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Novel CPVT-Associated Calmodulin Mutation in *CALM3* (CALM3-A103V) Activates Arrhythmogenic Ca Waves and Sparks

Nieves Gomez-Hurtado, PhD[1,*], **Nicole J. Boczek, PhD**[2,*], **Dmytro O. Kryshtal, PhD**[1,*], **Christopher N. Johnson, PhD**[3], **Jennifer Sun, BS**[3], **Florentin R. Nitu, PhD**[4], **Razvan L. Cornea, PhD**[4], **Walter J. Chazin, PhD**[3], **Melissa L. Calvert, BS**[2], **David J. Tester, BS**[2], **Michael J. Ackerman, MD, PhD**[2,5,6], and **Bjorn C. Knollmann, MD, PhD**[1]

[1]Division of Clinical Pharmacology, Oates Institute for Experimental Therapeutics, Vanderbilt University School of Medicine, Nashville, TN

[2]Department Molecular Pharmacology & Experimental Therapeutics, Windland Smith Rice Sudden Death Genomics Laboratory

[3]Departments of Biochemistry and Chemistry & Center for Structural Biology, Vanderbilt University, Nashville, TN

[4]Department of Biochemistry, Molecular Biology & Biophysics, University of Minnesota, Minneapolis, MN

[5]Department of Cardiovascular Diseases (Division of Heart Rhythm Services)

[6]Department of Pediatrics (Division of Pediatric Cardiology), Mayo Clinic, Rochester, MN

Abstract

Background—Calmodulin (CaM) mutations are associated with severe forms of long QT syndrome (LQTS) and catecholaminergic polymorphic ventricular tachycardia (CPVT). We recently reported that CaM mutations were found in 13% of genotype-negative LQTS patients, but the prevalence of CaM mutations in genotype-negative CPVT patients is unknown. Here, we identify and characterize CaM mutations in 12 patients with genotype-negative but clinically-diagnosed CPVT.

Methods and Results—Mutational analysis of *CALM1*, *CALM2* and *CALM3* coding regions, in vitro measurement of CaM-Ca^{2+} (Ca) binding affinity, RyR2-CaM binding, Ca handling, L-type Ca current (LTCC) and action potential duration (APD). We identified a novel CaM mutation –

Correspondence: Björn C. Knollmann, MD, PhD, Professor of Medicine and Pharmacology, Division of Clinical Pharmacology, Vanderbilt University School of Medicine, Medical Research Building IV, Rm. 1265, 2215B Garland Ave. Nashville, TN 37232-0575, Tel: [Office] (615) 343-6493, Tel: [Lab] (615) 936-7303, Fax: (615) 343-4522, bjorn.knollmann@vanderbilt.edu; Michael J. Ackerman, MD, PhD, Windland Smith Rice Cardiovascular, Genomics Research Professor, Professor Medicine, Pediatrics, & Pharmacology, Mayo Clinic Windland Smith Rice Sudden, Death Genomics Laboratory, Guggenheim 501, Mayo Clinic, 200 First Street SW, Rochester, MN 55905, Tel: 507-284-0101 / Fax: 507-284-3757, ackerman.michael@mayo.edu.
[*]Drs. Gomez-Hurtado, Boczek, and Kryshtal contributed equally as first authors

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A103V – in *CALM3* in 1 of 12 patients (8%), a female who experienced episodes of exertion-induced syncope since age 10, had normal QT interval, and displayed ventricular ectopy during stress testing consistent with CPVT. A103V modestly lowered CaM Ca-binding affinity (3-fold reduction vs WT-CaM), but did not alter CaM binding to RyR2. In permeabilized cardiomyocytes, A103V-CaM (100 nM) promoted spontaneous Ca wave and spark activity, a cellular phenotype of RyR2 activation. Even a 1:3 mixture of A103V-CaM:WT-CaM activated Ca waves, demonstrating functional dominance. Compared to LQTS D96V-CaM, A103V-CaM had significantly less effects on LTCC inactivation and APD, and caused delayed after depolarizations (DADs) and triggered beats in intact cardiomyocytes.

Conclusions—We discovered a novel CPVT mutation in the *CALM3* gene that shares functional characteristics with established CPVT-associated mutations in *CALM1*. A small proportion of A103V-CaM is sufficient to evoke arrhythmogenic Ca disturbances via RyR2 dysregulation, which explains the autosomal dominant inheritance.

Keywords

calmodulin; catecholaminergic polymorphic ventricular tachycardia; calcium; ryanodine receptor; calcium channel

Introduction

Calmodulin (CaM) is an essential Ca-binding protein with multiple cellular targets. CaM has 4 EF-hand Ca-binding motifs located in 2 N-terminal (CaM-N) and 2 C-terminal (C-CaM) globular domains, which are connected by a flexible linker[1]. At high [Ca], Ca binds cooperatively to CaM, inducing a conformational change that transduces Ca signals in a wide range of biological processes including inflammation, muscle contraction, memory, metabolism, and immune responses. Recently, CaM missense mutations have been identified in patients with severe ventricular arrhythmia and sudden death susceptibility[2-4]. Humans have 3 CaM genes (*CALM1*, *CALM2*, and *CALM3*) encoding for a perfectly conserved sequence of amino acids, and all three CaM genes are expressed in the heart[5].

CaM regulates a variety of ion channels in cardiac myocytes. Among them, changes in CaM regulation of the L-type calcium channel (LTCC) and RyR2 have been associated with autosomal dominant syndrome of sudden death that can present with clinical features of long QT syndrome (LQTS) or catecholaminergic induced polymorphic ventricular tachycardia (CPVT)[2-4]. CaM is the main Ca sensor for the LTCC[6, 7] and mutations that lead to a reduction in CaM Ca binding affinity impair Ca-dependent inactivation of LTCCs and cause action potential prolongation, which can explain the LQTS phenotype[8]. CaM also binds to RyR2 stoichiometrically (4 CaMs per tetrameric RyR2)[9] and inhibits RyR2 opening at all [Ca], which regulates SR Ca release[10]. Mutations that cause defective CaM-mediated RyR2 inactivation may contribute to RyR2 leakiness promoting significantly higher spontaneous Ca wave and spark activity, a typical cellular phenotype of CPVT.

Here, we tested the prevalence of CaM mutations in a cohort of 12 patients with genotype-negative CPVT. We identified a novel CaM mutation – A103V – in *CALM3*, in one of the 12 patients. We then characterized the functional effects of the novel A103V mutant CaM on

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Ca binding affinity, RyR2 binding, Ca handling, and the LTCC and compared the effects of A103V to those of the only two other CaM mutants associated with CPVT (N54I and N98S). Our data suggest that the CALM3-A103V mutation underlies the patient's CPVT.

