Calmodulin Mutations Associated with Recurrent Cardiac Arrest in Infants

Running title: Crotti et al.; Calmodulin mutations in recurrent cardiac arrest

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Abstract:

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 two unrelated infants presenting with recurrent cardiac arrest to discover a genetic cause.

Methods and Results—We ascertained two unrelated infants (probands) with recurrent cardiac arrest and dramatically prolonged QTc interval who were both born to healthy parents. The two parent-child trios were investigated using 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 two genes encoding calmodulin. We discovered three heterozygous de novo mutations in either CALM1 or CALM2, two of the three human genes encoding calmodulin, in the two probands and in two 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.

Key words: arrhythmia, sudden cardiac death, exome, calcium signaling
Introduction

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 (SIDS).\(^3,6\) When a genetic cause has been identified in these early onset and highly malignant conditions, mutations are often \emph{de novo},\(^3,7,9\) but many cases do not have a clear genetic or molecular explanation.

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 two genes encoding calmodulin, a ubiquitous and essential calcium signaling protein involved critically in a myriad of physiological events, are associated with life-threatening cardiac arrhythmias accompanied variably by neurological complications. We made this discovery by performing exome sequencing on two unrelated infants with recurrent cardiac arrest and subsequently by examining calmodulin genes for mutations in a cohort of LQTS cases without a defined genetic etiology. 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 two probands (one Caucasian, one Hispanic) with highly malignant ventricular arrhythmia syndromes and their respective unaffected parents, and 82 additional unrelated cases of LQTS without an identified genetic cause. Control subjects were from two sources. A panel of Hispanic Americans (n=92) was obtained from the Coriell Institute for Medical Research, while a panel of Caucasian Europeans (n=1800) for whom exome data were available 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 base pairs) sequencing was performed on the Illumina HiSeq2000 platform. Proband 1 was sequenced at the Institute of Human Genetics, Helmholtz Zentrum München, whereas 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 using BWA (version 0.5.8),\textsuperscript{11} then sequences were processed using the Genome Analysis TookKit (GATK)\textsuperscript{12} 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 (v130), 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 using custom scripts. Further, based on the hypothesis that disease causing mutations in the probands are de novo, we excluded all inherited variants (i.e., that were observed in either parent). Non-excluded variants were validated in proband and parental DNAs using conventional Sanger sequencing, then further annotated based upon evolutionary nucleotide conservation (GERP),13 predicted impact on protein function (Polyphen2, SIFT).14,15

Additional Mutation Detection
Mutation screening was performed in 82 unrelated LQTS subjects (Schwartz Score ≥3.516 and/or resting QTc ≥480 ms) without a previously identified genetic cause to search for additional variants in the candidate disease-causing genes identified in the two probands. Specifically, the coding exons of CALM1, CALM2 and CALM3, three genes encoding identical calmodulin proteins, were amplified by polymerase chain reaction (PCR; primer sequences provided in supplemental Table S1) then sequenced using 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 Data Supplement.
Results

Study Subjects

We ascertained two unrelated infants (probands) with recurrent cardiac arrest. Proband 1 was a Caucasian female from Italy who suffered cardiac arrest due to ventricular fibrillation (VF) at age 6 months. Her birth and prenatal history were unremarkable, family history was negative for sudden cardiac death, and both parents were asymptomatic with normal electrocardiograms (ECG). There had been no evidence of fetal bradycardia. Following successful external defibrillation, an ECG demonstrated a markedly prolonged QTc interval (630 ms), frequent episodes of T-wave alternans and intermittent 2:1 atrioventricular (AV) block (Fig. 1). Echocardiogram revealed normal cardiac anatomy and contractile function. The patient was treated with propranolol and an internal cardioverter defibrillator (ICD) 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 ICD shocks. Propranolol dosage was progressively increased to 10 mg/kg/day without arrhythmia suppression. Left cardiac sympathetic denervation (LCSD) was performed at age 12 months, but QTc remained prolonged (550-630 ms) and episodes of T-wave alternans continued to occur. A loading dose of mexiletine did not shorten the QT interval. Verapamil reduced the frequency of T-wave alternans, but persistent cardiac electrical instability prompted right cardiac sympathetic denervation (RCSD). Despite all treatments, there were 16 episodes of VF during the first 2 years of life, mostly induced by adrenergic stimulation and not pause-dependent or preceded by torsades de pointes (TdP). Verapamil was replaced with flecainide and in the
following year she had only one VF episode triggered by strong emotional stress. A mild delay in language development was noted.

