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Novel Calmodulin (*CALM2*) Mutations Associated with Congenital Arrhythmia Susceptibility

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Abstract

Background—Genetic predisposition to life-threatening cardiac arrhythmias such as in congenital long-QT syndrome (LQTS) and catecholaminergic polymorphic ventricular tachycardia (CPVT) represent treatable causes of sudden cardiac death in young adults and children. Recently, mutations in calmodulin (*CALM1*, *CALM2*) have been associated with severe forms of LQTS and CPVT, with life-threatening arrhythmias occurring very early in life. Additional mutation-positive cases are needed to discern genotype-phenotype correlations associated with calmodulin mutations.

Methods and Results—We employed conventional and next-generation sequencing approaches including exome analysis in genotype-negative LQTS probands. We identified five novel *de novo* missense mutations in *CALM2* in three subjects with LQTS (p.N98S, p.N98I, p.D134H) and two subjects with clinical features of both LQTS and CPVT (p.D132E, p.Q136P). Age of onset of major symptoms (syncope or cardiac arrest) ranged from 1–9 years. Three of five probands had cardiac arrest and one of these subjects did not survive. Although all probands had LQTS, two subjects also exhibited electrocardiographic features consistent with CPVT. The clinical severity among subjects in this series was generally less than that originally reported for *CALM1* and *CALM2* associated with recurrent cardiac arrest during infancy. Four of five probands responded to β-blocker therapy whereas one subject with mutation p.Q136P died suddenly during exertion despite this treatment. Mutations affect conserved residues located within calcium binding loops III (p.N98S, p.N98I) or IV (p.D132E, p.D134H, p.Q136P) and caused reduced calcium binding affinity.

Conclusions—*CALM2* mutations can be associated with LQTS and with overlapping features of LQTS and CPVT.

Keywords

calmodulin; long QT syndrome; ventricular arrhythmia; catecholamineric polymorphic ventricular tachycardia

Introduction

Congenital long QT syndrome (LQTS) is a recognizable and treatable genetic predisposition to sudden cardiac death in children and young adults.[1] Considerable genetic heterogeneity underlies this syndrome, although a large fraction of successfully genotyped LQTS subjects belong to three major subtypes (LQT1, LQT2, and LQT3) associated with mutations in three genes encoding plasma membrane ion channels (*KCNQ1*, *KCNH2*, and *SCN5A*, respectively). Distinct genotype-specific patterns of T-wave morphology,[2,3] triggers for cardiac events,[4] clinical outcomes[5] and response to the epinephrine provocation test[6,7] have been observed. Importantly, genotype can also influence the response to specific drug therapy.[8–11] Genetic testing for known arrhythmia susceptibility genes has become standard-of-care for some disorders including LQTS, but despite the rapid progress in understanding the genetic basis, an etiology remains unknown in many cases.[12] Additional studies are needed to reveal the missing heritable factors and to elucidate genotype-phenotype correlations.

Recently, mutations in two of three genes encoding identical peptide sequences for the essential calcium signaling protein calmodulin were associated with life-threatening arrhythmia predisposition including malignant forms of LQTS, catecholaminergic polymorphic ventricular tachycardia (CPVT) and idiopathic ventricular tachycardia (IVF).[13–15] Nyegaard and colleagues identified two distinct missense *CALM1* (p.N54I, p.N98S) mutations in association with CPVT.[13] Crotti, *et al*. used exome sequencing and targeted re-sequencing to discover novel *CALM1* (p.D130G, p.F142L) and *CALM2* (p.D96V) missense mutations in subjects with infantile or perinatal presentations of severe LQTS associated with recurrent cardiac arrest.[14] Most recently, Marsman and colleagues identified a novel *CALM1* mutation (F90L) segregating with IVF and sudden death in a Moroccan family.[15] Although this limited number of calmodulin mutations suggests preliminary genotype-phenotype correlations, additional mutations are needed to establish the spectrum of clinical features and severity of arrhythmia phenotypes associated with calmodulin mutations.

Here we report the discovery of five novel *de novo* missense *CALM2* mutations associated with congenital arrhythmia susceptibility in probands of varying ancestry. The mutations alter conserved residues that directly coordinate calcium ions in the carboxyl-terminal domain of calmodulin and cause significant reductions in calcium binding affinity. Clinical and electrophysiological findings in these subjects suggested that *CALM2* mutations can be associated with less severe forms of LQTS compared to our previous report[14] as well as with overlapping clinical features of LQTS and CPVT.

Methods

Study subjects

The QT interval was corrected for heart rate using Bazett's formula (QTc = QT/√RR), and the diagnosis of LQTS was made by the Schwartz criteria.[1] All individuals who participated in the study gave written informed consent prior to genetic and clinical investigations in accordance with the standards of the Declaration of Helsinki and the local ethics committees at each participating institution. We studied two Japanese cohorts including one consisting of 12 unrelated LQTS subjects who were without a genetic diagnosis after sequencing genes previously associated with life-threatening arrhythmias (*KCNQ1, KCNH2, SCN5A, SCN1B, SCN2B, SCN3B, SCN4B, KCNE1, KCNE2, KCNJ2* and *CAV3)*,[13,14,16] and another cohort consisting of 190 unrelated LQTS patients in whom whole exome sequencing was performed. Exome sequencing was performed on a parent-child trio in which the proband was a child who suffered cardiac arrest at age 17 months. A German mutation-negative LQTS proband and a Moroccan girl with sudden cardiac death were also screened for mutations in *CALM1, CALM2 and CALM3*.

Candidate gene and exome sequencing

Targeted exon capture was performed for 240 candidate arrhythmia susceptibility genes (Supplemental Table S1) using the SureSelect Target Enrichment System according to the manufacturer's suggestions (Agilent Technologies, Inc. Santa Clara, CA). The captured DNA was sequenced on the Genome Analyzer IIx platform (Illumina Inc. San Diego, CA) with paired-end reads of 101 base pairs (bp) for insert libraries consisting of 150–200 bp fragments. On average for targeted capture sequencing, 1.1 Gbp of short read sequence data were generated and 98.9% were mapped to the reference human genome. Whole exome capture was performed using Agilent SureSelect Human All Exon V4 reagent and captured DNA was sequenced on Illumina HiSeq2000 (performed at RIKEN) or HiSeq2500 (performed at Vanderbilt University) platforms. For data obtained on the HiSeq2000, an average of 6.4 Gbp of short read sequence data were generated with 98.6% mapped successfully to the reference human genome and 66-fold average coverage for all capture exons. For data obtained on the HiSeq2500, an average of 5.8 Gbp was generated per subject with 99.8% mapped and 50-fold average coverage.

