals to influence the activity of ion channels, kinases, and other target proteins that contribute importantly to cardiac function. Given that calmodulin is essential to fundamental cell processes, mutations are expected to have severe consequences. Here, we report calmodulin mutations in 2 unrelated infants with recurrent cardiac arrest and features of severe long-QT syndrome who were both negative for mutations in known arrhythmia-predisposing genes. Using next-generation sequencing technologies to scan coding exons across the genome (eg, exomes), we discovered de novo mutations in 2 distinct genes (*CALM1*, *CALM2*) encoding identical calmodulin proteins. Screening of calmodulin genes in a cohort of long-QT syndrome patients without a defined genetic origin identified 2 additional subjects with de novo mutations in *CALM1*. Mutation carriers had recurrent cardiac arrest secondary to ventricular tachyarrhythmias, severely prolonged QTc interval, and evidence of electrically unstable myocardium (T-wave alternans). Most carriers exhibited neurological deficits (epilepsy, neurodevelopmental delays) of variable severity that could be attributed to brain injury secondary to cardiac arrest or possibly to enhanced susceptibility to neuronal injury in the setting of circulatory insufficiency. Mutant calmodulin proteins exhibited reduced affinity for Ca^{2+} predicted to disrupt critical functions of calmodulin that would be arrhythmogenic. Our findings provide a genetic origin for ventricular arrhythmias during infancy and illustrate the phenotypic and biochemical consequences of human calmodulin mutations. Calmodulin genes should perhaps be screened in severe, early-onset cardiac arrhythmias.

Calmodulin Mutations Provoke Recurrent Cardiac Arrest in Infants

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SUPPLEMENTAL MATERIAL

PATIENT 3 is a 3 year-old Caucasian male from Greece who had cardiac arrest at age 1 month. His parents and two siblings were asymptomatic with normal ECGs. After resuscitation, his ECG demonstrated a markedly prolonged QTc interval (610 ms) with late peaking T-waves. Echocardiogram revealed normal cardiac anatomy and contractile function. He was treated with propranolol and then discharged from the hospital after 6 days with a normal neurological exam, but had recurrent VT-VF cardiac arrest 20 days later treated successfully with defibrillation and lidocaine. After this second cardiac arrest, his neurological condition was profoundly different with clinical features consistent with severe encephalopathy along with generalized tonic-clonic seizures. He remained hospitalized in an intensive care unit for the subsequent 8 months and suffered 15 additional episodes of VF. Ventricular fibrillation typically occurred immediately after an episode of T-wave alternans and was not pause-dependent. The addition of mexiletine to his medical regimen resulted in complete control of arrhythmias. Screening for mutations in *KCNQ1*, *KCNH2*, *SCN5A*, *KCNE1* and *KCNE2* (Molecular Cardiology Laboratory, Fondazione IRCCS Policlinico S. Matteo, Pavia, Italy) was negative. His neurological condition gradually improved but a brain MRI study revealed moderate cortical atrophy, and he was subsequently noted to have a delay in language development but no focal neurological deficits. An ICD was implanted at age 2.5 years.

PATIENT 4 is a 14-year-old Caucasian male from Italy who was adopted at age 8 with limited clinical information available prior to that time. Apparently, he had been treated with propranolol since birth and had multiple episodes of loss of consciousness associated with convulsive movements and incontinence. He had severe cognitive disability, and was treated for epilepsy with valproate and carbamazepine. Evaluation at age 10 revealed a markedly prolonged QTc (>600 ms), episodes of T-wave alternans and non-sustained ventricular tachycardia. Echocardiogram revealed normal cardiac anatomy and contractile function. Screening for mutations in *KCNQ1*, *KCNH2*, *SCN5A*, *KCNE1* and *KCNE2* (Molecular Cardiology Laboratory, Fondazione IRCCS Policlinico S. Matteo, Pavia, Italy) was negative. Escalating propranolol dosage was limited by nocturnal bradycardia (32 beats per minute). Mexiletine did not shorten the QT interval, reduce frequency of T-wave alternans or suppress ventricular arrhythmias. At age 11 he underwent LCSD and had two more syncopal episodes associated with incontinence and absence of a central pulse but while he was not taking propranolol. An ICD was implanted following a recurrent episode of ventricular fibrillation.