Methods

Study Subjects

The study population consisted of 12 unrelated patients with genotype negative but clinically diagnosed CPVT who were referred to the Windland Smith Rice Sudden Death Genomics Laboratory at Mayo Clinic, Rochester, Minnesota for genetic testing with robust evidence of CPVT with either an abnormal stress test or documented ventricular ectopy (Table 1). The 12 patients were genotype negative after CPVT mutational analysis of the two major CPVT genes, *RYR2* and *CASQ2*. This study was approved by the Mayo Foundation Institutional Review Board and informed consent was obtained for all patients.

Mutational analysis

Comprehensive open reading frame and splice junction mutational analysis of the *CALM1*, *CALM2*, and *CALM3* coding regions was performed on genomic DNA extracted from fresh blood of 12 CPVT patients (Puregene DNA Isolation Kit, Qiagen, Inc, Valencia, CA) using PCR, denaturing high performance liquid chromatography (DHPLC; WAVE DNA Fragment Analysis System, Transgenomic Inc., Omaha, NE), and direct DNA sequencing (ABI Prism 377, Applied Biosystems Inc., Foster City, CA). Primer sequences are shown in Supplementary Table 1

Animal use

The use of animals in this study was approved by the Animal Care and Use Committees of Vanderbilt University, Nashville, TN, USA, and the University of Minnesota, MN, USA and performed in accordance with NIH guidelines.

Measurement of Ca Binding to CaM

To study the Ca binding affinity of the mutant A103V-CaM, we bacterially expressed and purified recombinant wild-type (WT) and mutant CaMs (N54I, N98S, D96V and A103V), as reported previously[3]. To summarize, the recombinant CaM cDNA sub-cloned into a pET15b vector was mutated using QuikChange site directed mutagenesis. WT and mutant CaMs were expressed in E. coli BL21 (DE3) cells and purified by hydrophobic chromatography using a phenyl sepharose column. To minimize Ca, the proteins were dialyzed 4 times 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. Ca-binding affinities for WT and A103V-CaM proteins were determined as described[3]. Briefly, macroscopic-binding constants for the pairs of Ca-binding sites in CaM-N and CaM-C were measured by monitoring the intrinsic tyrosine and phenylalanine fluorescence of the protein during the course of a Ca titration. Free [Ca] was determined using the fluorescent dye fluo-5N. Data were analyzed by plotting the normalized fluorescence signal versus free [Ca] and fitting to the model-independent, 2-site Adair function. The dissociation constants (K_d)

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for each domain are reported as the average value for the pair of sites by taking the square root of K2 from the Adair equation.

CaM Binding to RyR2

To resolve any differences in CaM/RyR2 binding due to the A103V-CaM mutation, we used a FRET-based assay that detects acceptor-labeled CaM (A-CaM) binding within FRET range (<10 nm) of a donor-labeled variant of FKBP12.6 (D-FKBP)[11], which is pre-targeted to RyR2 in porcine cardiac SR vesicles, as previously described in detail[4]. We measured the competitive inhibition of A-CaM binding by unlabeled A103V or WT-CaM. The FRET samples consisted of 3mg/ml SR prelabeled with D-FKBP[11], WT or A103V-CaM, 100 nM A-CaM, 20 mM K-PIPES (pH 7.0), 150 mM KCl, 5 mM GSH, 1 mM EGTA, 0.1 mg/ml BSA, 1 μg/ml Aprotinin/Leupeptin, and sufficient $CaCl_2$ to yield the indicated free [Ca^{2+}] (as calculated using MaxChelator, http://maxchelator.stanford.edu).

Ventricular myocytes isolation

Single ventricular myocytes from 10- to 16-week-old C57BL/6 mice were isolated by enzymatic digestion using collagenase as previously described[12]. Briefly, the ascending aorta was cannulated and the heart was perfused with collagenase (Worthington type II) and protease at 36-37°C. After perfusion, the hearts were cut up, chopped into small pieces, filtered through a 250 μm mesh and washed twice in a standard Tyrode solution containing 0.2 mM of $CaCl_2$ to disperse the isolated left ventricular myocytes. Finally, the cells were resuspended in a solution containing 0.6 mM of $CaCl_2$.

Ca sparks and SR Ca content

For Ca sparks measurements freshly isolated mouse ventricular myocytes were permeabilized with saponin (40 μg/mL) for 60 seconds and placed in internal solution composed (in mM) of K-aspartate 120, KCl 15, KH_2PO_4 5, $MgCl_2$ 5.6, dextran 4%, HEPES 10, MgATP 5, phosphocreatine di-Na 10, creatine phosphokinase 10 U/mL, glutathione (reduced) 10, and Fluo-4 0.025. Free [Ca] was 50 nM (calculated with MaxChelator). To allow equilibration of CaM binding to cellular targets, measurements were done after 30-minute incubation with either WT or CPVT mutant CaMs (A103V, N54I or N98S). Free [CaM] was kept at the physiological concentration of 100 nM[13]. Ca sparks were imaged with a 510 Zeiss inverted confocal microscope in line-scan mode for 3 seconds. Image analysis was performed with ImageJ and Ca spark master. SR Ca load was assessed as the Ca transient amplitude induced by rapid application of 10 mM caffeine.

Ca wave measurement

For Ca wave measurements, cardiomyocytes were first exposed to a Ca free relaxing solution and then permeabilized with saponin (40 μg/mL) for 60 seconds and placed in internal solution composed (in mM) of 120 K-aspartate, 15 KCl, 5 KH_2PO_4, 0.75 $MgCl_2$, 4% dextran, 10 HEPES, 5 Mg_2ATP, 10 glutathione (reduced), 0.025 Fluo-4 and 10 phosphocreatine (di-Na). These solutions also contained 10 U/ml creatine phosphokinase and had free [Ca] = 120 nM. To allow equilibration of CaM binding to cellular targets, all Ca wave recordings were done after 30-minute incubation either WT or CPVT mutant CaMs

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(A103V, N54I or N98s). Free [CaM] was kept at the physiological concentration of 100 nM[13]. Ca waves in myocytes were imaged with a confocal microscope (LSM 510 Zeiss) in line-scan mode. Ca wave analysis was performed as described[4]. For the mixing studies cells were incubated with WT-CaM at a final concentration of 100 nM or with a mixture of 75% WT-CaM and 25% CPVT-CaM mutants A103V, N54I or N98S (final concentration 100 nM). Given the variability between different experimental days, the Ca wave frequency and amplitude data were normalized to the mean of WT group obtained on the same day.

