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Published in:
Biochimica et Biophysica Acta - Molecular Cell Research

DOI:
10.1016/j.bbamcr.2021.119119

Publication date:
2021

Document version
Publisher's PDF, also known as Version of record

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Citation for published version (APA):
Berchtold, M. W., Munk, M., Kulej, K., Porth, I., Lorentzen, L., Panina, S., Zacharias, T., Larsen, M. R., & la Cour, J. M. (2021). The heart arrhythmia-linked D130G calmodulin mutation causes premature inhibitory autophosphorylation of CaMKII. Biochimica et Biophysica Acta - Molecular Cell Research, 1868(12), [119119]. https://doi.org/10.1016/j.bbamcr.2021.119119
The heart arrhythmia-linked D130G calmodulin mutation causes premature inhibitory autophosphorylation of CaMKII

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ABSTRACT

The Ca2+/calmodulin (CaM)-dependent kinase II (CaMKII) is well known for transmitting Ca2+-signals, which leads to a multitude of physiological responses. Its functionality is believed to involve CaMKII holoenzyme dynamics where trans-autophosphorylation of the crucial phosphorylation site, T286 occurs. Phosphorylation of this site does not occur when stimulated exclusively with the arrhythmia associated D130G mutant form of CaM in vitro. Here, we present evidence that the loss-of-CaMKII function correlates with premature phosphorylation of its inhibitory phosphosite T306 in CaMKIIα and T307 in CaMKIIβ as this site was up to 20-fold more phosphorylated in the presence of D130G CaM compared to wildtype CaM. Indeed, changing this phosphosite to a non-phosphorylatable alanine reversed the inhibitory effect of D130G both in vitro and in live cell experiments. In addition, several phosphosites with so far undescribed functions directing the Ca2+-sensitivity of the CaMKII sensor were also affected by the presence of the D130G mutation implicating a role of several additional autophosphosites (besides T286 and T306/T307) so far not known to regulate CaMKII Ca2+ sensitivity. Furthermore, we show that introducing a D130G mutation in the CALM2 gene of the P19CL6 pluripotent mouse embryonic carcinoma cell line using CRISPR/Cas9 decreased the spontaneous beat frequency compared to wildtype cells when differentiated into cardiomyocytes supporting an alteration of cardiomyocyte physiology caused by this point mutation. In conclusion, our observations shed for the first time light on how the D130G CaM mutation interferes with the function of CaMKII and how it affects the beating frequency of cardiomyocyte-like cells.

1. Introduction

CaM acts as an important Ca2+ regulated molecular signaling device in a large number of cellular processes including cell division, proliferation, motility and metabolism among others [1,2]. One well-investigated function of CaM is its involvement in the regulation of the activity of protein kinases. Since the surprising discovery of a linkage between mutations in the CALM1 gene (N54I, N98S) and catecholaminergic polymorphic ventricular tachycardia (CPVT) [3] a number of other amino acid substitutions in the CaM protein, originating from all three CALM genes has been associated with either CPVT, long QT syndrome (LQTS) or idiopathic ventricular fibrillation (IVF) [4-9]. Several of the mutations are either part of or directly adjacent to an EF-hand and have been shown to strongly reduce the Ca2+-binding affinity of the C-terminal lobe of CaM, up to over 50-fold for D130G [5]. CaM has multiple targets essential for cardiomyocyte function including the Ryanodine Receptor 2 (RyR2). It has been proposed that mutated CaM causing CPVT (N54I, N98S, A103V, D132E) interferes with the function of wild type (wt) CaM by preventing the CaM mediated closing of the RyR2 Ca2+ release channel thus leading to uncontrolled Ca2+ release from the sarcoplasmic reticulum (SR) [4,10,11]. Furthermore, CaM mutations have been associated with small conductance calcium-
activated potassium channel (SK) 2 [11] as well as with SK3 channels [12] and changes in both Na+ and Ca2+ currents [4,5,8,13,14]. Several of the known CaM regulated proteins are also targets of CaMKII including the RyR2 and other ion channels, indicating that CaM can either regulate targets by direct interaction or via its effect through CaMKII. It is therefore relevant to ask how the activity of CaMKII is affected by the CaM mutations. CaMKII belongs to a family of autoactivating enzymes phosphorylating serine/threonine (T) residues of multiple target proteins [15]. Many of these target proteins are well known, especially for their roles in both muscle and neuronal tissue [16]. Activation of CaMKII is induced by binding of Ca2+-loaded CaM to a stretch of amino acids (K293-F313 in CaMKIIα) in the regulatory domain of this enzyme [17]. This, in turn, leads to phosphorylation of T286 in the α isofrom (T287 in the δ isofrom which is the major cardiac CaMKII isoform). Whereas T286 will be phosphorylated as a consequence of CaM binding, the two phosphosites, T305/T306 (α) (T306/T307 (δ)), when phosphorylated, prevent CaM binding [18]. Initial experiments by Huang et al. [10] showed that D130G CaM and other mutant CaM would bind to the FRET based CaMKII sensor Camui with similar apparent affinity as the wt CaM at 200 μM Ca2+ [10]. More recent research from our laboratory indicated that whereas binding of D130G CaM (genetic numbering as opposed to previously used D129G CaM, the CaM protein numbering without the start codon) to CaMKII still occurs, activation of CaMKII appears to be impaired, as T286 is not phosphorylated and CaMKII target peptide phosphorylation is decreased [19]. Furthermore, a dominant negative effect of D130G CaM on the activation kinetics of Camui was found, which could be a link to understanding how mutant CaM can affect heart functions. This is especially interesting as an identical CaM protein is encoded by three genes located on different chromosomes in humans [20], and mutation of only one allele may lead to heart arrhythmia.

