The Arrhythmogenic Calmodulin Mutation D129G Dysregulates Cell Growth, Calmodulin-dependent Kinase II Activity and Cardiac Function in Zebrafish

Running title: CPVT and LQTS calmodulin mutations

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ABSTRACT

Calmodulin (CaM) is a Ca²⁺ binding protein modulating multiple targets of which several are associated with cardiac pathophysiology. Recently, CaM mutations were linked to heart arrhythmia. CaM is crucial for cell growth and viability, yet the effect of the arrhythmogenic CaM mutations on cell viability as well as heart rhythm remains unknown and only a few targets with relevance for heart physiology have been analyzed for their response to mutant CaM. We show that the arrhythmia-associated CaM mutants support growth and viability of DT40 cells in the absence of wt CaM except for the LQTS mutant CaM D129G. Of the six CaM mutants tested (N53I, F89L, D95V, N97S, D129G, F141L) three showed a decreased activation of Ca²⁺/CaM-dependent kinase II (CaMKII), most prominently the D129G CaM mutation, which was incapable of stimulating T286 autophosphorylation. Furthermore, the CaM D129G mutation led to bradycardia in zebrafish and an arrhythmic phenotype in a subset of the analyzed zebrafish.

Ca²⁺ is the main trigger for muscle contraction (reviewed in (1,2)). Ca²⁺ signaling is highly regulated in cardiomyocytes and controls a number of important proteins governing release and uptake of Ca²⁺ into the sarcoplasmic reticulum (SR) as well as cardiomyocyte contraction. Impaired contractibility and lethal cardiac arrhythmias may result from dysregulated Ca²⁺-signaling and cycling. One of the main players in Ca²⁺-mediated effects is
calmodulin (CaM), a ubiquitous intracellular Ca\textsuperscript{2+} receptor involved in a vast number of physiological processes including cell proliferation and muscle activity (reviewed in (3)). Identical CaM proteins are expressed in humans from three independent genes located on different chromosomes (4). It is well known that malfunctioning of several CaM regulated proteins may cause heart failure including among many others the cardiac Ca\textsuperscript{2+} release channel (RyR2) and the L-type Ca\textsuperscript{2+} channel (reviewed in (2)). In addition, CaM regulated protein kinases, in particular the Ca\textsuperscript{2+}/CaM-dependent kinase II (CaMKII), have been found to be dysregulated in diverse cardiac disorders (reviewed in (5-8)).

CaM plays a critical role in catecholaminergic polymorphic ventricular tachycardia (CPVT) as well as in long QT syndrome (LQTS) (9,10). Nyegaard et al. identified a mutation in the CaM gene 1 locus on Chromosome 14 of a Swedish family, segregating with a dominantly inherited form of CPVT. This mutation changes residue 53 in the CaM protein from an asparagine to an isoleucine. In addition, a de novo mutation in CaM gene 1 changing residue 97 from asparagine to serine was found by screening CPVT patients. Thus, it was concluded that the CaM genes may be candidates for genetic screening of patients with tachycardia (9).

Using whole exome sequencing of patients with LQTS Crotti et al. found three other de novo mutations (D130G, represented in two patients; D96V and F142L (these mutations are numbered D129G, D95V and F141L respectively in this work in line with the nomenclature used in the first article describing arrhythmogenic CaM mutations (9)) in the CaM genes 1 and 2 (10). An additional inherited CaM 1 mutation F90L (referred to as F89L in this work) was discovered in a family with a history of idiopathic ventricular fibrillation (11). Five novel CaM mutations in the CaM gene 2 have been found in three patients with LQTS (N97S, N97I, D133H) and two with both LQTS and CPVT features (D131E, Q135P) (12). Two arrhythmogenic CaM mutations, D129G associated with LQTS (13) and A102V associated with CPVT (14) were found in the CaM 3 gene. A recent investigation on the whole exome of 38 elusive LQTS patients revealed five CaM positive cases, of which one had a novel mutation (E140G) (15) In addition, two novel mutations (D131V and D131H), both associated with LQTS were recently identified (16). Figure 1 summarizes the currently available information on mutated CaM amino acids associated with arrhythmia. The CPVT mutations exhibit either moderately higher (N53I) or slightly reduced (N97S, A102V) Ca\textsuperscript{2+} affinities (9,14), whereas the CaM mutations in LQTS patients all have a high impact on the CaM Ca\textsuperscript{2+} affinity, likely due to disruption of EF-hand 3 or 4 Ca\textsuperscript{2+}-binding (10) (Figure 1).

A number of cardiac target proteins are regulated by CaM. So far, the effect of CaM mutants on the interaction with and modulation of the RyR2 and the cardiac L-type channel have been investigated in some detail (17,18). FRET studies with cardiac SR membranes indicated that CPVT CaM mutants increase binding to the RyR2, enhance its channel opening probability and lead to spontaneous Ca\textsuperscript{2+} waves and spark activity, features not seen for LQTS causing CaM mutants (17). A study using guinea pig ventricular myocytes showed that LQTS mutants induce increased action-potential prolongation, increase Ca\textsuperscript{2+} transient amplitudes and suppress the CaM dependent inactivation of the of L-type channel Cav1.2 (18).