Sequence data analysis

Sequence reads were mapped to the human reference genome (GRCh37) using the Burrows-Wheeler Aligner (BWA: version 0.6.1).[17] Possible duplicate reads were removed using SAMtools[18] and custom software leaving an average of 0.8 Gb and 5.5 Gb for targeted capture and exome sequencing, respectively. More than 93% of targeted regions were covered by at least 10 reads. After filtering by pair mapping distance, mapping uniqueness and orientation between paired reads, the mapping result files were converted into the pileup format using SAMtools.[18] Variant calling was conducted in part on the basis of published methods.[19–21] We, further, used the following quality control filters: (i) alignments near putative indels were refined using GATK,[22] and (ii) a strand bias filter excluded variants whose alternative allele was preferentially found on one of the two available read orientations at the site. Variants found in dbSNP Build 137, 1000 Genomes,[23] or Exome

Variant Server (EVS)[24] databases were excluded from further analyses. Synonymous and intronic (other than canonical splice sites) variants were also excluded. Three other exome databases (RIKEN database of 731 non-cardiac disease Japanese exomes, Human Genetic Variation Browser [HGVD] database including exome data obtained from 1208 Japanese subjects [http://www.genome.med.kyoto-u.ac.jp/SnpDB] and the Institute of Human Genetics Helmholtz Zentrum München database of >3,000 exomes of European ancestry) were also queried for the candidate mutations.

Additional mutation detection

Targeted PCR-Sanger sequencing was performed as described previously[14] on DNA from a German female with clinical features of LQTS and CPVT, as well as in a Moroccan girl with sudden cardiac death and a presumptive diagnosis of CPVT to search for variants in the coding exons of *CALM1*, *CALM2*, and *CALM3*. Variants discovered by exome sequencing were also confirmed by Sanger sequencing using an automated capillary electrophoresis DNA sequencing platform (Applied Biosystems, Foster City, CA), then further annotated based upon evolutionary amino acid conservation (Mutation Taster)[25] and predicted impact on protein function (Polyphen2, SIFT).[26,27] Mutation position in calmodulin was based upon RefSeq NP_001734 counting the predicted translational start codon (Met) as position 1.

Expression of recombinant calmodulins and measurement of calcium affinity

Biochemical studies of recombinant calmodulin proteins were performed as previously described.[14] Briefly, recombinant wildtype and mutant calmodulins were expressed in *E. coli* and purified by standard chromatographic approaches. Macroscopic affinity constants for calcium binding in the amino-terminal and carboxy-terminal domains were determined by measuring changes in intrinsic fluorescence as reported by Shea and coworkers.[28,29] The data were analyzed by plotting the normalized fluorescence signal *vs* free Ca^{2+} concentration and fitting to a two-site Adair function for each domain.[30,31]

Results

Case presentations

Case 1—A 6-year-old Japanese girl was admitted to the hospital for evaluation of syncope and a markedly prolonged QT interval. She had a history of fetal bradycardia but had an uneventful birth. She had her first episode of syncope at age 19 months. An electrocardiogram (ECG) at that time showed marked QT prolongation (QTc = 579 ms) with atypical notched, late peaking T waves (Fig. 1A). Atrial pacing at 100 bpm prolonged QTc from 596 ms to 619 ms, whereas mexiletine shortened QTc from 596 ms to 550 ms (Fig. 1B). Subsequently, she experienced multiple episodes of cardiac arrest during exertion when she failed to take mexiletine prompting placement of an implantable cardioverter defibrillator (ICD) at age 14 years. Medical therapy with mexiletine and a β-adrenergic receptor blocker atenolol was generally effective in preventing ventricular arrhythmias, although there was an episode of appropriate ICD discharge that occurred during exertion. The patient had no history of seizures or developmental delay. Genetic testing for mutations in *KCNQ1*, *KCNH2*, *SCN5A*, *SCN1B*, *SCN2B*, *SCN3B*, *SCN4B*, *KCNE1*, *KCNE2*, *KCNJ2*

and *CAV3* was negative. There was no family history of LQTS or sudden death, and both parents had normal QTc intervals (father 369 ms, mother 394 ms) as did her two brothers (368, 388 ms).

Case 2—A 5-year-old Japanese boy had an episode of syncope with seizure while running. Two months later, he had a similar episode and was evaluated in an emergency room. An ECG exhibited QTc prolongation (478 ms, Fig. 2A), whereas an echocardiogram, electroencephalogram, and brain magnetic resonance imaging were normal. He showed no developmental delay. There was no family history of arrhythmias or sudden death, and both parents (father 364 ms, mother 396 ms) and his brother (340 ms) had normal QTc intervals. Epinephrine infusion test did not induce ectopic beats but caused marked QTc prolongation (baseline HR/QT/QTc, 56 bpm/484 ms/466 ms; peak HR/QT/QTc, 94 bpm/446 ms/558 ms; steady-state HR/QT/QTc, 73 bpm/484 ms/535 ms; Fig. 2B). This subject did not tolerate exercise testing because of dizziness. Genetic testing for mutations in *KCNQ1*, *KCNH2*, *SCN5A*, *KCNE1*, *KCNE2*, *KCNJ2* and *AKAP9* was negative. Treatment with propranolol alone or in combination with mexiletine shortened the QTc interval to 471 – 473 ms (Fig. 2C), but he continued to experience syncope and dizziness while running. The drugs were replaced with metoprolol at age 11 years. Subsequently, he had no further episodes of syncope, and there was normalization of the QTc (449 ms) with elimination of the notch in the descending limb of the T wave (Fig. 2C).

Case 3—A 29 year-old German female, who was previously diagnosed with perinatal bradycardia and neonatal LQTS, had been treated with pindolol. Her family history was negative for cardiac arrest and sudden death. On β-blocker therapy she remained asymptomatic until age 9 years when she suffered syncope while swimming following an interruption of therapy. At that time, there was evidence of exercise-induced polymorphic ventricular ectopy. She became asymptomatic for several years after resumption of treatment with various β-blockers (pindolol, propranolol, atenolol). Her resting ECG exhibited QT prolongation (465 – 578 ms) with persistent biphasic T waves in leads III, aVF, aVL, V3 and negative T waves in V4-V6 (Fig. 3A). The patient never suffered seizures, and she had normal physical and mental development. At age 22 years, exercise-induced polymorphic ventricular ectopy and one 3-beat run of polymorphic ventricular tachycardia was documented (Fig. 3B, Supplemental Fig. S1). Echocardiographic evaluation was normal, but MRI revealed features consistent with non-compaction of the left ventricle myocardium (LVNC). Both parents had normal QTc intervals (father 407 ms, mother 377 ms) with no signs of polymorphic ventricular arrhythmias. Directed screening of genes involved with LQTS and CPVT (*KCNQ1, KCNH2, SCN5A, KCNE1, KCNE2, KCNJ2, ANK2, CAV3, KCNE3, SNTA1, RYR2, CASQ2*) was negative for mutations.