SUPPLEMENTAL METHODS

CALMODULIN GENE EXPRESSION ANALYSIS

Expression of *CALM1*, *CALM2* and *CALM3* in human heart was examined by real-time quantitative RT-PCR using gene specific primers and fluorescent Taqman probes (sequences provided in supplemental Table S1) employing previously described methods and tissues.^{1,2} Relative expression levels were calculated by normalizing cycle threshold (C_T) values of the three genes to that of β -actin expression ($2^{-\Delta C_T}$). All tissues were assayed 6 times for each gene. Differences in expression among the three genes within each developmental group were assessed by one-way ANOVA and normality of the data was confirmed using a Shapiro-Wilk test (Origin Pro 8.0, OriginLab, Corp, Northampton, MA).

RECOMBINANT PROTEIN EXPRESSION

Recombinant calmodulin cDNA was sub-cloned into the *NcoI* and *BamHI* restriction sites of the pET15b vector (Novagen). Mutations were made using QuikChange site directed mutagenesis (Stratagene). Proteins were expressed in *E. coli* BL21 (DE3) cells (Novagen) transformed and grown overnight at 37°C. A single colony was selected, then cells were grown in lysogeny broth at 37°C up to an OD_{600} of 0.6 and the culture was induced for 3 hours at 37°C using 1 mM isopropyl 1-thio- β -D-galactopyranoside. Production of ^{15}N enriched protein was performed in minimal media with $^{15}\text{NH}_4\text{Cl}$ (0.5 g/l) as the sole nitrogen source and induction of cells overnight at 25°C. Cell pellets were re-suspended in Buffer A (50 mM Tris at pH 7.5, 500 mM KCl, and 1 mM EDTA), then lysed by sonication. The solution was centrifuged at 20,000 x g for 10 minutes then supernatant was filtered and loaded onto a phenyl sepharose chromatography column pre-equilibrated with Buffer A. The protein was collected in the flow through, and the column was cleaned with 0.1 M NaOH. Calcium chloride was added to the collected elutant to 10 mM, which

was then loaded onto the clean phenyl sepharose column pre-equilibrated with 50 mM Tris at pH 7.5, 500 mM KCl and 10 mM CaCl_2 . After washing with buffer, the protein was eluted by reducing KCl to 150 mM and adding 1 mM EDTA to the mobile phase. Fractions were pooled and samples were dialyzed overnight at 4°C twice in 50 mM HEPES at pH 7.4, 100 mM KCl, and 5 mM EGTA, and twice more with the same buffer except EGTA was lowered to 0.05 mM. The molecular mass of all proteins was confirmed using negative electro-spray mass spectroscopy.

NMR SPECTROSCOPY

NMR samples were prepared by concentrating to ~1.0 mM in the final dialysis buffer with 10% D_2O added for a lock signal. The protein concentration was determined using the previously determined extinction coefficient of calmodulin ($3006 \text{ M}^{-1} \text{ cm}^{-1}$).^{3,4} Two-dimensional ^{15}N - ^1H HSQC experiments were acquired on a Bruker 600 MHz AV-III spectrometer with 128 points over 2000 Hz and 2048 points over 7700 Hz in the ω_1 and ω_2 dimensions. The data were processed with a sinc window function for both dimensions using Bruker Topspin 3.0 software.

FLUORESCENCE SPECTROSCOPY AND MEASUREMENT OF CALCIUM AFFINITY

Macroscopic binding constants for calcium binding sites I and II in the N-terminal domain, and sites III and IV in the C-terminal domain were determined by titrating proteins (6 μM in 50 mM HEPES at pH 7.4, 100 mM KCl, 0.05 mM EGTA, 5 mM NTA, and 1 mM MgCl_2) as described by Shea and coworkers.^{5,6} To summarize, measurement were made by following the intrinsic phenylalanine fluorescence for the N domain (excitation wavelength = 250 nm, emission wavelength = 280 nm,) and tyrosine fluorescence for the C domain (excitation wavelength = 277 nm, emission wavelength = 320 nm) after each step during a Ca^{2+} titration. Spectra were collected at 22°C using a Jobin Horiba FluoroMax-3 spectrometer with excitation and emission slits of 5.5 and 6.0 mm, respectively. The free Ca^{2+} concentration at each point in the titration

was determined by the extent of saturation of the commercial calcium indicator dye fluo-5N (Invitrogen, 4 nM, excitation wavelength = 467 nm, emission wavelength = 514 nm). The K_d for fluo-5N was determined to be 85 μ M in our titration buffer. Data analysis was performed by plotting the normalized fluorescence signal vs free Ca^{2+} concentration and fitting to the model-independent two site Adair function:^{7,8}