Little is known about the effect of CaM mutants on the function of CaMKII, an important player in cardiac Ca\textsuperscript{2+}-signaling. CaMKII regulates the RyR2, Cav1.2 and Nav1.5 in a strictly Ca\textsuperscript{2+}/CaM dependent manner (reviewed in (19)). Ca\textsuperscript{2+}/CaM activates CaMKII by displacing the autoinhibitory domain of the CaMKII allowing trans-phosphorylation on position T286, which renders the enzyme active (20). T286 phosphorylation increases the affinity of CaM to the enzyme 1,000 fold, keeping it active even at low Ca\textsuperscript{2+} concentrations during a certain time period, which allows the enzyme to act as a chemical memory of previous heartbeats by integrating individual Ca\textsuperscript{2+} signals (21). Increased activity of CaMKII is implicated in several heart diseases including dilated cardiomyopathy, cardiac hypertrophy and arrhythmia, mainly due to increased leak of...
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Ca<sup>2+</sup> from the SR and Ca<sup>2+</sup>-mediated arrhythmias (5, 22).

In this study our aim was to examine the effect of six CaM mutants (N53I, F89L, D95V, N97S, D129G and F141L) in an in vitro system with conditional CaM expression in DT40 cells (23). In addition, the goal was to investigate whether the CaM mutants are able to activate CaMKII and to analyze how the mutant with the most pronounced effect in vitro (CaM D129G) affects the heart rhythm of zebrafish. Our study shows that the arrhythmogenic CaM mutants affect the examined functions differentially and indicates that the mutation changing the first Ca<sup>2+</sup> coordinating residue from D to G in EF-hand 4 of CaM affects the in vitro parameters to the highest degree and causes an arrhythmogenic phenotype in vivo.

**RESULTS**

The CaM D129G mutant cannot rescue DT40 cells depleted of wt CaM-

We have previously established a cellular CaM knock out/knock-in system with conditional CaM expression (tet off system) (23). This is a unique cellular model system as it allows expressing CaM variants in the absence of endogenous CaM in a vertebrate cell line after four to five days of tetracyclin treatment (Fig. 2A). This enables us to study the effect of CaM mutations on basic cellular processes such as cell growth, viability and cell cycle regulation as well as regulation of CaM dependent signaling pathways and interaction of CaM with its numerous target proteins. In the present study we used this system to investigate the known cardiac failure causing CaM mutants for their potential to support cell growth. Stable expression of the different mutants in a wt CaM background did not affect growth, whereas, removing wt CaM in the absence of ectopically expressed CaM blocked cell growth and viability completely. HA tagged CaM was fully able to restore these features as reported before. Remarkably, among the tested CaM mutations, the D129G mutant decreased cell growth and viability to a large degree whereas among the other mutations only CaM F89L or N97S showed an effect on these parameters, however to a much lesser degree as compared to CaM D129G (Figs. 2C and D). As none of the mutant proteins were expressed to a higher extent as compared to the wild type and most of them still were able to support cell growth to a major degree (Fig. 2B) we assume that the observed effects were not the result of different levels of mutated CaM. In addition, earlier work (24) shows that lowering CaM concentrations down to 40% does not alter growth properties of DT40 cells. To exclude that the lack of viability was a clonal artifact multiple independent D129G expressing clones were tested (data not shown). The viability did not reflect the clonal diversity in HA-CaM expression as different cell lines with various CaM expression levels gave similar results.

CaMKII activity is affected by CaM mutations to a various degree, most prominently with CaMD129G-CaMKII plays a central role in a large number of Ca<sup>2+</sup>/CaM-signaling pathways and is essential for cardiomyocyte functions (25). We therefore asked whether the arrhythmia causing CaM mutants were capable of activating CaMKII. To do this we used the CaMKII sensor Camui (26), which is based on the CaMKII-alpha isoform and has been shown to mimic the heart tissue predominant delta-isoform of CaMKII (27). Camui exploits the structural and functional features of CaMKII by containing a fused fluorescent protein on the N- as well as on the C-terminus of CaMKII within Förster radius in the inactive state. CaMKII activation leads to separation of the fluorophores and thereby unquenching of the donor mCerulean (CFP). Adding wt CaM increased CFP fluorescence, as did the N53I, N97S and F141L mutants, whereas the maximal fluorescence as compared to wt CaM was strongly decreased for the F89L and D95V and most prominently for the D129G as well as for the Ca<sup>2+</sup>-binding deficient mutants (Fig. 3A). The strongly reduced ability of D129G to activate CaMKII was similar to that of the Ca<sup>2+</sup>-binding deficient mutants (EF12, EF34 and EF1234 (numbers indicate inactivated Ca<sup>2+</sup> binding sites)) (Fig. 3A). In cardiomyocytes the mutant CaMs are expected to exert their effects by competing with wt CaM since only one allele of the three genes expressing CaM is changed (reviewed in (28)). To mimic this situation for CaM D129G, which
in all our assays yielded the strongest effect we mixed CaM D129G and wt CaM in different ratios and measured Camui activation following addition of Ca$^{2+}$ (Fig. 3B). Exchanging wt for D129G CaM reduced the Camui activation as compared to only reducing wt CaM with buffer (dilution effect) indicating a dominant negative effect of the D129G (Fig. 3B). The CaM mutant with both EF-hands 3 and 4 mutated had an even more severe dominant negative effect (Fig. 3B). As the speed of Ca$^{2+}$ fluxes in the sarcoplasm regulates the dynamics of the Ca$^{2+}$ receptor CaM and its interactions with targets and thereby the heart rhythm we investigated whether the CaM D129G mutant would affect CaMKII in an initial activation phase. Kinetic measurements comparing the activation speed of CaMKII activation in the presence of wt CaM or CaM D129G alone or mixed in a 2:1 ratio showed that CaMKII activation by CaM was significantly reduced when CaM D129G was present (Figure 3C). These combined data show that the D129G CaM mutant can exert a dominant negative effect on the CaMKII function in vitro.