Case 4—A Moroccan female from a family with no history of cardiac arrhythmia was hospitalized at age 8 years after an episode of syncope associated with prolonged period of unconsciousness. At that time, she had a prolonged QTc interval (500 ms) with ventricular bigeminy. A Holter recording demonstrated prolonged QTc interval (ECG images were not available). Echocardiographic evaluation was normal. Both parents and four female siblings were asymptomatic. The subject was treated with nadolol (40 mg/day) and she remained

asymptomatic with QTc intervals ranging from 420 – 450 ms without ventricular ectopy. Unfortunately, she died suddenly in at age 11 years while dancing at a wedding in Morocco. The initial diagnosis was LQTS, and later a diagnosis of catecholaminergic polymorphic ventricular tachycardia was considered due to clinical circumstances and ventricular ectopy. No exercise stress test was performed. No neurologic dysfunction was reported and a head CT scan was normal. Genetic testing was negative for *KCNQ1*, *KCNH2*, *SCN5A*, *KCNE1*, *RYR2*, *CASQ2*, and *TRDN*.

Case 5—A previously healthy Caucasian boy from England suffered cardiac arrest secondary to ventricular fibrillation at age 17 months and he was promptly resuscitated. Electrocardiogram showed bradycardia and a prolonged QTc interval (555 ms; Fig. 4). There was no family history of cardiac arrhythmia and both parents were healthy with normal QTc interval duration. There were no siblings. An ICD was placed soon after the cardiac arrest and no discharges were documented over the ensuing 13 months. The subject was also treated with β-blockers. Genetic testing for mutations *KCNQ1*, *KCNH2*, *SCN5A*, *KCNE1* and *KCNE2* was negative.

A summary of clinical features observed in the five cases is presented in Table 1.

Discovery of novel *CALM2* mutations

To identify mutations in candidate arrhythmia susceptibility genes, a custom targeted exon capture probe panel interrogating 240 genes (Supplemental Table S1) was used to screen 12 unrelated mutation-negative Japanese LQTS probands using a next generation sequencing platform resulting in an average 187-fold coverage of targeted regions (additional details of the method will be reported elsewhere). A heterozygous nonsynonymous single nucleotide variant (c.400G>C, Fig. 5A) in exon 5 of *CALM2* was identified in a 6 year old girl (described above as Case 1). The nucleotide change predicts the substitution of a conserved aspartic acid residue with histidine (p.D134H) within the fourth EF-hand calcium-binding motif in the C-terminal domain of the encoded calmodulin protein. The location of this variant within the protein was four residues away from a mutation (p.D130G) previously associated with a very severe form of infantile LQTS (Fig. 5B,C).[14] This variant was not found in her parents nor her two brothers, and is absent in dbSNP, 1000 Genomes, Exome Variant Server (EVS), RIKEN and HGVD exome databases consistent with a novel *de novo* mutation.

Motivated by this finding, we searched for other calmodulin gene (*CALM1*, *CALM2*, *CALM3*) mutations in exome sequence data (coverage was 35X, 92X and 59X for the three calmodulin genes, respectively) obtained from 190 unrelated mutation-negative Japanese LQTS probands. A second heterozygous nonsynonymous variant (c.293A>G, Fig. 5A) was found in *CALM2* exon 5 in a 5-year-old boy (described above as Case 2) diagnosed with LQTS. This variant is predicted to replace a conserved asparagine at position 98 with serine (p.N98S) within the third EF-hand calcium-binding motif in calmodulin (Fig. 5B,C). Interestingly, *de novo* p.N98S mutation in a different calmodulin gene (*CALM1*) was previously associated with CPVT in an Iraqi female without QT prolongation.[13] This variant was not found in his parents or brother, and was absent in exome data of the other 189

LQTS probands as well as in databases of genetic variation (dbSNP, 1000 Genomes, EVS, RIKEN and HGVD exome databases) consistent with a *de novo* missense mutation. The probability that two private non-synonymous mutations occurred in *CALM2* among 190 Japanese samples by chance was estimated as 0.0258 (see Supplemental Material).

Exome sequencing was also performed on a 17 month old English boy who suffered cardiac arrest in the context of LQTS (Case 5) and his healthy parents (coverage averaged 30X for the three calmodulin genes). After excluding all variants shared with at least one parent, synonymous variants and common variants, the proband was found to have *de novo* nonsynonymous variants in 4 protein-coding genes (*CALM2*, *OBSCN*, *DLG1*, *GOLGA3*). However, only the variant identified in *CALM2* (c.A293>T, Fig. 5A) predicting substitution of asparagine at position 98 in calmodulin with isoleucine (p.N98I, Fig. 5B,C) was predicted to be 'deleterious' by SIFT and 'probably damaging' by PolyPhen-2. This variant occurs at the same position as the *CALM2* mutation discovered in Case 2 (LQTS) and a previous reported *CALM1* mutation (N98S) found in a child with CPVT.[13] *CALM2*-p.N98I was absent in the previously mentioned databases.

By candidate gene screening of *CALM1*, *CALM2* and *CALM3*, we identified two other heterozygous missense *CALM2* variants. One variant was discovered in a 29-year-old German female who was diagnosed initially with neonatal LQTS and later exhibited exercise-induced polymorphic ventricular ectopy (Case 3). The variant (c.396T>G, Fig. 5A) predicted the replacement of a conserved aspartic acid residue at position 132 with glutamate (p.D132E) within the fourth EF-hand calcium-binding motif in calmodulin (Fig. 5B,C). The location of the variant is two amino acids N-terminal of p.D134H (Case 1), and two residues C-terminal of the previously identified p.D130G.[14] This variant was predicted to be damaging by SIFT and Mutation Taster, while it was predicted to be benign by Polyphen2. The mutation was not found in the aforementioned databases of genetic variants (dbSNP, 1000 Genomes, EVS) and was also absent in the Helmholtz exome database in which mean coverage of *CALM2* was greater than 95-fold.[14] The variant was not found in her parents and therefore D132E was considered a likely novel *de novo* missense *CALM2* mutation.