Proband 2 was an Hispanic female infant living in the United States who presented with sinus bradycardia, T-wave alternans, markedly prolonged QTc (690 ms) and 2:1 AV block occurring 2 hours after a normal delivery (Fig. 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 90-95 bpm. An episode of 2:1 AV 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 propranolol treatment restored 1:1 AV 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 age 3 weeks following cardiac arrest and multiple episodes of VF. She was successfully defibrillated but suffered a right parietal lobe cerebral infarction that was documented by brain MRI. Treatment at that time included propranolol, mexiletine and an ICD. In the first two years of life she was hospitalized numerous times for episodes of VF that were successfully terminated by ICD shocks. At 2 years of age she developed seizures that were attributed to the prior brain injury. At age 3, seizures were well controlled with levetiracetam and her QTc was 500-510 ms on a combination of mexiletine and propranolol, but she exhibited developmental delays (10-33%) 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. Further, 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 two probands and their parents (parent-child trios), 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 (104X) for the 3 samples, with 20X 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 (Fig. 2). However, only two of these variants were novel and only one was validated by Sanger DNA sequencing (see below).

For proband 2, greater than 98% of high quality reads mapped uniquely to hg19 and the mean coverage depth was 58X with at least 20X 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 two were validated by Sanger sequencing (Fig. 2). One of the two validated variants occurred in a non-cardiac expressed gene with no known function (C6orf108) at a nucleotide position with poor evolutionary conservation (E17K; GERP score −6.1), and therefore, was deemed unlikely to be pathogenic. The remaining novel, nonsynonymous de novo variant is discussed further.
Among the validated, de novo variants discovered in the probands, two were in genes encoding the ubiquitous calcium signaling protein calmodulin (Fig. 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 replace acidic residues within the carboxyl terminal domain (C-domain) (Fig. 3B). Neither mutation was found by direct screening of DNA from ethnically matched control subjects nor 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 mean coverage of CALM1 and CALM2 was >95X. To further illustrate the extreme rarity of calmodulin gene mutations, only two nonsynonymous coding variants in CALM1 (T10I, and L143V) and none in CALM2 were called in 8599 alleles of European ancestry by the Exome Sequencing Project (http://evs.gs.washington.edu/EVS/). Further, no CALM1 or CALM2 nonsynonymous variants were identified in the Helmholtz exome data. Therefore, we concluded that mutations of CALM1 or CALM2 were likely responsible for the life-threatening syndrome observed in the probands.

Human calmodulin is encoded by three separate genes each located on a different chromosome (Fig. 4A), but the protein products of each gene have identical amino acid sequences. We examined the expression of each calmodulin gene in human heart (left ventricle) from three developmental stages (fetal, infant, adult) using quantitative RT-PCR and gene-specific fluorogenic TaqMan probes. All calmodulin genes are expressed throughout
development with the rank order of expression being \textit{CALM3} > \textit{CALM2} > \textit{CALM1} (Fig. 4B). These data demonstrate cardiac expression of the two calmodulin genes in which we discovered mutations.

The discovery of mutations in calmodulin genes in the setting of a severe cardiac arrhythmia syndrome associated with markedly prolonged QT interval prompted us to examine a cohort of other cases of congenital long-QT syndrome (LQTS) for which no genetic cause had been found. We performed a directed search for mutations in \textit{CALM1}, \textit{CALM2}, and the closely related \textit{CALM3} in a cohort of 82 LQTS cases with no identified mutations. \textit{CALM1} mutations were discovered in two subjects within this cohort, both with early onset and severe clinical presentations.

The same mutation discovered in proband 1 (\textit{CALM1}-D130G) was found in a 3 year-old Caucasian male from Greece (Case 3) who had suffered multiple cardiac arrests beginning at age 1 month. We also discovered a novel \textit{CALM1} missense mutation in an adopted 14 year-old Caucasian male from Italy (Case 4) with recurrent episodes of non-sustained ventricular tachycardia, T-wave alternans, markedly prolonged QTc interval and cardiac arrest due to ventricular fibrillation. The mutation discovered in this subject predicted substitution of a highly conserved phenylalanine residue at position 142 with leucine (F142L; Fig. 3A). This mutation was absent in Caucasian controls of western European ancestry and was not observed in the aforementioned databases of genetic variants (see Methods). Additional clinical information about these two additional mutation carriers is available in the online Data Supplement.