CaM D129G cannot stimulate phosphorylation of CaMKII T286 and phosphorylation of downstream targets is markedly reduced-A small yet consistent Camui activation change by CaM D129G in our in vitro experiment indicated some contribution of the mutant in the activation. To investigate the phosphorylation status of CaMKII in the presence of wt or D129G CaM we purified ectopically expressed rat CaMKII from HEK cells. CaMKII is well known to become activated by Ca$^{2+}$/CaM through binding and steric removal of a regulatory domain leading to autophosphorylation of T286 (29). Incubation of recombinant CaMKII with D129G CaM led to a diminished autophosphorylation of CaMKII T286, as compared to incubation with wt CaM (Fig. 4A and B). To test whether D129G CaM affected the CaMKII-dependent phosphorylation of downstream targets we analyzed the in vitro phosphorylation of the CaMKII substrate autocamtide-2 (KKALRRQETVDAL) and found that in the presence of D129G CaM phosphorylation of the peptide was significantly reduced as compared to wt CaM (Fig. 4C). These results along with the Camui data indicate that D129G is able to bind to CaMKII but cannot cause its activation. CaM D129G expression in zebrafish causes decreased heart rate-To examine whether wild type CaM and CaM D129G would elicit different effects on the development and/or function of the embryonic heart, we injected either wild type CaM (CaM-WT) or CaM D129G synthetic RNA into myl7:GFP embryos. In myl7:GFP transgenic zebrafish embryos, GFP expression is driven by the cardiac-specific promoter myl7 (30), allowing in vivo phenotypic analysis and quantitative measurement of cardiac parameters (31). As shown in Fig. 5A, wt or D129G CaM overexpression did not affect the overall development of zebrafish embryos or the morphogenesis of the hearts. After two days of development, well-patterned hearts capable of propelling circulation through the body were observed in uninjected controls and D129G or CaM-WT RNA injected embryos (Fig. 5A). While CaM-WT RNA injected embryos had a heart rate similar to that observed in uninjected control embryos, D129G overexpression reduced the heart rate to ~83% of the level observed in controls This is a moderate difference, however statistically significant (n=128, p<0.001) (Fig. 5B) considering that endogenous CaM is present as well. Interestingly, ~8% of D129G RNA injected embryos exhibited an abnormal cardiac rhythm where the atrium beats twice per ventricular contraction (Figs. 5C and D). Overall these results demonstrate a significant impact of D129G on the heart rate and conduction of the developing heart in zebrafish.

**DISCUSSION**

In this study we tested the arrhythmogenic CaM mutations N53I, F89L, D95V, N97S, D129G and F141L in a cell based system and in a CaMKII activation assay. We found that the CaM mutant D129G stands out from six investigated cardiac arrhythmia related CaM mutations in interfering with the viability of a genetically modified vertebrate cell line expressing exclusively mutant CaM. Further, CaM D129G showed the strongest effect on CaMKII activity measured in a FRET based assay and also diminished CaMKII substrate
phosphorylation as compared to wt CaM. Based on these results we tested the D129G mutant in vivo. Expression of CaM D129G in zebrafish led to decreased heart rate and in a subpopulation of animals to a 2:1 ratio of atrium to ventricle heart rate denoted as breakdance phenotype.

CaM is the major sensor of Ca\(^{2+}\) signals in all eukaryotic cells regulating a multitude of physiological processes including muscle contraction. In cardiac tissue CaM is involved in the tuning of ion channels including the SR Ca\(^{2+}\) release channel RyR2, potassium and sodium channels in the sarcolemma directly involved in excitation-contraction coupling (reviewed in (28,32)). Several of these ion channels are further regulated indirectly through Ca\(^{2+}\)/CaM modulation of cytosolic enzymes such as CaMKII and calcineurin. In addition, CaM also regulates transcriptional processes important for proper heart function. One example is the phosphorylation of class II histone deacetylases by the CaM/CaMKII pathway. In the phosphorylated stage these deacetylases are excluded from the nucleus and incapable of repressing genes that are involved in heart hypertrophy (reviewed in (5)). CaM and CaMKII were also shown to affect the heart rhythm by regulating the expression of ion channels involved in excitation contraction coupling (reviewed in (33,34)). The recent finding that CaM mutations in one out of six expressed CaM alleles, which all encode the same protein, may cause several types of heart arrhythmias, mainly CPVT and LQTS came as a surprise as CaM is perfectly conserved in vertebrates underlining the importance of maintenance of an unchanged primary structure. Even though several laboratories have initiated work to investigate the molecular basis of how CaM mutation cause heart arrhythmia little is known about the molecular mechanisms affected by CaM mutations in the heart.

We replaced wt CaM with six arrhythmia causing CaM mutants and found that only CaM D129G affected cell growth and viability to a major degree. This indicated that exchanging aspartic acid at position 129 with glycine renders CaM incapable of supporting basic vertebrate cell functions. One would therefore expect that such a mutation would not be compatible with life. However, it has to be considered that three genes code for an identical CaM protein in mammals and that the D129G mutation was only found in one allele of all the three CaM genes (10,13,15). All three CaM genes are transcribed in the heart as measured by qPCR (10). Highest mRNA levels were found for the CaM 3 gene followed by the CaM 2 and the CaM 1 gene in the human left ventricle. However, it is not known how much each transcript contributes to the expression of CaM on the protein level. In addition, it is as well possible that the different transcripts are used for translation in different subcellular locations in cardiomyocytes in analogy to findings in neurons (35). Therefore, it seems that this mutation can be tolerated for normal human development but would affect heart physiology by a dominant effect over wt CaM. D129 is a very important residue as it coordinates Ca\(^{2+}\) in the 4th EF hand Ca\(^{2+}\) binding loop. All other LQTS related mutations investigated in this report even though directly at Ca\(^{2+}\) coordinating positions showed considerably milder effects on Ca\(^{2+}\) affinity (10). Changing an aspartate to a glycine at position 129 may have severe structural consequences as glycine allows highest flexibility. This is corroborated by an earlier study where we showed that changing D129 to A did not abolish cell growth and viability (23). In addition, NMR data of CaM D129G from a study by Crotti et al. (10) support the notion that a local structural change along with reduced Ca\(^{2+}\) binding may explain the effect of this mutant in the functions analyzed in our study.