The second *CALM2* variant discovered by targeted sequencing was found in an 8 year old Moroccan girl (Case 4) with presumptive diagnoses of LQTS and CPVT who died suddenly during exertion despite ongoing treatment with β-blockers. The variant (c.A407>C, Fig. 5A) predicted the replacement of glutamine at position 136 with proline (p.Q136P) in the fourth EF-hand calcium-binding motif (Fig. 5B,C). The mutation was not found in the aforementioned databases of genetic variants (dbSNP, 1000 Genomes, EVS), and was absent in the parents and four siblings consistent with a *de novo* mutation.

CALM2 mutations confer impaired calcium affinity

We previously demonstrated that calmodulin mutations associated with early onset LQTS confer reduced affinity for calcium.[14] Similarly, Nyegaard and colleagues examined calcium affinity for *CALM1* p.N98S, which they observed in a *de novo* case of CPVT, and found a slight depression in C-domain calcium binding affinity.[13] To determine the biochemical consequences of the four novel *CALM2* mutations we identified, recombinant calmodulin

proteins were generated and purified, and *in vitro* Ca^{2+} binding affinities were measured. None of the four mutations significantly affected Ca^{2+} affinity in the N-domain, but substantial effects on affinity in the C-domain were observed (Fig. 6). Dissociation constants for Ca^{2+} (Kd) of 2.1 ± 0.1, 15 ± 1, 48 ± 10, 27 ± 5 and 19 ± 2 μM, were determined for wildtype, N98I, D132E, D134H and Q136P, respectively, corresponding to a 7 to 23-fold reduction in Ca^{2+} binding affinity to the C-domain. These data demonstrate a significant functional impairment caused by the novel calmodulin variants consistent with disease-causing mutations that will likely disrupt the ability to transduce intracellular Ca^{2+} signals leading to cardiac arrhythmia susceptibility.

Discussion

The identification of new arrhythmia susceptibility genes and mutations will facilitate the prevention of sudden cardiac death through the rapid identification of at-risk populations, and may illuminate new molecular targets for therapy. Here we expand the spectrum of mutations in calmodulin, a recently demonstrated cause of life-threatening heart rhythm disorders.

Calmodulin functions as a Ca^{2+} sensor in a wide range of intracellular Ca^{2+} signaling pathways. The protein sequence is completely conserved among all vertebrates, and in humans three unique genes (*CALM1*, *CALM2*, *CALM3*) encode for identical calmodulin protein.[32] In the recent reports of human calmodulin gene mutations, there was only one *CALM2* allele identified compared to five *CALM1* mutations.[13–15] The previously identified calmodulin mutations associated with LQTS phenotypes along with those we report here affect conserved residues within the two EF-hand motifs of the C-domain and cause substantially impaired Ca^{2+} affinity. The mutations with the greatest impact on Ca^{2+} affinity involve substitutions of conserved aspartic acid residues (D130G, D132E, D134H) known to be directly involved in coordinating Ca^{2+} ions in Ca^{2+} binding loop IV.[33,34] Notably, even substitution with the highly similar glutamic acid side chain in D132E has an influence on Ca^{2+} affinity. Similar effects of this subtle Asp to Glu mutation on the Ca^{2+} affinity of calmodulin have been reported.[35] Although a functional effect was not predicted *in silico* by PolyPhen2, it is well established that each residue in the Ca^{2+} binding loops of calmodulin and other EF-hand proteins contribute to the biochemical functions of the protein.[36]

The cellular mechanisms responsible for arrhythmia susceptibility in the setting of calmodulin mutations are likely to be complex given the multitude of molecular interactions possible for this critically important signaling molecule. As previously speculated for LQTS,[14] dysfunctional calmodulin may disrupt Ca^{2+} dependent inactivation of L-type calcium channels leading to increased depolarizing current during the plateau phase of the cardiac action potential. Impaired regulation of voltage-gated sodium channels may also be evoked by certain LQTS-associated calmodulin mutations. For calmodulin mutations associated with CPVT, aberrant regulation of the sarcoplasmic reticulum ryanodine receptor/Ca^{2+} release channel (*RYR2*) is a plausible mechanism based on previous studies.[37] Interestingly, the CPVT mutations do not impair Ca^{2+} affinity to the same extent as those associated with LQTS.[13] The molecular and cellular pathophysiology of arrhythmia

susceptibility in the setting of calmodulin mutations is currently under intense investigation.[38–40]

Our findings further expand the phenotypic spectrum of cardiac arrhythmias associated with calmodulin mutations. Three of the probands (Cases 1, 2, 5) had a later onset of LQTS compared to what was described in the study by Crotti, *et al.*, in which calmodulin mutation-positive subjects had highly malignant ventricular arrhythmias beginning very early in life.[14] Further, none of the *CALM2* mutation positive subjects we report here had significant neurological findings, other than syncope-associated seizures (Case 1), in contrast to the original report in which most subjects had seizures and/or developmental delays. The previously observed neurological impairments were speculated to be the result of brain injury secondary to hypoxia in the setting of recurrent cardiac arrest. The minimal or absent neurological symptoms in the probands we describe here may reflect fewer episodes of cardiac arrest or more rapid resuscitation. These new observations further imply that neurological symptoms may not be an intrinsic manifestation of calmodulin mutations.

Genotype-phenotype correlations among the calmodulin mutation positive subjects we described may provide clues to the pathophysiological mechanisms. In particular, *CALM2*-p.D132E was identified in an adult with a history of neonatal LQTS who later developed exercise-induced polymorphic ventricular arrhythmia consistent with CPVT. Similarly, *CALM2*-p.Q136P was identified in a child with LQTS and ventricular ectopy somewhat suggestive of CPVT. We speculate that the combined clinical features of LQTS and CPVT reflect the impact of p.D132E and possibly p.Q136P on two principal molecular targets. Abnormal calmodulin regulation of L-type Ca^{2+} channels would account for impaired myocardial repolarization similar to Timothy syndrome,[41] while dysregulation of *RYR2* would lead to altered regulation of intracellular calcium homeostasis as expected in CPVT.[42,43]

Our study also revealed that an identical amino acid substitution in two distinct calmodulin genes can present with different clinical phenotypes. Whereas *CALM1*-p.N98S was originally found in an Iraqi female with CPVT,[13] we identified *CALM2*-p.N98S in a Japanese male with an unambiguous LQTS phenotype (Case 2). The physiological basis for this genotype-phenotype disparity is unknown, but may involve differences in the corresponding proteomes of different probands due to sex or ethnicity, or differences in regional or cell-type specific expression of *CALM1* and *CALM2*.