\textbf{Biochemical Consequences of Calmodulin Mutations}

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 investigation of the effect of 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 bipyrimidal coordination sphere;\(^{20}\) Fig. 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 wildtype 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 all three mutations have reduced Ca\(^{2+}\) affinity in the C-domain (5-53 fold) (Fig. 5B,C), and no significant effect on N-domain Ca\(^{2+}\) affinity (data not shown). The structural integrity of the mutant proteins was then validated using heteronuclear nuclear magnetic resonance spectroscopy (see online Data Supplement, Fig. S1). These biochemical data predict a significant alteration in the ability of mutant calmodulins to transduce Ca\(^{2+}\) signals and 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 a myriad of intracellular signaling processes in eukaryotic cells.\(^{24}\) In electrically excitable tissues such as heart and brain, calmodulin transduces Ca\(^{2+}\) signals to influence activity of ion channels, kinases and other target proteins that contribute importantly to physiological functions of these organs.\(^{25-27}\) Calcium ion binding by four highly conserved EF-
hand domains promotes conformational changes that are integral to calmodulin function.\textsuperscript{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 Table 1. 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 ms), and intermittent 2:1 AV block. Ventricular fibrillation was typically triggered by adrenergic activation, and either occurred spontaneously or was preceded by a short period of polymorphic ventricular tachycardia that was not pause-dependent. Treatment with β-adrenergic antagonists was of some value albeit not sufficient to prevent all arrhythmic events. All mutation carriers were treated with a second anti-arrhythmic agent (most often mexiletine or flecainide) and either an implantable defibrillator, cardiac sympathetic denervation or both of these interventions.

The clinical syndrome we describe bears many similarities with congenital LQTS, but the intensity and frequency of arrhythmic events as well as the very early age of onset are not entirely typical of LQTS. Further, all cases 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, cases 3 and 4). Seizures were present in 3 of the 4 cases, and one subject (proband 2) suffered a cerebrovascular accident following 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.\textsuperscript{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 four 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.\(^{31,32}\) Over expression of calmodulin mutants with defective Ca\(^{2+}\) binding causes dramatic prolongation of ventricular action potentials in guinea pig myocytes,\(^{33}\) a plausible mechanistic link with 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 K\(_{v}7.1\)) 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 \(I_{Ks}\) and this 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 (CPVT) in a RYR2 and CASQ2 mutation-negative Swedish family to chromosome 14q31-32, a locus that includes CALM1 among nearly 70 genes. 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 other mutation-negative CPVT cases identified a single de novo missense mutation (reported as N97S, EF-hand domain III) in an Iraqi child. Both mutations impair Ca2+ 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 three genes encoding calmodulin, but the transcriptional regulation, tissue-specific expression and intracellular distribution of the three gene products are incompletely understood. We demonstrated the expression of all three 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 wildtype protein alleles encoded by
the three calmodulin genes. This suggests that haploinsufficiency is unlikely to account for the severe phenotype observed in heterozygous carriers of either \textit{CALM1} or \textit{CALM2} mutations and raises the possibility of a dominant-negative mechanism. Further, 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 life-saving measures are successful. Finally, the reason why mutations in ubiquitously expressed calmodulin genes present predominantly with a cardiac phenotype, albeit with notable neurological deficits following cardiac arrest, is not clear. We speculate that the heart may simply have less physiological reserve with respect to Ca\textsuperscript{2+} signaling than other tissues.

In conclusion, 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\textsuperscript{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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**Conflict of Interest Disclosures:** The authors have no potential conflicts of interest related to the work reported in this manuscript. Author Contributions: L.C., P.J.S. and A.L.G. conceived the experiments and wrote the manuscript. G.M.D.F., B.C., M.O., J.P., S.K., and D.W.B. ascertained study subjects and compiled clinical data. C.N.J., M.D.F., S.G.K, J.D.K., M.P., T.M.S., E.G., T.W., P.L., B.B., T.C., and C.S. performed critical experiments or performed data analysis. T.M., W.J.C. and A.L.G. planned and supervised critical experiments, and reviewed data.

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unexpected eminence of Ca\textsuperscript{2+} channel inactivation in controlling heart excitation. *Proc Natl Acad Sci U S A.* 2002;99:17185-17190.


Table 1. Summary of clinical features of calmodulin mutation carriers.