We tested the CaM mutants for their capability to bind and stimulate CaMKII autophosphorylation using the Camui FRET system (36) CaMKII functions in the heart by regulating a number of Ca\(^{2+}\), K\(^{+}\) and Na\(^{+}\) channels through phosphorylation. Its chronic activation may cause heart failure through a variety of mechanisms (5,32). One crucial target relevant for heart rhythm control and phosphorylated by CaMKII is the RyR2. Phosphorylation of the RyR2 enhances Ca\(^{2+}\) release from the SR disturbing normal Ca\(^{2+}\)-cycling (reviewed in (5)). In the heart, the major CaMKII isoform is CaMKII\(\delta\) with the three

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main splice forms mostly differing in the presence of a nuclear localization signal: CaMKIIδ is mainly found in the sarcoplasm and acts in regulation of ion channels and Ca²⁺ handling whereas CaMKIIβ is found predominantly in the nucleus and regulates hypertrophic gene activity (5). As in other CaMKII isoforms the active form of CaMKIIδ is stabilized by phosphorylation on Thr 286 as well as other posttranslational modifications such as oxidation and O-linked glycosylation rendering the enzyme Ca²⁺/CaM independent (27). Recent investigations indicate that T286 phosphorylation is necessary but not sufficient for full CaMKII activation. Coultrap et al. found that higher than 15-25% activation of CaMKII by preincubation with Ca²⁺/CaM is an exception and needs a special type of binding by the substrate (37). Full activation towards regular substrates such as tyrosine hydroxylase and GluR1 requires additional regulation by Ca²⁺/CaM. Thus, incomplete and substrate-dependent autonomy prevents uncoupling from subsequent regulation. The same group later showed that further stimulation of partially autonomous CaMKII is physiologically important as autonomous CaMKII must be further stimulated by Ca²⁺/CaM to enhance synaptic strength (38). Molecular mechanisms of this additional Ca²⁺/CaM dependent activation of partially autonomous CaMKII have not been established so far.

Hwang et al. tested the available CaM mutants for their potential to affect the RyR2 calcium release channel (17). As CaMKII is known to phosphorylate and activate the RyR2 channel the arrhythmia causing CaM mutants were also compared to the wt for their potential to regulate CaMKII and thereby having an effect on RyR2 activity. Neither of the mutants led to a decreased CaMKII activity as compared to the wt (17). This is in contrast to our results carried out with the same CaMKII sensor by Hwang et al. used. Not only did we see a strong decrease in CaMKII activity with the D129G but also, the D95V (D96V) and N97S (N98S) gave significantly reduced (23 and 63% respectively) CaMKII peak activity (Fig. 3A). Earlier literature indicates that EF hand 3 and 4 in CaM are of crucial importance for CaMKII activation (39) and as position D129 is the first Ca²⁺-coordinating residue in EF loop 4 our results are not surprising. Based on these data it is surprising that Hwang et al. (17) did not find a difference between mutants affecting EF hand 3 and 4 and wt CaM in CaMKII activation.

Crotti et al. (10) found that the C-domain of CaM D129G had a 54-fold lower Ca²⁺-affinity (kd 150 µM) as compared to the intact wt CaM C-domain, which would make it unlikely that CaM D129G would be able to fully activate CaMKII at low Ca²⁺ concentration. In the work by Hwang et al., ATP is not added in the CaMKII activation assay. It is therefore questionable whether the CaMKII-sensor is autophosphorylated. Our experiments show that addition of CaM D129G causes a small decrease in Camui FRET indicative of CaM binding, yet binding only occurs under saturating Ca²⁺ conditions and does not involve T286 phosphorylation. From this, we hypothesize that CaM D129G can bind, but, not stimulate CaMKII autophosphorylation. This leads to a competitive binding responsible for the dominant negative delay in the activation profile of CaMKII (Figures 3C and 6). The pleiotropic nature of CaMKII, targeting both function and expression of proarrhythmic as well as anti-arrhythmic molecules (reviewed in (40)) makes it difficult to derive a conclusion on the consequences of the D129G CaM defect at the organismal level. It is however, safe to conclude that the autonomous activation of CaMKII is affected. Studies into molecular mechanism of how CaM mutations affect CaMKII and in particular how they can exhibit a dominant negative effect on CaMKII should be done on a structural level in the future.

Based on results obtained in vitro we then asked whether the D129G CaM, which exhibited the most prominent effect in vitro would also have an effect in vivo. D129G CaM RNA injected in zebrafish embryos exhibited a major effect on zebrafish heart physiology. Expressing CaM D129G in zebrafish eggs not only altered the heart rhythm but also led to the "breakdance" phenotype characterized by an atrium to ventricle beating ratio of 2:1 of two days old zebrafish. This indicates for the first time that this mutant affects the heart function in an in vivo vertebrate model system. The D129G-induced 2:1 beat phenotype resembles
LQTS. This phenotype is comparable to the human Long QT Syndrome, an arrhythmia caused by a modification of ion channels involved in cardiac repolarization (41). The low penetrance (only 8% of the investigated animals show this phenotype) is likely to be the result of the labile nature of injected RNA. Clearly, generating stable transgenic fish or knock-in D129G fish would help clarify the possible causative relationship between D129G and LQTS. As it is known that CaM regulates K+ channels involved in repolarizing current by moving K+ ions outward (42) and mutations in zebrafish K+ channels induce the bradycardia/LQT phenotype (43) it is possible that the observed phenotypes are correlated with failure in Ca2+/CaM regulation of K+ channels either directly or through CaMKII.