Except during periods of medication non-compliance, all *CALM2* mutation positive probands described in this report were responsive to β-blockers administered alone or in combination with mexiletine. However, because of recurrent cardiac arrests during treatment lapses, Cases 1 and 5 had implantation of an ICD. In our prior report of calmodulin mutations in severe LQTS, probands with *de novo CALM1* or *CALM2* mutations experienced arrhythmia recurrence on pharmacological therapy and were eventually treated with ICD implantation to reduce the risk for sudden cardiac death.[14] Similarly, symptomatic mutation-positive subjects with IVF reported by Marsman, et al., had ICD implantation.[15] By contrast, the Swedish family segregating CPVT with mutation *CALM1*-N54I described by Nyegaard, *et al.* exhibited variable responses to β-blockers alone and only 1 of 10 living

mutation-positive subjects received an ICD.[13] None of the cases we report here underwent left cardiac sympathetic denervation.

In conclusion, we report discovery of five novel *de novo CALM2* mutations in association with LQTS and exertion-induced arrhythmias. The encoded mutant calmodulin proteins have impaired C-domain Ca^{2+} binding affinity that will presumably cause dysfunction in Ca^{2+} signaling with resulting adverse effects on plasma membrane ion channels, intracellular membrane ion channels or possibly both. Therapy with β-blockers was successful in preventing life-threatening exertion-triggered arrhythmias. Our study provides new evidence of congenital arrhythmia susceptibility caused by calmodulin mutations, provides further information regarding genotype-phenotype correlation and expands the allelic diversity within *CALM2*. Calmodulin gene mutations should be sought in pediatric cases of LQTS and CPVT for whom other genetic candidates have been excluded. Because of the predominance of *de novo* mutations, calmodulin genes could be considered especially when both parents are unaffected.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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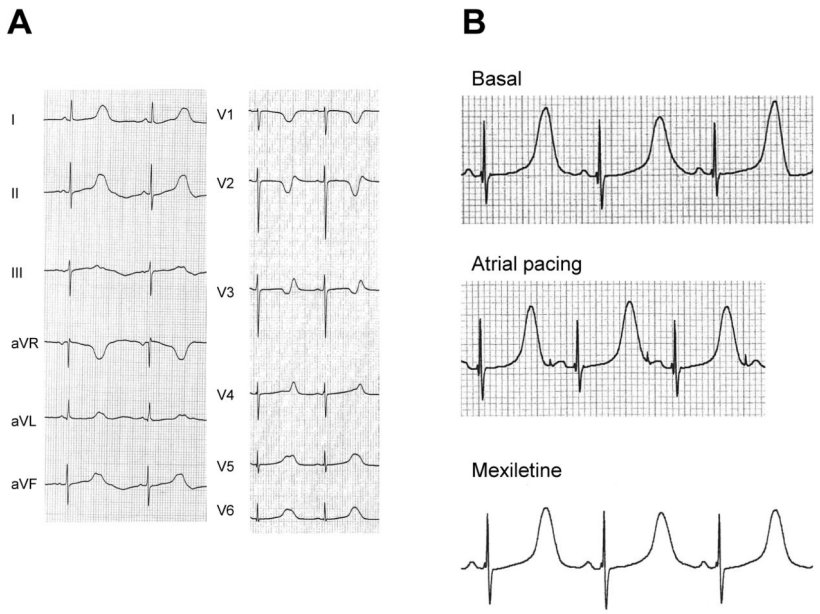


Figure 1.

Electrocardiographic abnormalities in Case 1. A) Standard 12-lead ECG recorded at age 6 years showing marked QTc prolongation (579 ms) with atypical T wave morphology (late-peaking with notch on the descending limb). B) Atrial pacing at 100 bpm prolonged QTc from 596 ms to 610 ms. By contrast, mexiletine treatment shortened QT interval from 596 ms to 550 ms.