$$\bar{Y} = \frac{K_1[X]^1 + 2K_2[X]^2}{2(1 + K_1[X]^1 + K_2[X]^2)}$$

where X is the concentration of free Ca^{2+} , K_1 is a sum of intrinsic microscope equilibrium constants ($k_1 + k_2$) for pairs of Ca^{2+} binding sites (I and II in N domain; or III and IV in C domain), and K_2 is the product of intrinsic microscopic equilibrium constants and a cooperativity constant ($k_1 \cdot k_2 \cdot k_c$). The dissociation constants (K_d) for the N domain and C domains are reported as the average value for the pair of sites by taking the square root of K_2 .

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SUPPLEMENTAL FIGURE S1

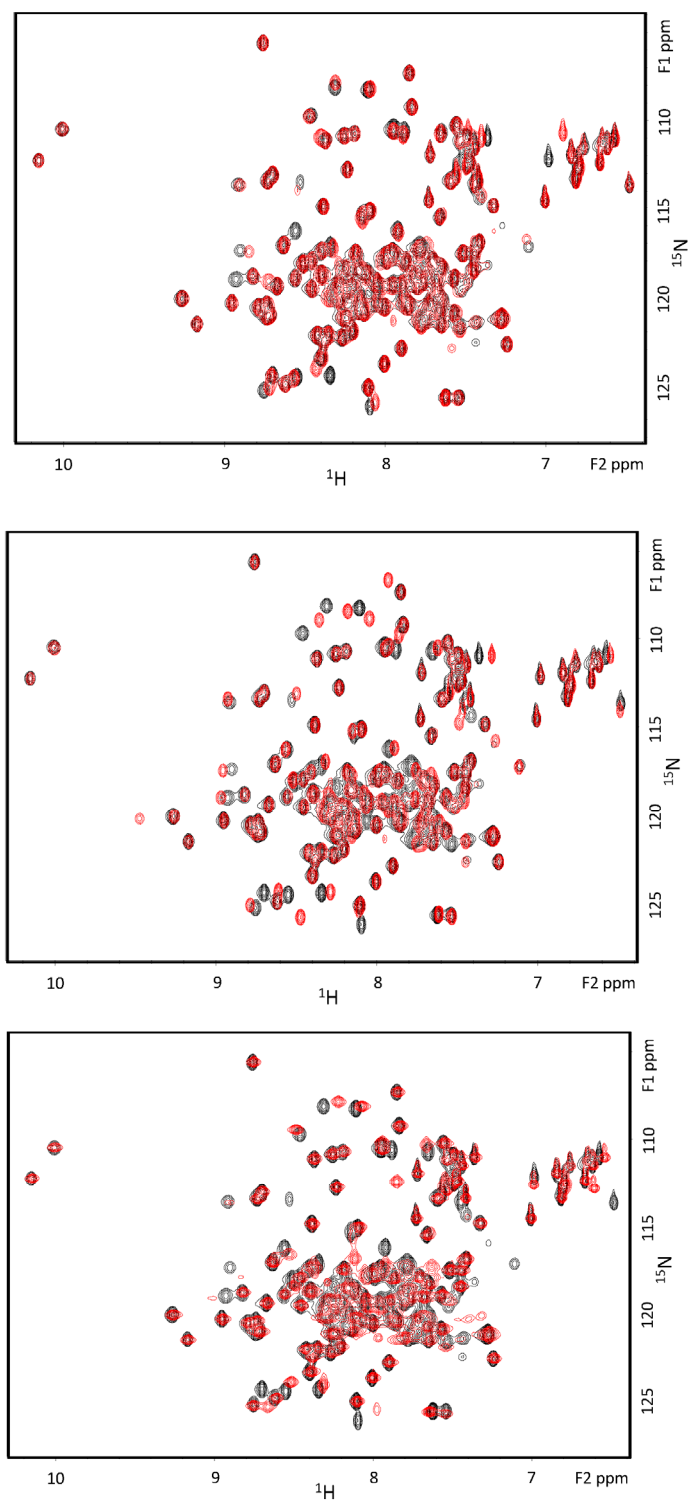


Fig. S1 - Overlays of 600 MHz 2D ^{15}N - ^1H HSQC spectra for apo wild type mammalian calmodulin (black), and mutants (red) D96V (top), D130G (middle) and F142L (bottom).