Here, we investigated possible mechanisms of how the D130G CaM mutation affects CaMKII by comparing the autophosphorylation status of CaMKII at residues T306α and T307δ in the α and δ isoforms, respectively, in the presence of wt or D130G CaM by mass spectrometry. This was followed by analysis of structural changes occurring in the CaMKII sensor Camui with alterations of these phosphosites. Our data show that the D130G CaM mutation leads to a premature phosphorylation of the T306α and T307δ sites and to a changed phosphorylation pattern in a group of phosphosites in the kinase-hub linker region 330–337 (for the δ isofrom) that we find to be associated with Camui sensitivity to Ca2+/CaM mediated activation in vitro. We demonstrate by in vitro and live cell experiments that replacing the T305/T306 (α) residues by alanine counteracted the negative effect of D130G CaM on CaMKII activity. Our data link the heart arrhythmia associated mutation to a possible mechanistic dysfunction of CaMKII. In order to get further insights into how D130G CaM affects the function of cardiomyocytes we replaced the wt CALM2 gene by a D130G mutant CALM2 gene using CRISPR/Cas9 in the P19CL6 embryonic carcinoma cell line and found that this led to a reduction of spontaneous beat frequency after differentiation into cardiomyocytes.

2. Results

2.1. D130G CaM causes T306 hyperphosphorylation of purified CaMKII

One of the hallmarks of CaMKII activation is the phosphorylation of T286 leading to an exposure of the regulatory segment of CaMKII causing a cascade of CaMKII activation and what is termed an autonomous Ca2+/CaM independent activity (reviewed in [21]). Autophosphorylation of T305/T306 (α), which are completely buried in the presence of wt CaM, on the other hand causes inhibition of CaM binding to CaMKII and prevents activation [18]. Previously, we have shown that T286 phosphorylation of CaMKII following stimulation by D130G CaM was decreased as compared to that by wt CaM [19]. Here, we demonstrate by nano-liquid chromatography coupled to tandem mass spectrometry (nano-LC–MS/MS) that both CaMKIIα and δ were phosphorylated to a higher degree at T306α and T307δ following D130G CaM mediated stimulation as compared to wt CaM (Fig. 1a). Phosphorylation of these residues could explain the poor response of the Camui sensor to D130G CaM. To investigate whether this was the case we mutated T305α and T306α in the Camui construct harbouring CaMKIIα, each mutation alone and in combination, to alanine (A) and examined to what degree these sites would rescue the D130G CaM-induced Camui activation profile (Fig. 1b). Indeed, when both of these sites were mutated the Camui activity was restored, however only under high Ca2+ conditions (10–100 μM). Converting position 306, but not 305, from T to A alone was sufficient to restore Camui activity at 100 μM Ca2+.