Inactivation of LTCCs

Inactivation of L-type Ca currents (I_{CaL}, LTCCs) was studied in freshly isolated murine ventricular myocytes using whole-cell patch clamp technique. CaM (final concentration 6 μM) was added to the internal solution composed (in mM) of 110 CsCl, 1$MgCl_2$, 5 MgATP, 0.2 cAMP, 14 EGTA and 20 Hepes and then dialyzed into the cell via patch pipette using positive pressure. The external solution was composed (in mM) of 134 NaCl, 5 CsCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 Glucose and 10 Hepes. Currents were elicited with 500-ms depolarizing pulses to 0 mV from holding potential H=-70 mV applied every 2 min to track the effect of CaM over time, as it diffuses into the cell. Usually, the effect of CaM on I_{CaL} inactivation was reaching its maximum in 4-6 min after start of infusion. A 15-ms pre-pulse to -40 mV was applied prior to the test pulse in order to inactivate Na currents. Cells were pre-treated with ryanodine (50 μM) and thapsigargin (10 μM) for 30 min prior to every experiment to prevent SR Ca release. All experiments were conducted at room temperature.

Action potential measurements

For these experiments cells were studied in current-clamp mode. Ventricular action potentials were measured using pipette solutions containing (in mmol/L): potassium gluconate, 110; NaCl, 5; KCl, 10; EGTA, 0.5; Hepes, 10; MgATP, 5; cAMP, 0.2; pH adjusted to 7.2 with KOH. 6 μM of WT, A103V mutant or D96V mutant CaM was added to the pipette solution and dialyzed into the cell via patch pipette using positive pressure. Whole-cell patches were established in control Tyrode solution containing (mmol/L): NaCl, 134; KCl, 5.4; MgCl2, 1; CaCl2, 2; Hepes, 10; glucose, 10; pH adjusted to 7.4 with NaOH. A train of four action potentials (1 Hz) was triggered by application of a 2-ms current injection 20 % above threshold (usually 0.3-0.4 nA). Resting potential and action potential (AP) duration measured at 50% and 90% repolarization (APD50 and APD90, respectively) were measured from the last paced AP for every cell. The incidence of early after depolarizations (EADs) was quantified for each cell during the pacing train. The incidence of delayed after depolarizations (DADs) and spontaneous beats triggered by DADs was calculated during the 45-s period after the pacing train.

Statistical analysis

Data are presented as means±SD. Gaussian distribution of the samples was assessed by D'Agostino-Pearson test. Then, statistical significance was evaluated by one-way ANOVA followed by Tukey post-test in those samples that followed a normal distribution or by Kruskal-Wallis followed by Dunn's post-test. For statistical comparison of EADs activity occurrence, the Fisher's exact test was used and the data are presented as percentage. A value of $P<0.05$ was considered statistically significant.

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Results

Clinical studies

Our genotype negative CPVT cohort consisted of 12 individuals, of which 25% were males and the average age at diagnosis was 20 ± 12 years. Overall, 83% experienced syncope, 17% cardiac arrest, and 25% had a positive family history of cardiac arrhythmias or sudden unexplained death (Table 1). DHPLC revealed one novel CaM missense mutation, p.A103V in *CALM3*, in one patient.

The A103V-CALM3 positive subject is currently a 31-year-old female who presented at 10 years of age with a loss of consciousness during exertion. Subsequently, between the ages of 10 and 14, she experienced a dozen additional syncopal episodes, many of which took place during exertion. Two of the syncopal events resulted in loss of bladder function and one required CPR. Due to these episodes, she had an ECG taken, which was unremarkable except for a prominent U wave. Her initial stress test in 1994 recorded single premature ventricular contractions (PVCs) in isolation during exercise. The ectopy increased to PVCs in bigeminy. During epinephrine infusion, she also had bigeminal PVCs and a brief run of non-sustained ventricular tachycardia triplets. In 1994, she was diagnosed originally with "atypical LQTS" and treated with beta blockers. Treadmill stress tests while on beta blockers still evidenced PVCs in bigeminy and couplets but with lower frequency (Figure 1). For the past 20 years, she has been event free while being fully compliant on beta blockers. In 2000, CPVT instead of atypical LQTS was considered as the likely diagnosis but the patient tested negative for the known CPVT-susceptibility genes.

There is no family history of arrhythmias or sudden deaths. However, her mother described several fainting episodes when she was school age. Her mother and father underwent stress testing and while her father's test was normal, her mother's test revealed exercise-induced PVCs. Mutation specific testing established that her mother was also heterozygous for A103V-CALM3 while her father was negative.

Effect of A103V-CaM on Ca binding

CaM binds Ca at two higher affinity Ca binding sites situated in the C-domain (EF-hand III and EF-hand IV) and two lower affinity sites in the N-domain (EF-hand I and EF-hand II)[14]. As expected based on the site of the mutation in EF-hand III, this novel CALM3 A103V mutant has reduced Ca binding affinity in the C-domain (3-fold reduction) whereas the mutation did not significantly alter Ca binding to the N-domain (Figure 2). This effect in Ca binding affinity was different from that found with N54I, an established CPVT-related CaM mutant located in the EF-hand II that had no effect on Ca binding affinity in either domain. However, the modestly reduced Ca binding affinity was similar to that of the only other known CPVT-linked CaM mutant that is also located in EF-hand III (N98S).[4]

A103V-CaM promotes Ca sparks activity and reduces SR Ca load

Our previous studies have shown that the two known CPVT-linked CaM mutants N54I and N98S both cause RyR2 activation[4]. Hence, we next measured the effect of A103V-CaM on Ca sparks – a measure of RyR2 Ca release channel activity in cardiomyocytes[15] – and

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compared the results with the CPVT-linked N54I-CaM and N98S-CaM (Figure 3). To avoid wave propagation, free [Ca] was kept at 50 nM with strong buffering by 0.5 mM EGTA. The cardiomyocytes were permeabilized with saponin to prevent any influence of membrane ion channels. Compared to WT-CaM, A103V-CaM increased spark frequency (Figure 3B) to a similar level as N54I-CaM or N98S-CaM without modifying any other spark parameter such as amplitude, duration or width (Figure 3C-E). As a result of increased SR Ca leak, all three CaM mutants reduced SR Ca content assessed by rapid caffeine application (Figure 3 F-G).

A103V-CaM promotes Ca waves

We next examined the consequences of CPVT-CaMs on arrhythmogenic Ca waves. Experiments were performed at physiological free [CaM] of 100 nM and diastolic free [Ca] of 120 nM. Under those conditions, cardiomyocytes incubated with A103V-CaM exhibited increased spontaneous Ca release in the form of regular propagated Ca waves compared with WT-CaM (Figure 4A and C). The effect was of A103V-CaM was similar to the effect obtained upon incubation with N54I or N98S. A103V-CaM but not N54I or N98S-CaM also increased the amplitude of Ca waves (Figure 4).