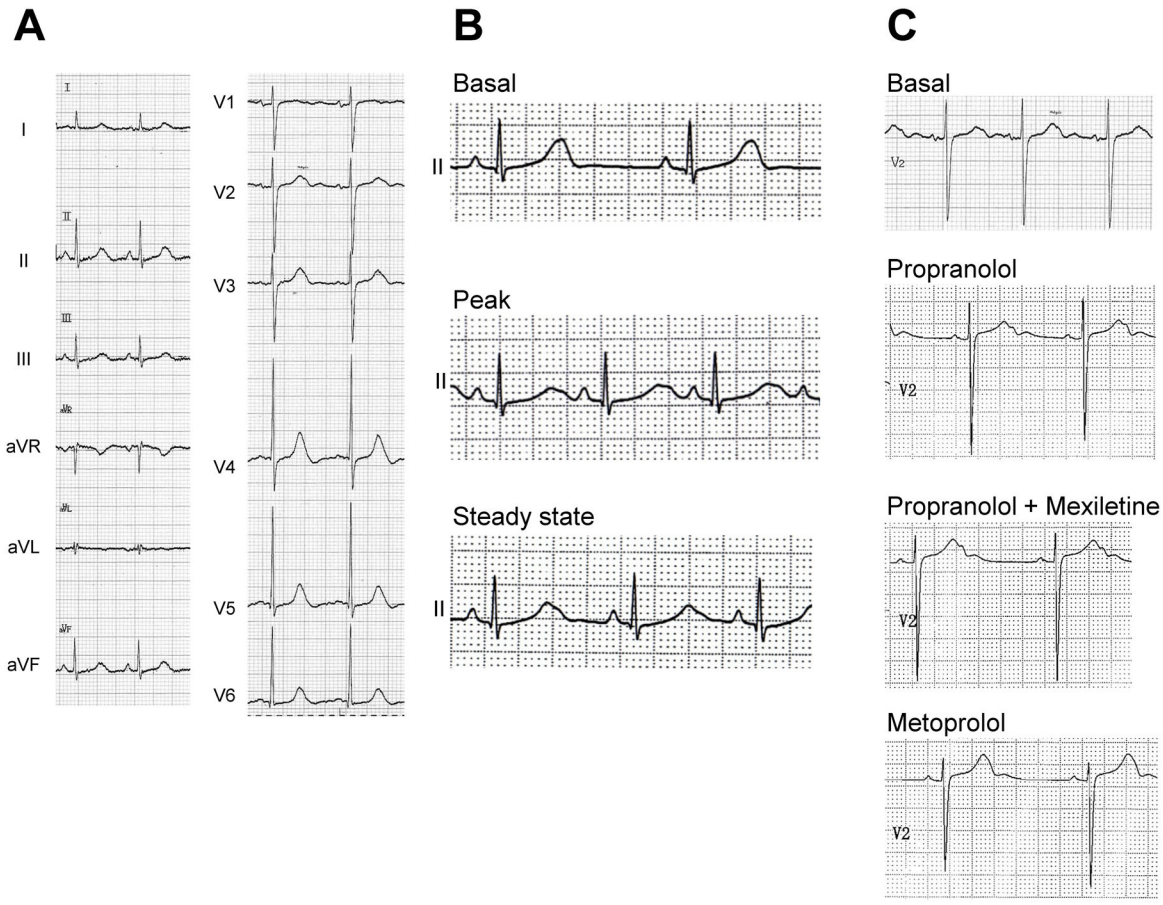
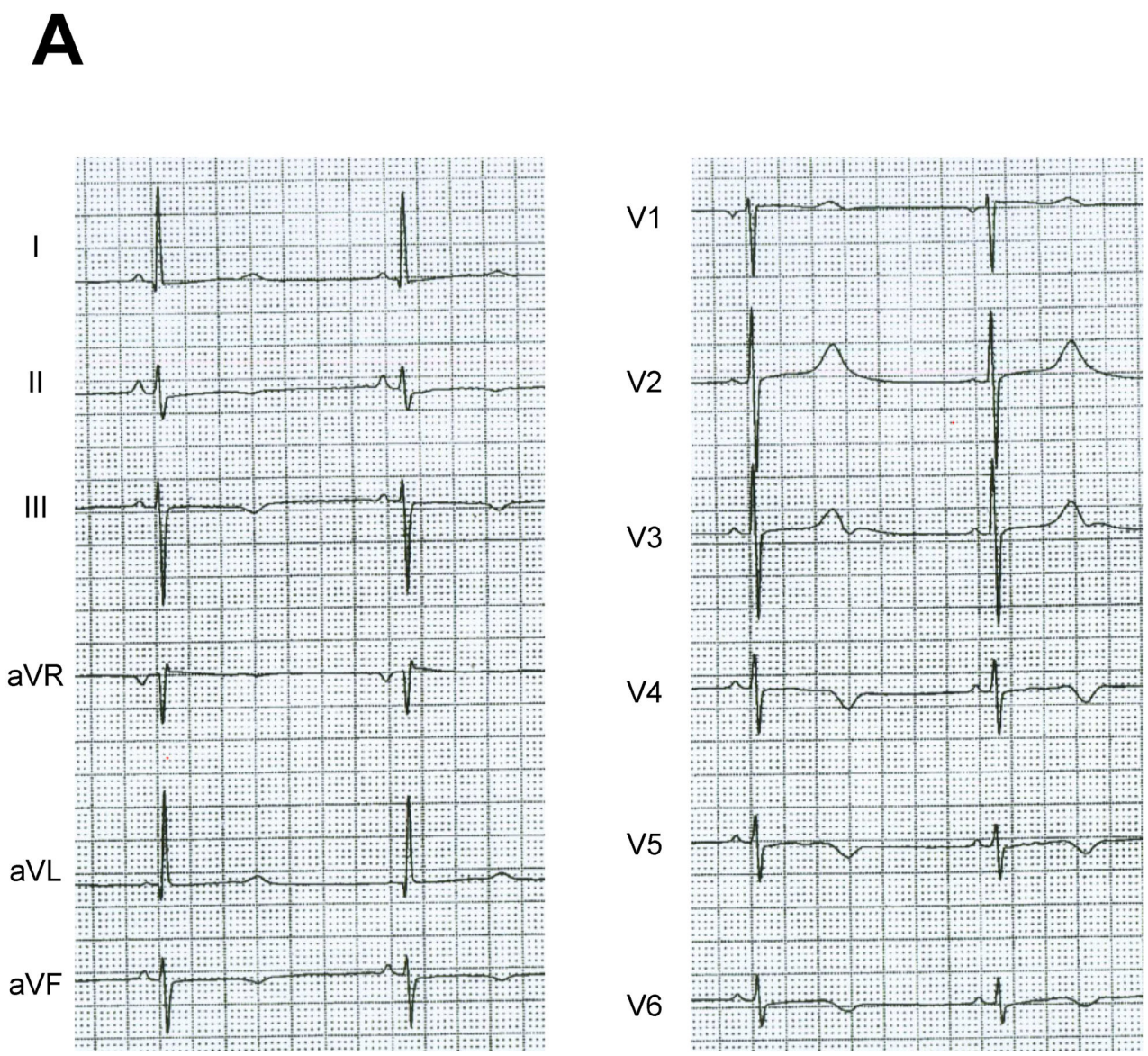


Figure 2.

Electrocardiographic abnormalities in Case 2. A) Standard 12-lead ECG recorded at age 5 years showing QTc prolongation (478 ms). B) Epinephrine challenge test prolonged QTc at peak (466 ms to 558 ms) and at steady-state (535 ms). C) Propranolol or propranolol with mexieltine caused QTc shortening from 517 ms to 471 ms and 473 ms, respectively. Metoprolol normalized the QTc to 449 ms.