**EXPERIMENTAL PROCEDURES**

**DNA constructs**—In this study, six different heart arrhythmia linked CaM mutations CaM N53I, CaM F89L, CaM D95V, CaM N97S, CaM F129L and CaM F141L were analyzed. The numbers indicate the position of amino acid exchange without the initial methionine. Site directed mutagenesis was performed in the pCMV_HA-CaM_wt_Eco vector, described in (23).

For protein expression CaM N53I, CaM F89L, CaM D95V, CaM N97S, CaM F129L and CaM F141L mutations were made by the quick change method in a pGemex vector. For DT40 cell expression, the pCMV-HA Eco vector providing a short protein tag, earlier shown not to interfere with the function of CaM (23) was used.

Mutant Camui constructs were made by PCR techniques in the Camui vector containing full length CaMKIIalpha kindly provided by Yasunori Hayashi (36).

**Cell lines**—DT-40 derived clone ET 1-50 (23) grown in RPMI with 10% fetal bovine serum (FBS) and 1% chicken serum served as the parental cell line for generating clones expressing HA-tagged mutant CaMs. Transfection of 5,000,000 cells was performed with 15 µg linearized pCMV-HA CaM Eco-gpt in 100 µl electroporation buffer (20 mM HEPES, 135 mmol/L KCl, 2 mmol/L MgCl2, 0.5% Ficoll 400, 200 mmol/L ATP and GSH 500 mmol/L, adjusted to pH 7.4 with KOH). Electroporation was done with the Bio-Rad Gene Pulser System (Bio-Rad, CA) in 1 mm cuvettes at 124 V and 750 µF. Immediately afterwards, 500 µl of spent medium was added. After 2 minutes of incubation, the cells were transferred to 2 ml spent medium. Transfected cells were transferred to a 96 well plate in selection medium prepared by adding the following to the growth medium: 250 mg/l Xanthine, 20 mg/l Hypoxanthine and 15 mg/l Mycophenolic acid prepared according to manufactures recommendations (Sigma-Aldrich). After 6-days, colonies were transferred to fresh selection medium and clones were expanded for 3 – 7 days before freeze stocks were prepared. The clones were tested by Western blot analysis with mouse αHA and mouse αCaM antibody to confirm HA-CaM expression. The specificity of the used antibodies was verified by probing cells that do not contain native CaM or the HA epitope (23).

**Growth curve and viability assay**—DT40 cell growth was monitored for seven days with and without tetracycline treatment. Cells were adjusted to 50,000-75,000 cells/ml. Counting and FACS analysis was performed every day. Every second day, cells were reseeded at 50,000-75,000 cells/ml. Cells pellets were tested by Western Blot analysis for the expression of CaM and HA-CaM.

**Western blot analyses**—DT40 cell pellets were lysed in 50 mmol/L Tris HCl pH 7.5, 150 mmol/L NaCl, 0.5% NP-40, 2 mmol/L EDTA, 1 mmol/L DTT and protease inhibitor cocktail for 20 minutes on ice followed by centrifugation at 15,000 x g for 10 min at 4°C. Proteins in lysates were separated by SDS PAGE (12% polyacrylamide) After blotting, the membrane was treated for 10 min in 0.02% glutaraldehyde. The blot was incubated with the primary antibody mouse αCaM (Millipore 05-173) 1:8,000 or mouse αHA11 (Covance16B12) 1:1,000. For development the Amersham ECL Prime Western Blotting Detection Reagent was used and the blot was analyzed with Bio-Rad Chemie Doc MP (Bio-Rad, CA). A prestained broad range marker (#26619, Thermo Fisher Scientific) was used to verify the sizes of the observed bands.
Zebrafish—Zebrafish-husbandry and transgenic lines. Zebrafish of transgenic line, myl7:GFP were maintained and bred as described previously (44). Full length D129G and CaM-WT cDNA was cloned into pCS2+3XFLAG. For mRNA synthesis, plasmids were linearized by NotI and mRNA synthesized using the SP6 mMESSAGE mMACHINE kit according to the manufacturer’s manual (Ambion).

Zebrafish injections. 100 pg of CaM mutant and CaM-WT mRNA were injected into one-cell stage embryos collected from crosses of myl7:GFP zebrafish. Embryos were then maintained for further phenotypic analysis at two days post fertilization ( dpf).

Zebrafish cardiac imaging and analysis. Movies of GFP-labelled Tg(myl7:GFP) hearts were taken at 30 frames per second under a Zeiss Stemi SV11 microscope. A custom designed area-scan algorithm calculated pixel density in a user-specified area on the heart and the value was quantified as a unit of fluorescence intensity over time in frames (30 frames per second). This generated a trace of cardiac contractions, and the data was plotted as cardiac trace over time (seconds).

CaM expression and purification—pGemex-2-CaM constructs were transformed into Echerichia coli competent cells BL-21 (DE3). The cells were incubated at 37°C until the OD600 was 0.8-1 and CaM expression was induced with 100 µmol/L IPTG. CaM was detected in culture medium after overnight incubation. Culture medium with additional 5 mmol/L CaCl$_2$ was applied to pre-equilibrated HiTrap Phenyl Sepharose column (GE Healthcare) and CaM elution proceeded according to (45). All purified CaM proteins used in the Camui/CaMKII experiments were dialyzed overnight against a buffer containing 50 mmol/L Tris-HCl pH 8 and 0.1 mmol/L EGTA using 10,000 MWCO Snakeskin dialysis tubing (Thermo Fisher Scientific).