A103V-CaM exhibits dominant-activating effects on Ca Waves

As only 1 out of 6 CaM alleles of our proband hosts the A103V-CaM missense mutation, we next tested whether a small fraction of mutant CaM in the presence of WT-CaM (25% mutant, 75% WT, final concentration 100 nM) can exert a dominant effect on RyR2 function. We found that in the presence of 3-fold excess of WT-CaM (75%), A103V-CaM promoted significantly higher Ca wave frequencies[4] compared to WT-CaM. The dominant effect of A103V-CaM was as strong as that of N54I-CaM and N98S-CaM, the only two other CaM mutants implicated in CPVT.

RyR2 binding

We next used a FRET-based assay to specifically detect CaM/RyR2 binding in SR vesicles[11, 16]. RyR2-specific CaM binding was measured by FRET between D-FKBP and A-CaM. Figure 5 illustrates how A103V- and WT-CaM compete with 100 nM A-CaM and reduce FRET, at either 30 nM or 30 μM [Ca]. These results indicate that CaM-A103V binds to RyR2 with an affinity that is comparable to WT-CaM, both at nM and μM [Ca] relevant to diastolic and systolic conditions in the heart.

A103V-CaM modestly disrupts inactivation of LTCCs

Alterations in Ca binding to CaM may disrupt Ca-dependent inactivation of LTCC in the heart[6,17]. Thus, we next tested the effect of A103V in ventricular myocytes dialyzed with WT or mutant CaMs. A103V showed a trend to slightly impaired LTCC inactivation without affecting peak currents compared to WT in a similar way as N98S-CaM, whereas N54I had no effect on LTCC inactivation (Figure 6). However, the effect of A103V-CaM on LTCC inactivation was much smaller than that of D96V-CaM, a mutation associated with severe LQTS but not CPVT in humans.

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A103V-CaM does not modify action potential duration but promotes DAD and triggered beats

We next evaluated the effect of the mutant CaMs on the action potential duration in intact cardiomyocytes dialyzed with WT, A103V-CaM, or the LQTS-associated D96V-CaM. A103V-CaM did not have a significant effect on APD50 or APD90 compared to WT, whereas D96V dramatically increased both APD50 and APD90 (Figure 7A). Consistent with its clinical phenotype of LQTS, EADs were observed in 36% of cells dialyzed with D96V-CaM. EADs were not found in cells dialyzed with WT or A103V-CaM (Figure 7B). Finally, we quantified the incidence of DADs and triggered beats after the 1 Hz pacing train. Consistent with the activation of spontaneous Ca release observed in permeabilized myocytes (Figures 3 and 4), only the A103V-CaM mutant associated with CPVT caused spontaneous beats triggered by DADs (Figure 7C).

Discussion

The major finding reported here is the discovery of a novel CPVT mutation – CaM-A103V – in the *CALM3* gene; a gene that up to now had not been linked to CPVT. Furthermore, finding CaM mutations in 1 out of 12 patients (8%) with major CPVT genotype-negative but clinically diagnosed CPVT suggests that the prevalence of CaM mutations in CPVT may be higher than previously thought. Since CaM mutations have also been identified in 13 % of genotype-negative LQTS patients[18], and in a family with idiopathic ventricular fibrillation[19], CaM mutations should be considered as a significant contributor to genetic sudden death syndromes.

The cellular mechanisms involved in the arrhythmia susceptibility in the setting of CaM mutations are complex due to the multiple targets of this protein in the cell. For example, dysfunctional CaM mutants can impair Ca-dependent inactivation of LTCCs leading to increased depolarizing current during the plateau phase of the cardiac action potential[3]. On the other hand, CaM mutations can also lead to aberrant regulation of the ryanodine receptor (RyR2) Ca release[4, 9]. Our *in vitro* investigation demonstrated that CaM-A103V has an activating effect on RyR2 Ca release that is similar to that reported for two established CPVT-linked CaM mutations (N54I and N98S)[4] and matches also the defect in Ca-handling observed in animal models of CPVT–linked mutations residing directly within either the cardiac ryanodine receptor or calsequestrin[12, 20]. Given that models of human CPVT-linked mutations present higher rates of spontaneous Ca release and delayed after depolarizations[21] and that drugs that suppress spontaneous SR Ca release in single cells are effective in preventing CPVT in animal models and patients[22], it is generally accepted that spontaneous Ca release from the SR is the underlying pathophysiological mechanism responsible for CPVT. In this regard, previous studies using *CALM1* mutants (N54I and N98S) showed that both CPVT-CaMs bound with greater affinity to RyR2 than WT-CaM but at the same time both failed to inhibit or directly activated RyR2, rendering them hyperactive and generating arrhythmogenic Ca waves[4].

The novel *CALM3* mutation A103V studied here also activates RyR2 Ca release channels generating Ca waves and depleting SR Ca store. In fact, spontaneous Ca releases induced by this mutation generated DADs that triggered spontaneous APs (Figure 7C), which is

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consistent with the clinical CPVT phenotype. Similar to the previously reported *CALM1* mutations, A103V-CaM exhibited dominant-activating effects on Ca waves when mixed with WT-CaM, which is consistent with an autosomal dominant inheritance pattern in humans[4]. However, in contrast to the previously published CPVT CaM mutants, A103V did not exhibit increased RyR2 affinity. The significance of the latter finding is unclear, but could mean that only 1 mutant CaM out of 4 CaMs bound to the RyR2 complex is needed and sufficient to cause aberrant RyR2 regulation. The unchanged RyR2 binding affinity of A103V-CaM, compared to the increased affinity observed for the two other CPVT-linked CaM mutantsN54I and N98S, might explain the milder phenotype of our two patients compared with patients carrying the previously reported CPVT-linked *CALM1* mutations, especially since, CALM3 mRNA transcript levels are reportedly higher than CALM1 and 2 mRNA transcripts levels in human hearts [3].

Another important CaM target related to arrhythmogenesis is the LTCC (Cav1.2). Pre-association of Ca-free-CaM with Cav1.2 regulates Ca-dependent channel inactivation[23]. Thus, CaM mutations that show reduced Ca binding affinity can disrupt Ca-dependent inactivation of LTCC. While A103V-CaM mutant showed a trend to impair LTCC inactivation due to a small reduction in Ca binding affinity, the effect is very small compared to the published LQTS-associated CaM mutations (D96V, D130G F142L and E141G)[8,18]. Furthermore, LTCC measurements were done with blocked SR and unlike LQTS-associated CaM mutations, A103V enhances SR Ca release and consequently the Ca-dependent inactivation of LTCCs. In fact, we observed that the net effect of A103V-CaM on AP duration was minimal in contrast to the massive AP prolongation observed in cells dialyzed with the LQTS linked D96V-CaM (Figure 7A), which is consistent with the lack of QT prolongation in the A103V mutation-positive patient. Nevertheless, the subtle impairment of Ca binding affinity and LTCC inactivation (Figures 2 and 6) and the trend towards longer APD (Figure 7) caused by A103V-CaM may explain the U-waves observed in the ECG of our patient. Notably, the CPVT-linked N98S-CaM mutation also causes some impairment of Ca binding[4] and hence LTCC inactivation, with U-waves reported in the first index case[24], and a full LQTS phenotype reported in a second individual who carried the same N98S mutation in *CALM2*[25].