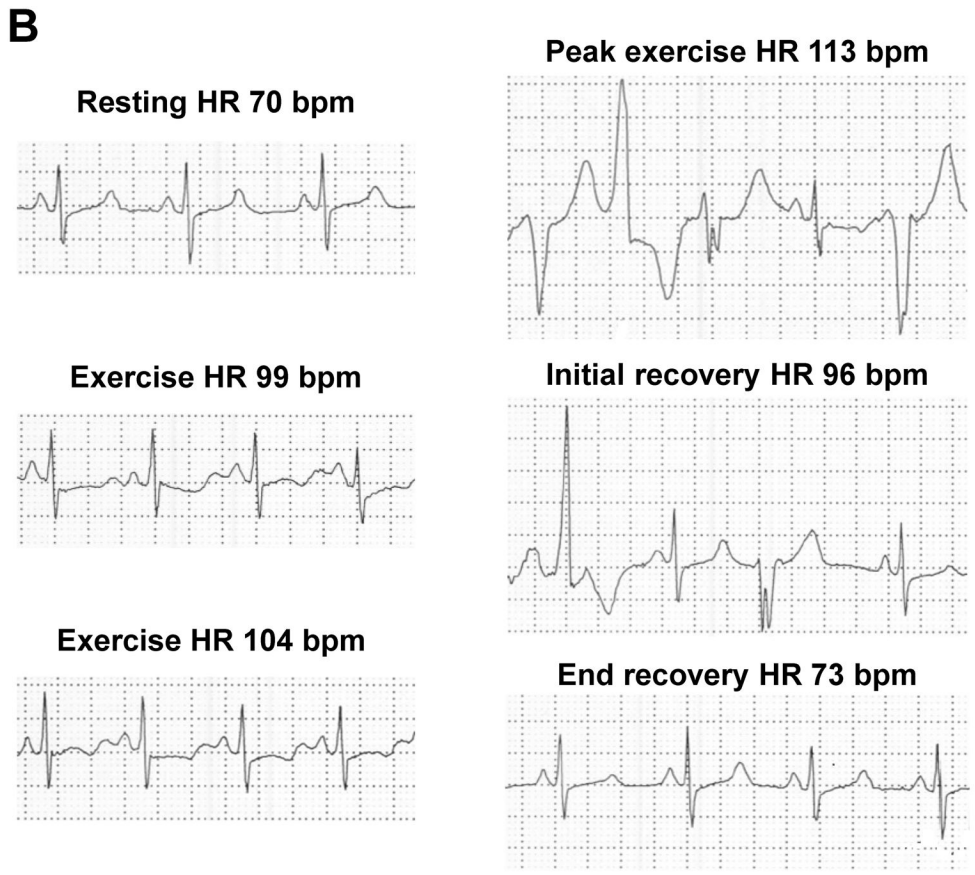


Figure 3.

Electrocardiographic features of Case 3. A) Standard 12-lead ECG recorded at age 27 years showing QTc prolongation (567 ms). B) Polymorphic ventricular ectopy recorded (lead III) during exercise (step test) at age 28 years. During exercise, a progressive increase of heart rate was observed with no arrhythmias until 110 bpm was reached. A 3-beat episode of polymorphic ventricular tachycardia was recorded at 113 bpm. A representative 12-lead ECG during exercise is provided as Supplemental Fig. S1.

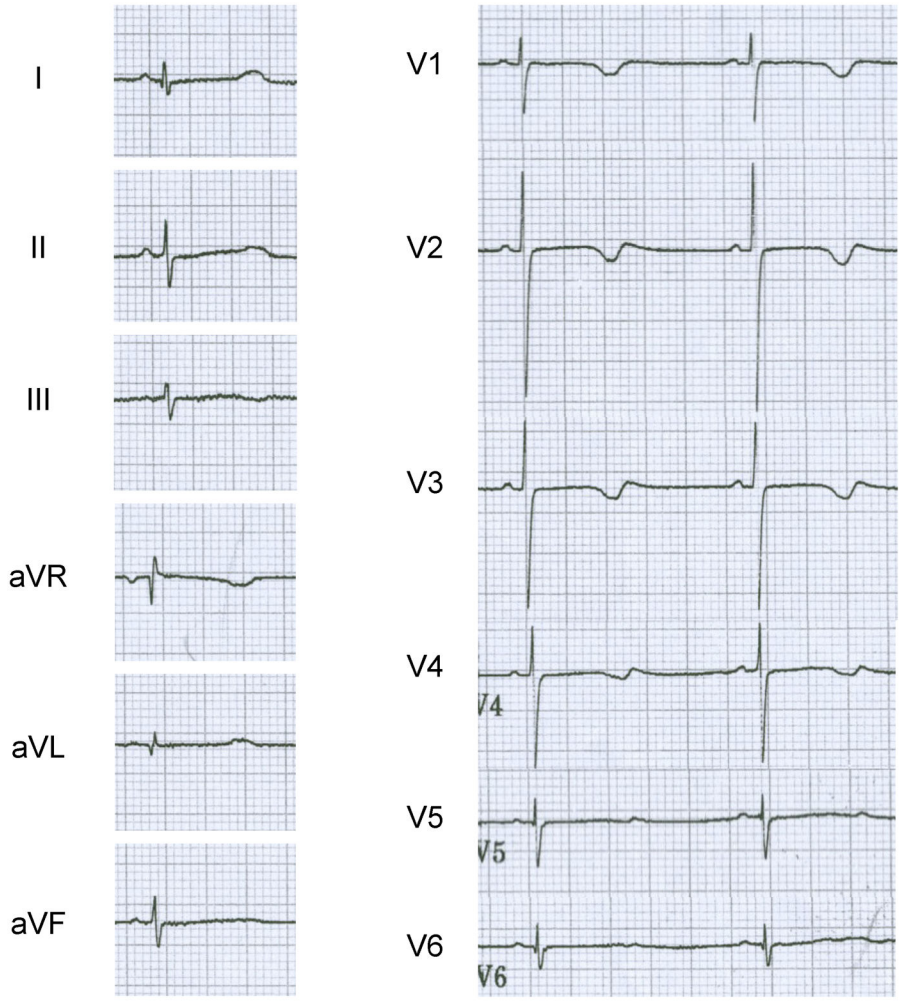


Figure 4.

Electrocardiographic features of Case 5. Standard 12-lead ECG recorded at age 2 years showing QTc prolongation (555 ms) and bradycardia (HR = 55 bpm) during β-blocker treatment. Because of bradycardia, only one beat was recorded in the limb leads.