Camui in vitro assay—The Camui mYpet-CaMKII alpha-mCerulean construct, kindly provided by Yasunori Hayashi (36), or derived mutants generated in our laboratory were transfected into HeLa cells using Lipofectamine (Invitrogen). HeLa cells grown under standard conditions (DMEM, 10% FBS, 1% penicillin/streptomycin) were seeded at 8,400,000 cells in a T175 flask the day before transfection and harvested two days after transfection. Cell lysis was done in 2 ml buffer (40 mmol/L Hepes NaOH pH 8, 5 mmol/L Magnesium acetate, 0.1 mmol/L EGTA, 0.01% Tween, 1 mmol/L DTT and protease inhibitor cocktail) and followed by dounce homogenisation. Lysates were cleared by 10 min centrifugation at 10,000 x g at 4°C, aliquots of the supernatant were shock frozen in liquid nitrogen and stored at -80°C. CFP fluorescence was measured at 37°C using a Tecan infinite F200 Pro (Thermo Fisher Scientific) and the ex448/em485 filter setting. For activation of Camui, 1 µmol/L CaM (unless otherwise indicated) and 40 µmol/L ATP were used. CaCl$_2$ was added to reach the indicated free Ca$^{2+}$ concentrations as calculated by the Max-chelator program (46). Background readings were deducted from all measurements to exclude contribution from lysate components.

Camui kinetics measurements—The velocity of the CaM/Camui-CaMKII reaction was calculated using a modified set up of the Camui in vitro assay. CFP fluorescence emitted from Camui containing HeLa cell lysate in a 96 well plate with 1 µM CaM and 40 µM ATP (final concentrations after stimulation) was measured every ~1 second. After ~10 seconds, an automated injector added CaCl$_2$ to reach an estimated final concentration of 200 µM free Ca$^{2+}$. Background measurements (prior to addition of calcium) were deducted and velocity of the CFP emission increase calculated as $V = [(Max-B)/2]/t$ where V is velocity, Max is the CFP emission (average of 10 measurements at the reaction saturation level), B = background (average of 9 measurements in the absence of Ca$^{2+}$), t = time (seconds). A logarithmic regression model equation $(y = \alpha *\ln(x) \pm \beta)$ calculated in Microsoft Office Excel was used in order to calculate the time (t) at the half maximum ((Max-B)/2) of the CFP emission.

CaMKII expression and purification—His tagged CaMKII was produced in HEK 293F cells by transfection of constructs using Polyethylenimine (Sigma). Cells were grown in DMEM supplemented with 10% Fetal Bovine Serum (FBS) (Biochrom AG), 1% penicillin/streptomycin for three days after
transfection when cells were harvested by centrifugation at 1000 x g for 4 min at 4°C, washed once in PBS then lysed in a buffer containing 0.3% NP-40, 0.5 M NaCl, 20 mM Na₃PO₄, 30 mM Imidazole and complete protease inhibitor cocktail (Roche). Following Dounce homogenization the lysate was cleared by centrifugation for 30 min at 20,000 x g. The supernatant was incubated overnight with Ni Sepharose (GE Health care) and washed with two volumes of lysis buffer before elution using buffer containing 500 mM imidazole to give a semi-purified His-CaMKII solution of unknown CaMKII concentration. The HeLa and HEK 293F cell lines were exclusively used for protein production, tested periodically for mycoplasma infection and phenotypic characteristics.

CaMKII in vitro activation - 11 µl of semi-pure His-CaMKII was mixed with 1 µM wild type or mutated CaM, 40 µM ATP and desired concentrations of Ca²⁺ to give a final volume of 12.5 µl and incubated at 37°C for 30 min. Proteins in the reaction mix were separated by SDS PAGE (10% polyacrylamide) and analyzed by Western blot using antibodies against pThr286 CaMKII (Abcam, ab32678) and CaMKII (Sigma-Aldrich C6974). Western blots were visualized and analyzed using the ChemiDoc MP bioimaging system (Bio-Rad, CA).

Mass spectrometry - All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise stated. Titanium dioxide (TiO₂) beads were from GL Sciences Inc. (Tokyo, Japan). Endopeptidase Lys-C was from Wako. Sequence-grade trypsin was from Promega (Madison, WI, USA). Recombinant human Histidine-tagged CaMKIIδ was obtained from Life Technologies (Carlsbad, CA, USA). Autocamtide-2 was from Santa Cruz Biotechnology Inc. (Heidelberg, Germany). Okadaic acid was purchased from MP Biomedicals (Thermo Fisher Scientific). Empore C18 extraction disk was from 3M Bioanalytical Technologies (St. Paul, MN). Poros Oligo R3 reversed phase chromatographic material was from PerSeptive Biosystems (Framingham, MA, USA). Reprosil-C18 3 µm beads were purchased from MikroLab Aarhus S/A (Højbjerg, Denmark).

All reagents used in the experiments were of sequencing grade. All solutions were made with ultrapure water (Elga Purelab Ultra water system, Bucks, U.K.).