<table>
<thead>
<tr>
<th>Subject</th>
<th>Sex</th>
<th>Age at Diagnosis</th>
<th>VF</th>
<th>QTc</th>
<th>TWA</th>
<th>2:1 AVB</th>
<th>Seizures</th>
<th>Developmental delay</th>
<th>Treatments</th>
<th>Mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proband 1</td>
<td>F</td>
<td>6 months</td>
<td>+</td>
<td>630 ms</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+/-</td>
<td>BB, MEX, VER, FLEC, ICD, LCSD, RCSD</td>
<td>CALM1-D130G</td>
</tr>
<tr>
<td>Proband 2</td>
<td>F</td>
<td>prenatal</td>
<td>+</td>
<td>690 ms</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>BB, MEX, ICD</td>
<td>CALM2-D96V</td>
</tr>
<tr>
<td>Case 3</td>
<td>M</td>
<td>1 month</td>
<td>+</td>
<td>610 ms</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>BB, MEX, ICD</td>
<td>CALM1-D130G</td>
</tr>
<tr>
<td>Case 4</td>
<td>M</td>
<td>? neonatal</td>
<td>+</td>
<td>&gt;600 ms</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>BB, MEX, LCSD</td>
<td>CALM1-F142L</td>
</tr>
</tbody>
</table>

1Mutation position is based on RefSeq NP_005175 and counting the predicted translational start codon (Met) as position 1. VF, ventricular fibrillation; QTc, rate-corrected QT interval; TWA, T-wave alternans; AVB, atrioventricular block; BB, β-blocker (propranolol); MEX, mexiletine; VER, verapamil; FLEC, flecainide; ICD, implantable cardioverter defibrillator; LCSD, left cardiac sympathetic denervation; RCSD, right cardiac sympathetic denervation.
Figure Legends:

**Figure 1.** Clinical phenotypes and pedigrees. Representative electrocardiographic recordings from two probands with early onset, life-threatening cardiac arrhythmias. Upper trace represents baseline ECG for both probands. Middle trace illustrates T-wave alternans. Lower trace for proband 1 illustrates onset of ventricular fibrillation following a period of T-wave alternans. Lower trace for proband 2 illustrates 2:1 AV 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 two probands. Total coding variants include all synonymous, nonsynonymous, nonsense, frameshift inducing insertions or deletions, and canonical splice site variants within captured exons, but exclude variants in 5’ and 3’ untranslated regions and introns. De novo variants are those found in 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 1202 exomes generated in the Institute of Human Genetics Helmholtz Zentrum München).

**Figure 3.** *De novo* calmodulin gene mutations in infants with severe cardiac arrhythmias. **A,** Nucleotide sequence traces indicating heterozygous calmodulin gene mutations in Proband 1 (same mutation as in Case 3), Proband 2 and Case 4. **B,** Amino acid sequence alignments for calmodulins from different species with location of missense mutations.

**Figure 4.** Expression of calmodulin genes in human heart. **A,** Chromosomal locations of the
three 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 RT-PCR 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 ± SEM. Differences in expression among the three genes were significant (p<0.05; one-way ANOVA) in fetal, infant and adult hearts.

Figure 5. Impaired Ca\textsuperscript{2+} binding by mutant calmodulin C-domains. A, Schematic model of the Ca\textsuperscript{2+} binding loops in the C-terminal EF-hand domains of calmodulin showing location of the mutations. B, Titration curves for Ca\textsuperscript{2+} binding to recombinant WT (black circles) and mutant calmodulins (F142L, blue triangles; D96V, grey diamonds; D130G, red squares). C, Calcium ion binding affinities for WT and mutant calmodulins.
Figure 2
Figure 3

(A) Sequence analysis of the CALM1 and CALM2 genes in Proband 1 (Case 3), Proband 2 (Case 2), and Case 4.

(B) Comparison of amino acid sequences across species for the CALM family. The amino acid sequence for each species is shown, with the mutation positions highlighted in red. The mutations in the EF-hand III and EF-hand IV domains are also indicated.
Figure 4
Figure 5
Calmodulin Mutations Associated with Recurrent Cardiac Arrest in Infants

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Calmodulin Mutations Provoke Recurrent Cardiac Arrest in Infants


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 (CT) values of the three genes to that of β-actin expression ($2^{-\Delta CT}$). 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 Ncol 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 \(^{15}\)NH\(_4\)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 CaCl₂. 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% D₂O added for a lock signal. The protein concentration was determined using the previously determined extinction coefficient of calmodulin (3006 M⁻¹ cm⁻¹).³,⁴ Two-dimensional $^{15}$N-$^1$H 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 $\omega_1$ and $\omega_2$ 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 MgCl₂) 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 Ca²⁺ 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 Ca²⁺ 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 $\mu$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

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

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 ($k_1*k_2*k_c$). 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 $K_2$. 
SUPPLEMENTARY REFERENCES


SUPPLEMENTAL FIGURE S1

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