Study Limitations

CaM also binds other targets in the heart and thus we cannot exclude that other mechanisms contribute to the autosomal dominant inheritance of A103V-CaM mutation. However, its dominant activating effect on RyR2-mediated Ca release is likely sufficient to explain the CPVT phenotype of humans positive for the A103V-CaM mutation.

Conclusions

We find that the *in vitro* phenotype produced by A103V-CaM is analogous to the two published and established CPVT-linked CaM mutations, which suggests that A103V should be considered a CPVT-susceptibility mutation. Our results further indicate that *in vitro* functional studies of Ca release in combination with action potential measurements in intact cardiomyocytes can be used to rapidly assess the molecular mechanism underlying the

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pathogenicity of CaM variants that will be discovered in the future. Just as *CALM3* has been implicated recently in the pathogenesis of LQTS thereby completing the CaM-mediated LQTS trilogy[26,27], now the CaM-mediated CPVT trilogy has been completed with the discovery of A103V-CALM3.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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What is Known

- Calmodulin mutations have been associated with severe forms of long QT syndrome (LQTS) and catecholaminergic polymorphic ventricular tachycardia (CPVT).
- In previous reports, calmodulin mutations were found in 13% of genotype-negative LQTS patients.

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What the Study Adds

- This report identifies and characterizes a novel calmodulin mutation in the *CALM3* gene (A103V-CaM) in 1 out of 12 patients with genotype-negative but clinically-diagnosed CPVT.
- A103V-CaM shares *in vitro* functional characteristics with the two published and established CPVT-associated mutations, suggesting that it should be considered a CPVT-susceptibility mutation.
- *In vitro* functional studies of spontaneous Ca^{2+} release events in combination with action potential measurements can be used to rapidly assess the molecular mechanism underlying the pathogenicity of calmodulin variants.

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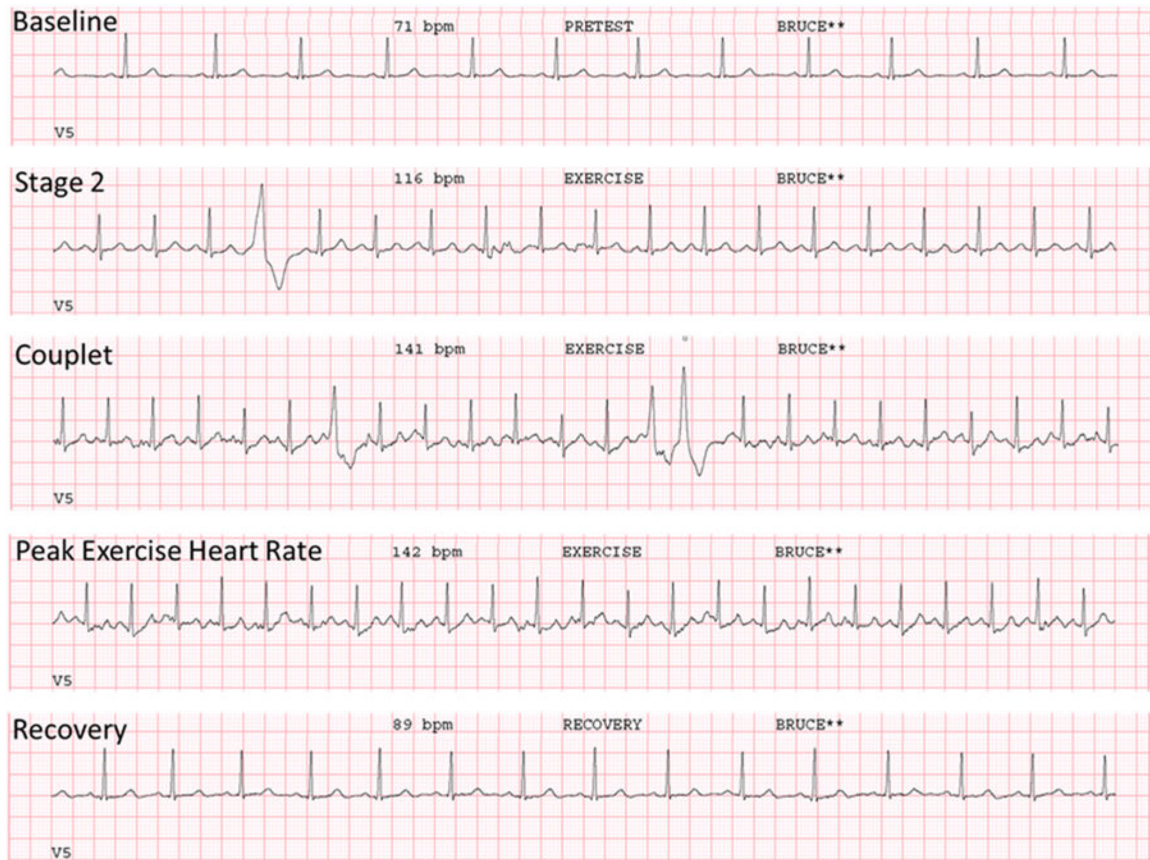

Figure 1.

Stress test for patient with A103V-CaM. A representative stress test for the patient harboring the A103V-CaM variant while on beta blocker therapy. Shown are the rhythm strips for the baseline, stage 2 with the onset of PVCs, a couplet, the peak exercise heart rate, and three minutes into recovery.