Stimulation of CaMKIIδ autophosphorylation - Protein concentration was determined by amino acid composition analysis using a Biochrom 30 amino acid analyzer (Biochrom, Cambridge, UK). Autophosphorylation of CaMKIIδ was induced by adding 1.5 µg CaMKIIδ to a reaction mix containing 50 mM Tris-HCl pH 7.5, 100 mM NaCl, 0.1% BSA, 1 mM CaCl₂, 10 mM MgCl₂, 100 µM ATP, 1 µM okadaic acid and 10 µg of wild type CaM or D129G CaM mutant, respectively with addition of 30 µg of substrate peptides (Autocamtide-2; K KALRQRKTVDAL). Stimulation of CaMKIIδ was performed for 2.5 min and 15 min at 37°C in a final volume of 54.8 µl. The reaction was stopped by adding reducing agent (10 mM dithiothreitol, DTT) and endopeptidase Lys-C to the mix, followed by 1h incubation at room temperature (RT). Each stimulation condition was performed in triplicate. Peptide digestion was performed by using either Lys-C or Lys-C in combination with trypsin. Lys-C only was used for peptides, which would have been too short if digested with trypsin. Lys-C generated peptides were alkylated in 20 mM iodoacetamide for 30 minutes at RT in the dark. Subsequently, samples were digested with trypsin at an enzyme to substrate ratio of approximately 1:50 for 12 hours at 37°C. The samples were acidified to 5% formic acid and desalted using Poros R3 RP column packed in a P200 stage tip with C18 3M plug. The purified peptide samples were dried by lyophilization and labeled using isobaric tagging labeling (iTRAQ™ 4-plex; Applied Biosystems, Foster City, CA) as described by the manufacturer. After labeling, all samples were pooled into one tube and dried by vacuum centrifugation to ~50 µl for phosphopeptide enrichment by titanium dioxide (TiO₂). The TiO₂ enrichment of phosphopeptides was performed essentially as previously described (47). Prior to nanoLC-MS/MS analysis, the phosphorylated peptide samples were resuspended in 0.1% TFA and desalted using Poros R3 RP column packed in a P200 stage tip with C18 3M plug. Purified
phosphopeptide samples were dried by lyophilization and stored at -80°C until further analysis.
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Authors contributions: MWB, JLC, TJ did the planning and directing of the experimental work, as well as writing and commenting; KK and MRL carried out phosphorylation assays and mass spectroscopy; KW and JNC performed the zebrafish work; RT made the DNA constructs, produced recombinant CaM and did the DT40 work; TZ performed the Camui in vitro assay and CaMKII in vitro activation.

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**FOOTNOTES**

The Abbreviations used were: CaM, Calmodulin; CaMKII, Ca/CaM kinase II; CPVT, catecholaminergic polymorphic ventricular tachycardia; HA, haemagglutinin; LQTS long QT syndrome; RyR2, Ryanodine receptor 2; SR sarcoplasmic reticulum

**FIGURE LEGENDS**

**FIGURE 1.** Representation of pathogenic CaM variants associated with CPVT, LQTS or IVF. The backbone is shown in grey except for the mutated residues where the side chains in stick
representation have been included and color coded either blue or red (red indicates the residue is directly involved in Ca\textsuperscript{2+}-coordination). Ca\textsuperscript{2+} is shown in yellow spacefill presentation. Textboxes show the amino acid conversion as well as the arrhythmia associated with the mutation and in which of the three calmodulin genes (CALM) the mutation has been identified and references to the discoveries. The CaM model is based on the 1.7Å structure of Ca\textsuperscript{2+} bound wt CaM (1CLL) modified from (48).

FIGURE 2. The CaM mutant D129G is not able to replace wt CaM in DT40 cells. The CaM1 gene of the chicken DT40 cells was silenced by disrupting exon 3 (red) by insertion of a tetracyclin-regulatable rat CaM cDNA (green) and the other allele by insertion of a selection marker (23). In this cell line both alleles of the CaM 2 gene had previously been disrupted (24) rendering the tetracyclin-regulatable rCaM (TetReg-CaM) as the only CaM expressed. Stable transfection of HA-tagged versions of CaM (blue) followed by down-regulation of rCaM enables exchange of wt for HA-tagged wt and mutant CaM. B. Western blot analysis of 25 µg total protein per lane from DT40 clones showing levels of TetReg-CaM and HA-tagged CaM non-treated (control, upper panel incubated with anti-CaM antibody) and four days of tetracyclin treatment showing almost complete exchange of wt for mutated HA-tagged CaM (lower panel incubated with both anti-CaM and anti-HA antibodies). As the CaM antibody has different affinities for the mutant versions of CaM signals shown for HA-CaM do not represent the precise amounts of these proteins. C. Growth curves of cells expressing HA-tagged CaM wt and mutants grown in the absence or D. presence of tetracyclin (tet). Error bars are SEM from five independent repetitions. E: Bar diagram showing % viable cells at 120 hours tet-treatment for all clones compared to the control without tet. Error bars are SEM from five independent repetitions.

FIGURE 3. CaM D129G activates CaMKII only to a minor degree and decreases wt CaM mediated activation. A. Recombinant heart disease related CaMs with the indicated amino acids mutated or wt CaM were incubated with the HeLa cell lysate containing the Camui CaMKII-sensor and peak unquenching of mCerulean was measured in the presence of 2.5 µM [Ca\textsuperscript{2+}]. CaM with disrupted Ca\textsuperscript{2+}-binding at EF-hands 1 and 2 (EF12), 3 and 4 (EF34) or 1, 2, 3 and 4 (EF1234) were used as negative controls. B. Bar diagram shows the effect of CFP emission on adding 1 µM wt CaM, CaM 129 or EF34 alone (left hand side), or mixing wt CaM with the mutant CaM proteins (right hand side). To test different ratios of wt:mutCaM the mutCaM was added to give a final total CaM concentration (wt + mutCaM) of 1 µM. Addition of buffer served as a control for the effect of reducing wt CaM. C. Activation kinetics of CaMKII in the presence of D129G CaM. P<0.05 as calculated by paired two-tailed analysis. Error bars represent SEM from three independent experiments.