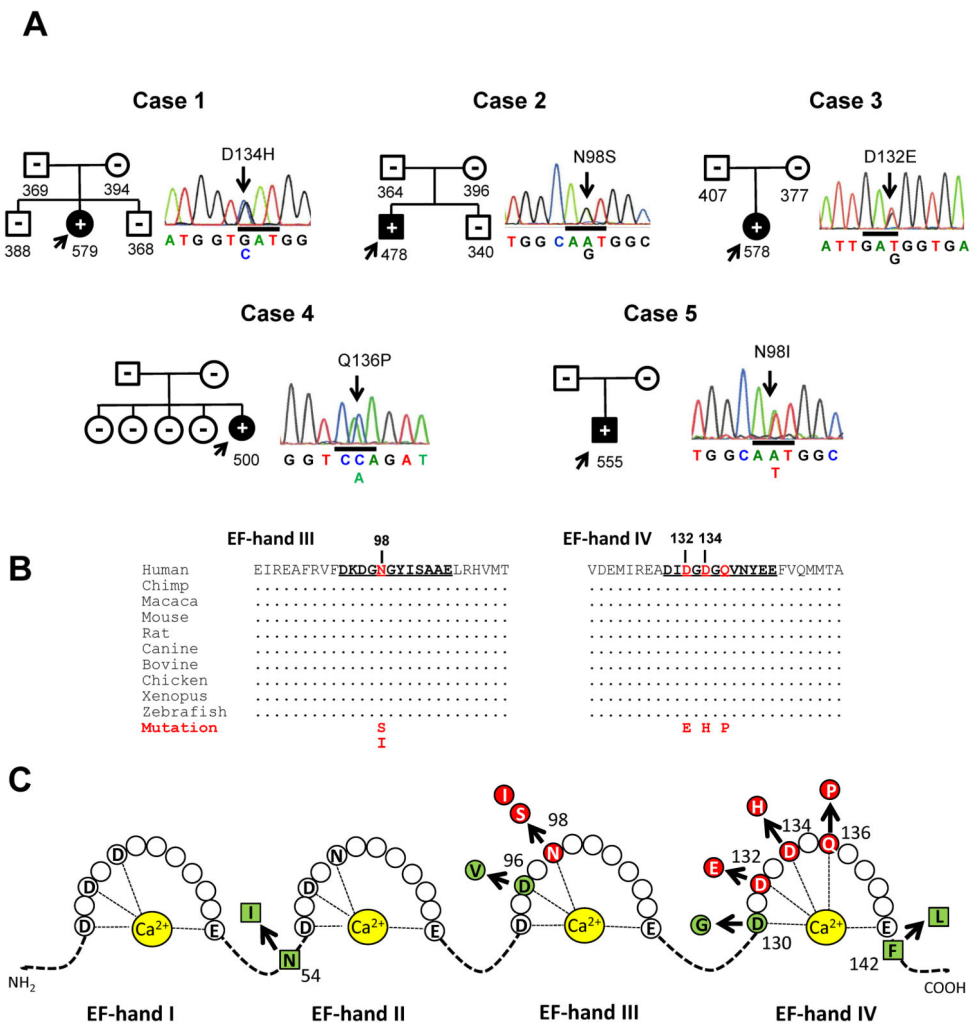


Figure 5.

Novel *de novo CALM2* mutations. A) Pedigrees and Sanger sequence electropherograms of each proband (marked by arrow). QTc values for each individual are shown underneath the corresponding pedigree symbol. B) Amino acid sequence alignments for calmodulins from different species with location of the five missense mutations. Amino acid sequence of EF-hands III and IV are underlined. C) Schematic model of Ca^{2+} binding loops in the N-terminal (I and II) and C-terminal (III and IV) domains of calmodulin showing the locations of mutations. Red circles represent the *CALM2* mutations (p.N98S, p.N98I, p.D132E, p.D134H, p.Q136P) identified in our present study, green symbols represents previously reported mutations.

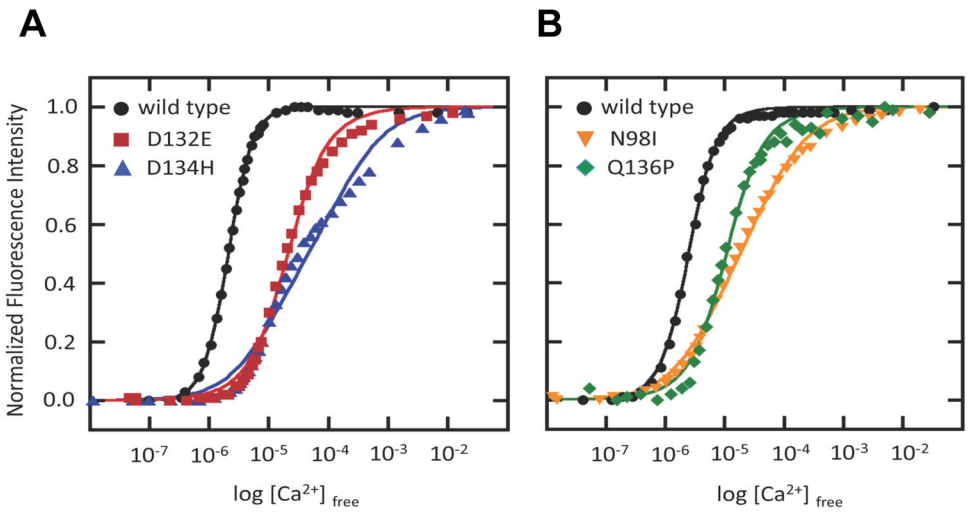


Figure 6.

Impaired Ca^{2+} binding by mutant calmodulin C-domains. A) Representative Ca^{2+} titrations monitored by intrinsic fluorescence for recombinant WT (black circles) and mutant calmodulins (D132E, blue triangles; D134H, red squares). K_d values derived by fitting data from three replicates to a two-site model were (mean ± SEM) 2.1 ± 0.1, 48 ± 10, and 27 ± 5 μM for wildtype, D132E and D134H, respectively. B) Representative Ca^{2+} titrations for WT (black circles) and mutant (N98I, inverted orange triangles; Q136P, green diamonds) calmodulins. K_d values derived by fitting data from three replicates to a two-site model were (mean ± SEM) 2.3 ± 0.1, 19 ± 2, and 15 ± 1 μM for wildtype, N98I and Q136P, respectively.

Table 1

Summary of clinical characteristics and the *CALM2* mutations of the probands

Subject	Sex	Age at Diagnosis (current age)	CA	QTc (ms)	Treatment	Mutation
Case 1	F	1 year (16 years)	Y	579	MEX, BB, ICD	*CALM2*-p.D134H
Case 2	M	5 years (12 years)	N	478	MEX, BB	*CALM2*-p.N98S
Case 3	F	Perinatal (29 years)	N	578	BB	*CALM2*-p.D132E
Case 4	F	8 years (died age 11 y)	SCD	500	BB	*CALM2*-p.Q136P
Case 5	M	17 months (30 months)	Y	555	BB, ICD	*CALM2*-p.N98I

Abbreviations: CA, cardiac arrest; MEX, mexiletine; BB: β-blocker; ICD, implantable cardioverter defibrillator; PVT, polymorphic ventricular tachycardia; PVC, premature ventricular contractions; SCD, sudden cardiac death.