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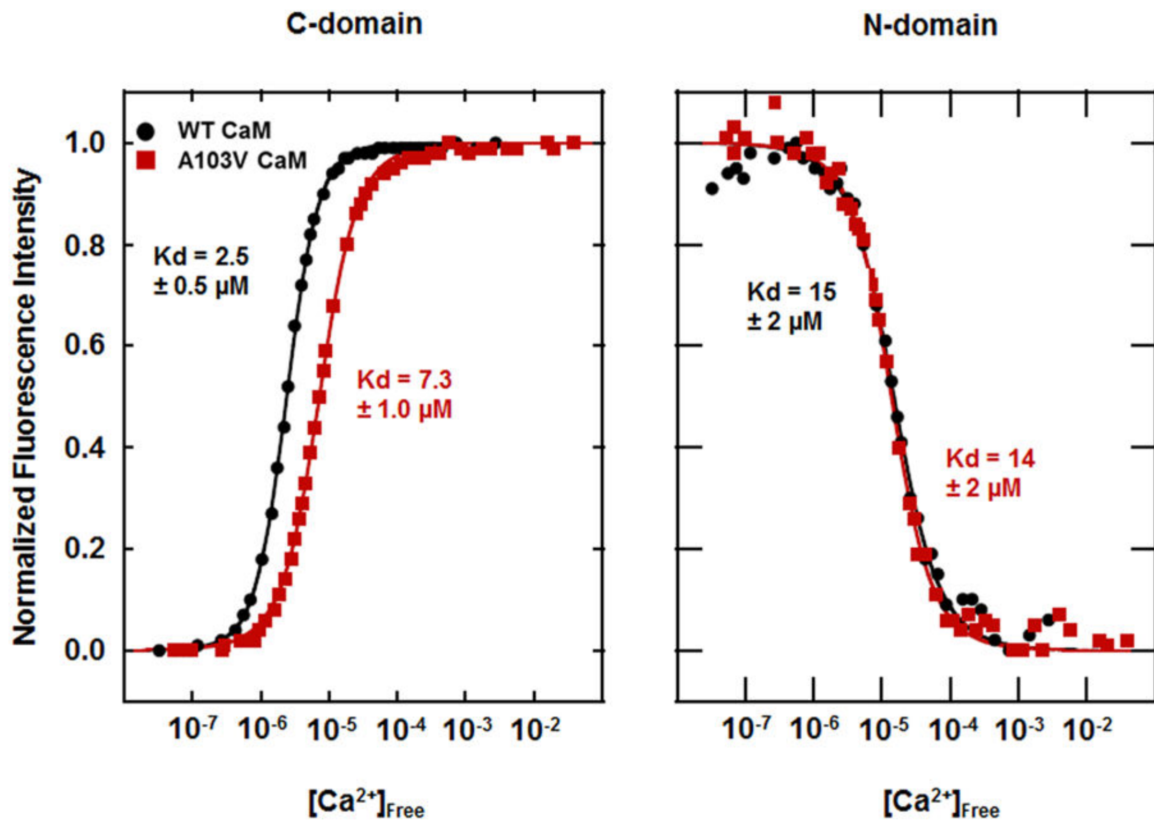

Figure 2.

Ca titration curves for wild-type (WT) CPVT CaM mutant (A103V) for the CaM-C and CaM-N domain. Dissociation constants (Kd, in μM) were derived for each domain by following intrinsic Tyr and Phe fluorescence for the N-domain and C-domain, respectively, and fitting to a standard binding equation. The A103V-CaM mutant reduced Ca binding affinity of CaM C-domain (3-fold reduction compared to WT-CaM), whereas N-domain Ca binding was unchanged. Values are averages of 3 experiments, and error was determined by analysis of the curve fits.

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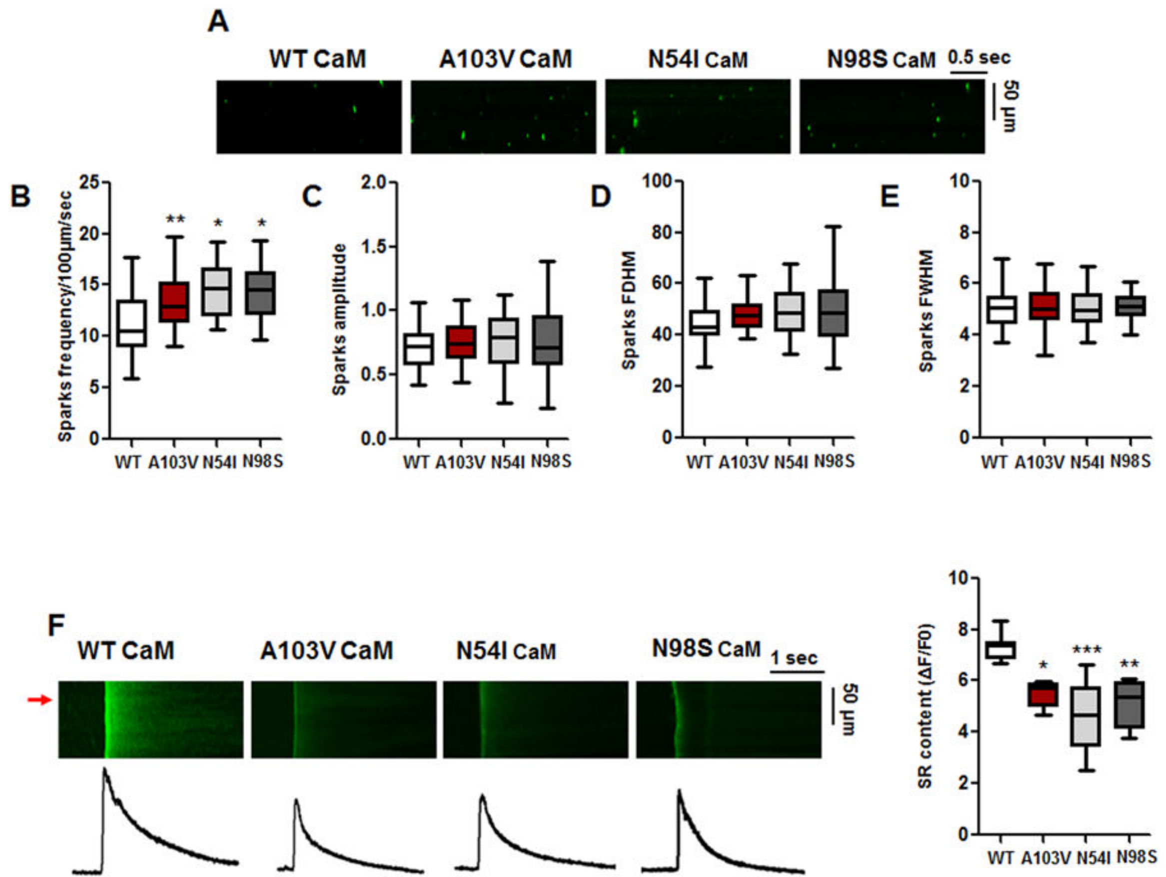

Figure 3.

A103V-CaM promotes Ca sparks activity and reduces sarcoplasmic reticulum (SR) Ca content. **A**. Representative line-scan images of Ca sparks in permeabilized mouse ventricular myocytes incubated for 30 minutes with WT-CaM or CPVT-CaMs (A103V, N54I and N98S) 100 nM **B**. Average Ca spark frequency/100μm/sec **C.** Ca spark amplitude **D.** Ca sparks full duration half max and **E.** Ca sparks full width half max. Data are presented as mean±SD. (n=30 *p<0.05; **p<0.01 *vs.* WT-CaM) **F.** Line scan (top) and (bottom) line plot (red arrow) examples of SR Ca content evaluated by 10 mM caffeine-evoked Ca transient in cardiomyocytes incubated with WT-CaM or CPVT-CaMs (A103V, N54I and N98S) 100 nM **G**. Average SR Ca content. Data are mean±SD. (n=6 *p<0.05; **p<0.01; ***p<0.001 *vs.* WT-CaM) free $[Ca]_i$ = 50 nM, EGTA = 0.5 mM.