FIGURE 4. Lack of CaMKII T286 phosphorylation in the presence of CaM D129G and reduced phosphorylation of a downstream target peptide. Purified CaMKII was stimulated with either wt CaM or D129G CaM under various [Ca\textsuperscript{2+}]. A. Representative Western blot analysis of phosphorylation analyzed with an anti-pT286/287 specific antibody. Ctr indicates control without added Ca\textsuperscript{2+}. B. Quantification of Western blot analysis showing pT286 CamKII to total CaMKII ratios relative to non-Ca\textsuperscript{2+} stimulated control from three independent experiments. Error bars represents SD. C. Quantitative mass spectrometry analysis of the autocamtide-2 target tryptic peptide (QET(phosp)VDAL, highlighted in blue) following recombinant CaMKIII stimulation with either wt or D129G CaM. Measurements were performed in triplicate at 2.5 and 15 minutes after the reaction was initiated. * indicates p<0.05; ** indicate p<0.005 calculated using either homoscedastic or heteroscedastic two-tailed t-test, depending on the statistical value of the F-test (heteroscedastic if p-value<0.05). Proteins with t-test p-value smaller than 0.05 were considered as significantly altered between the two tested conditions. Values are given as fold change of the D129G vs wt CaM. Detected CaMKIIδ tryptic T286-phosphopeptide is shown for comparison.

FIGURE 5. Impact of CaM D129G on the heart rate and conduction of the developing heart in zebrafish. A. Representative images of zebrafish injected with RNA coding for either wt CaM or
the mutant D129G as well as non-injected control (Upper panel). The lower panel shows GFP expression in the zebrafish hearts B. Bar diagram showing relative heart beat normalized to non-injected control embryos. Heart beat measurements were based on fluorescent intensities from cardiac contractions in non-injected control embryos, CaM-WT injected embryos, and D129G injected embryos. D129G injected embryos exhibit a heart rate of 83% compared to non-injected control and CaM-WT injected embryos. ***, p<0.001. C. Contractions in the atrium and ventricle of a non-injected animal. D. A representative trace from a D129G injected embryo exhibiting a 2:1 atrium to ventricle beat ratio, observed in ~8% of D129G injected embryos (n=128).

FIGURE 6. Hypothetical model for CaMKII activation delay. Half of the dodecameric holoenzyme has been sketched with the green units representing the regulatory domain and the blue units the hub domain. CaM is in yellow (wt) or orange (D129G) and the red dots represent phosphorylation sites T286. The presence of CaM D129G will lead to binding without T286 phosphorylation. As the D129G will occupy a CaM binding site (1) phosphorylation and full holoenzyme activation (2) will be delayed (figure 3C).

Please note that the figures are removed here as they may show up in unreadable ways with different Word versions but all the final versions are to be seen in the PDF version.
Figure 1

- N53P CPVT CALM1
- E140G LQTS CALM1
- F141L LQTS CALM1
- F89L IVF CALM1
- D131E H/V CPVT/LQTS CALM1
- D133H LQTS CALM2
- Q135P CPVT/LQTS CALM2
- A102V CPVT CALM3
- D95V LQTS CALM1
- N97S I2 CPVT/LQTS CALM1
- D129G LQTS CALM1
- D131E H/V CPVT/LQTS CALM1
- E140G LQTS CALM1
- F141L LQTS CALM1
- F89L IVF CALM1
- D131E H/V CPVT/LQTS CALM1
- D133H LQTS CALM2
- Q135P CPVT/LQTS CALM2
- A102V CPVT CALM3
- D95V LQTS CALM1
- N97S I2 CPVT/LQTS CALM1
- D129G LQTS CALM1
- D131E H/V CPVT/LQTS CALM1
- E140G LQTS CALM1
- F141L LQTS CALM1
- F89L IVF CALM1
- D131E H/V CPVT/LQTS CALM1
- D133H LQTS CALM2
- Q135P CPVT/LQTS CALM2
- A102V CPVT CALM3
- D95V LQTS CALM1
- N97S I2 CPVT/LQTS CALM1
Figure 2A

**HA-CaM (wt, N53I, F89L, D95V, N97S, D129G, or F141L)**

DT40 CaMI gene

High-tetO-rCaMIII-polyA

Figure 2B

**MW** Parental WT N53I F89L D95V N97S D129G F141L

**Control**

**Tet-treated**

17 kD

**HA-CaM**

**TetReg-CaM**

Figure 2C

Control

Cell number

Days

1.0E+10

1.0E+09

1.0E+08

1.0E+07

1.0E+06

1.0E+05

1 2 3 4 5 6 7

Figure 2D

**Tet-treated**

1.0E+10

1.0E+09

1.0E+08

1.0E+07

1.0E+06

1.0E+05

1 2 3 4 5 6 7

Figure 2E

% viable cells

Parental

wt

N53I

F89L

D95V

N97S

D129G

F141L

Control

Tet
Figure 3A

Figure 3B
Figure 3C

Activation kinetics relative to wt (RFU/time)

WT (2:3) WT, (1:3) elution buffer (2:3) WT, (1:3) 129 129

p<0.05
**CPVT and LQTS calmodulin mutations**

**Figure 4A**

- MW: wt 55 kD, 129 55 kD
- pT286 CaMKIIα
- CaMKIIα
- µM Ca

**Figure 4B**

- 2+ fold change (D129G/WT CaM)
- 2.5 min 15 min

**Figure 4C**

- Trypsin cleavage sites
- autocomtide-2
  m/z[Da] 500.230; charge state 2+
  N-Term(iTRAQ4plex); T3(Phospho)

- CaMKIIδ – canonical (Human)
  m/z[Da] 536.723; charge state 2+
  N-Term(iTRAQ4plex); T3(Phospho);
  C6(Carbamidomethyl); K8(iTRAQ4plex)
Figure 6

CPVT and LQTS calmodulin mutations