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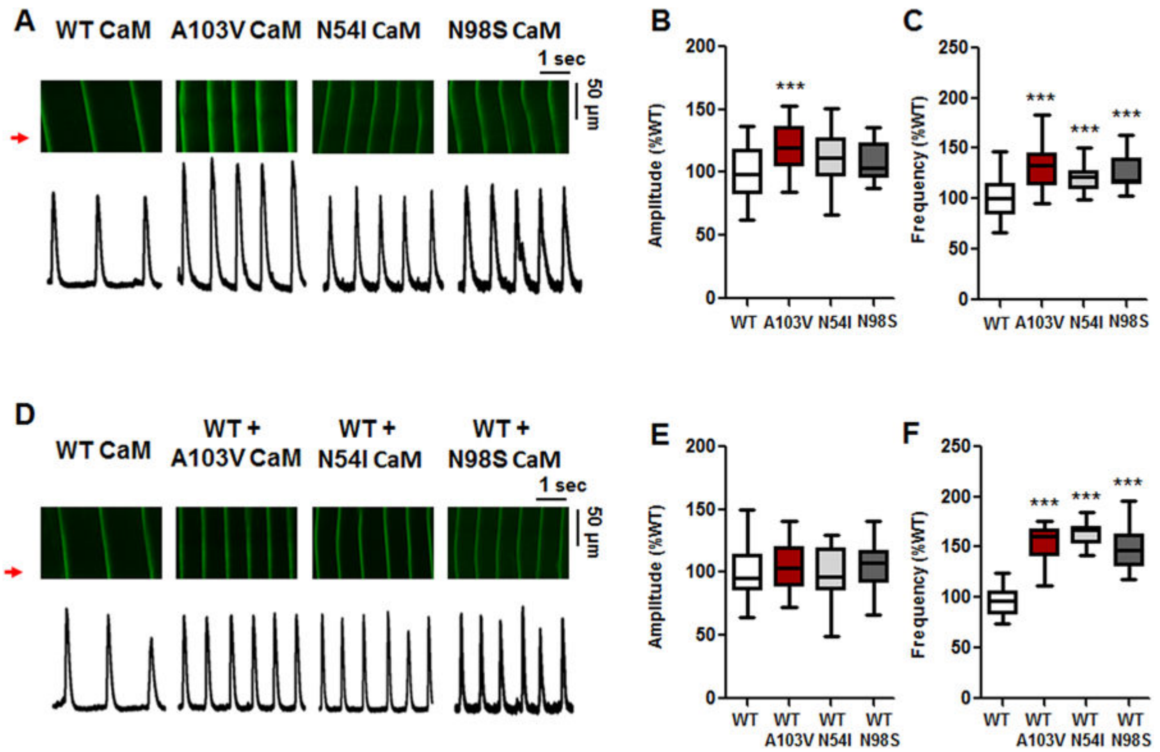

Figure 4.

A103V-CaM promotes Ca waves with dominant effect. **A**. Representative line scans (top) from permeabilized mouse ventricular myocytes after 30 minute incubation with wild-type (WT) or mutant CaMs (100 nM) and (bottom) line plot examples (red arrow) **B.** Averaged Ca waves amplitude and **C.** frequency. A103V-CaM promoted higher Ca wave frequency and amplitude compared to WT. Data are presented as mean±SD of values normalized by WT values on each experimental day (n = 30. N=3 ***$p<0.001$ *vs.* WT-CaM). **D.** Representative line scans (top) from permeabilized mouse ventricular myocytes after 30 minute incubation with either wild-type (WT) or mutant CaMs 25% mixed with 75% WT-CaM (final concentration 100 nM) and (bottom) line plot examples (red arrow) **E**. Averaged Ca waves amplitude and **F.** frequency. Even 25% A103V-CaM promoted Ca waves. Data are mean±SD of values normalized by WT values on each experimental day (n = 30. N = 3 ***$p<0.001$ *vs.* WT-CaM) free $[Ca]_i$ = 120 nM, EGTA = 100 µM.

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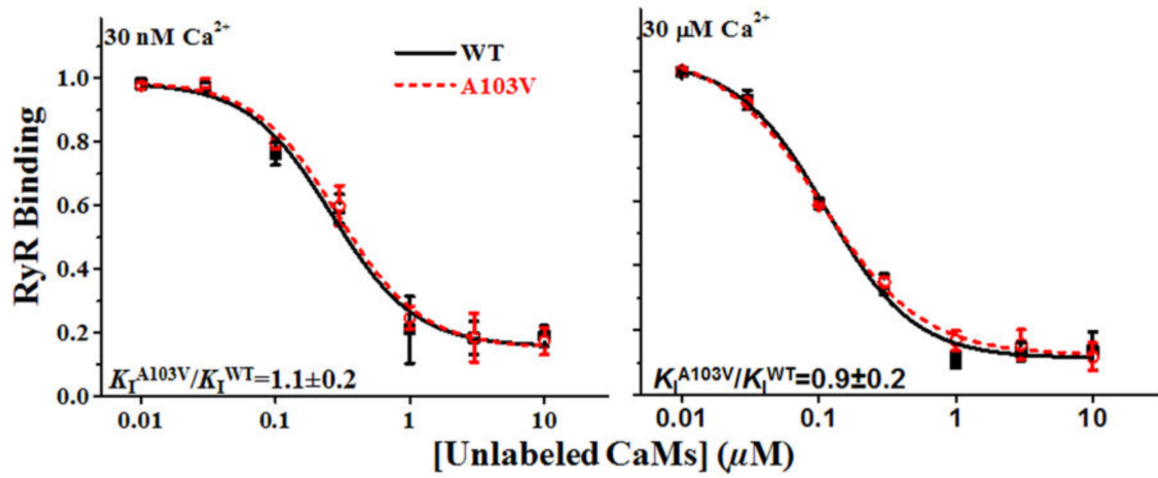

Figure 5.

The A103V mutation does not significantly change the affinity of CaM for RyR2. Both CaM species were equally effective in displacing A-CaM at nM and μM [Ca] relevant to diastolic and systolic conditions in the heart.

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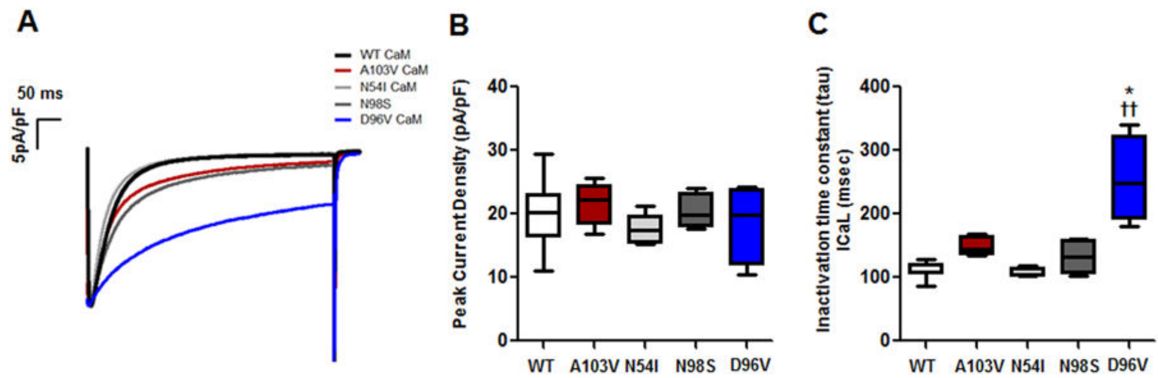

Figure 6.

A103V-CaM showed a trend to disrupt L-type Ca channels (LTCC) inactivation. **A.** Representative examples of traces for each experimental group. **B.** Average current densities (pA/pF) obtained in cells dialyzed with either WT-CaM, A103V-CaM, N54I-CaM, N98S-CaM (CPVT mutants) or D96V-CaM (LQTS mutant). CaM mutants had no effect on the peak current density. **C.** Effect of A103V-CaM, N54I-CaM, N98S and D96V-CaM on inactivation time constant of the LTCC compared to WT-CaM. A103V-CaM an N98S-CaM showed a trend to impair LTCC inactivation whereas N54I-CaM did not affect it at all. However, compared to a LQTS-associated CaM mutation (D96V-CaM), A103V-CaM had a smaller effect on LTCC inactivation. Data are mean ± SD. (WT-CaM, A103V-CaM and N54I-CaM n=6; N98S-CaM and D96V-CaM n=4. *$p<0.05$, *vs.* WT-CaM; $^{††}p<0.01$, *vs.* N54I-CaM)

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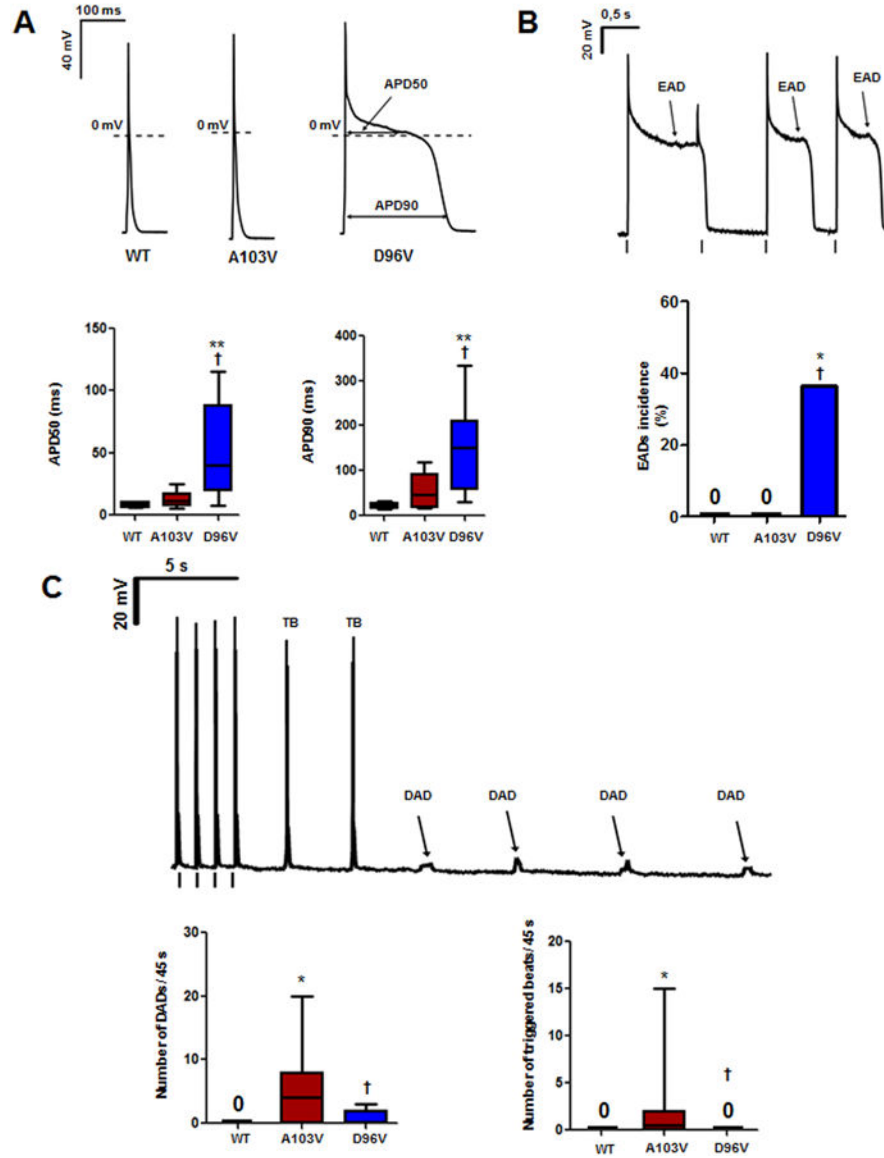

Figure 7.

A103V-CaM does not modify APD but induces spontaneous beats triggered by DADs. **A.** Top panel: Representative examples of AP records for each experimental group. Bottom panel: Average APD measured at 50% (APD50, left) and 90% (APD90, right) of repolarization. **B.** Top panel: Representative example of prolonged APs with EADs recorded from a cardiomyocyte dialyzed with D96V-CaM. Bottom panel: Percentage of cardiomyocytes exhibiting EADs during the pacing train. **C.** Top panel: Representative examples of DADs and triggered beats recorded from a cardiomyocyte dialyzed with A103V-CaM. Bottom panel: Averaged number of DADs (left) and triggered beats (right) during the first 45s after the pacing train in cardiomyocytes dialyzed with either WT or mutant CaMs. Data are mean ± SD. (WT-CaM n=7, A103V-CaM n=14 and D96V-CaM n=12 *p<0.05, **p<0.01 *vs.* WT-CaM; †p<0.05 *vs.* A103V-CaM)

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Table 1

Demographics of Genotype Negative CPVT Cohort

Number of probands	12
Age at Diagnosis, yrs ± SD	20 ± 12
Females (%)	9 (75)
QTc, ms ± SD	411 ± 33
Abnormal Stress Test with Ventricular Ectopy (%)	12 (100%)
Syncope (%)	10 (83)
Cardiac Arrest (%)	2 (17)
Positive Family History (%)	3 (25)