2.2. Functionally undescribed phosphosites influence CaMKII Ca2+ sensitivity

By in vitro screening of CaMKII for phosphosites we found 10 sites also identified in previous studies [22–25]. Functions of these phosphosites are not known and their structural relevance is difficult to study as most of them are situated in the flexible linker region connecting the regulatory segment of the kinase domain with the hub domain (Fig. 2a). Using bioinformatics, we found nine of these phosphosites to be highly conserved among different isoforms (Fig. 2a, lower part and Supplementary material Table S1) and across different species (Supplementary material Table S2). We show that both wt and D130G CaM are able to stimulate autophosphorylation of these nine sites, however, to a different degree. Three phosphosites in the border region of the kinase domain and the regulatory segment, S272/T276/S279 (CaMKIIα) were consistently phosphorylated to a lesser degree when activated by D130G CaM as compared to wt CaM (Fig. 2b).

Conversely, D130G CaM induced phosphorylation at six sites in the 330–337 (CaMKIIδ) linker region to a higher degree as compared to wt CaM (Fig. 2c). The degree of phosphorylation induced by D130G CaM compared to wt at the three regions analysed in this paper, are summarized in Fig. 2d.

Moreover, insertion of Ala in place of either Thr or Ser at eight sites (S275, T276, S279, S331, S333, T334, T336, T337) in the Camui construct resulted in a higher activity at low Ca2+ and a lower activity at high Ca2+ concentrations (Fig. 2e, and Supplementary material Fig. S3) compared to a construct with only six sites replaced by Ala (leaving T336 and T337 accessible for phosphorylation). This suggests that these two sites when phosphorylated have a negative effect on the activation of the CaMKII sensor Camui when CaM is not present, but activate CaMKII further when CaM is bound. When introducing phosphomimicking glutamic acid (Glu, E) or aspartic acid (Asp, D) in place of the Thr and Ser both constructs reverted to a more closed CaMKII indicating that these sites may be phosphorylated in the steady state wt CaMKII formation.

2.3. Generation of D130GCaM knock in P19CL6 cell lines by CRISPR/Cas9

We previously discovered that recombinant D130G CaM was incapable of fully activating CaMKII as shown both by the strongly reduced ability to induce a structural change in the CaMKII sensor Camui and the lack of T286 phosphorylation [19]. In order to test whether the above described effect of D130G CaM on CaMKII T305/T306 phosphorylation and on Camui activity could be reproduced in a cell-based system we knocked-in the D130G mutation in the CALM2 gene of the P19CL6 cell line using a CRISPR/Cas9 technology. The P19 cell line, subclone P19CL6, has been widely used as a model to study heart functions because the cells can differentiate into cardiomyocyte-like cells and create clusters of beating cells within the cell population upon stimulation with dimethyl sulfoxide (DMSO) [26,27].

We then analysed recombinant D130G CaM and wt CaM, as well as
lysates from the CALM2<sup>D130G/D130G</sup> cells and wt cells by Western blot analysis. Recombinant D130G CaM moved faster in the presence of EGTA and slower in Ca<sup>2+</sup> as compared to wt CaM (Supplementary material Fig. S1a, b). Interestingly, Wang et al. [28] found that another arrhythmogenic mutant F142L showed a slower electrophoretic mobility in the Apo form and no change in the Ca<sup>2+</sup> bound form compared to wt CaM. Analysis of the lysates confirmed the presence of two CaM bands in the CALM2<sup>D130G/D130G</sup> cell protein extract (Supplementary material Fig. S1c), one representing the D130G CaM and the other band, the wt CaM. D130G CaM produced a band with a higher intensity than wt when analysed with CaM antibodies most likely due to less steric hindrance for the antibody to the epitope. This difference is further examined in Supplementary material Fig. S1d,e, showing that an antibody recognising the C-terminal part of CaM (listed as binding to region AA 128–148) has an approximately 37-fold higher intensity to the D130G CaM protein as compared to wt. Interestingly when using an alternative antibody recognising the N-terminal part of CaM we also see a higher affinity, but only of approximately 4.5–x. (Supplementary material Fig. S1e). Based on measurements with these two antibodies we estimate that the mutated CaM is expressed at approximately 30% of the total CaM protein in our experimental setup (Supplementary material Fig. S1f,g). An RNA seq database gives an average expression from the CALM2 in mouse heart of 32% (Supplementary material S1h). The estimate that the mutated CaM is expressed at approximately 30% of the total CaM protein in our experimental setup (Supplementary material Fig. S1f,g). An RNA seq database gives an average expression from the CALM2 in mouse heart of 32% (Supplementary material S1h). The estimate that the mutated CaM is expressed at approximately 30% of the total CaM protein in our experimental setup (Supplementary material Fig. S1f,g). An RNA seq database gives an average expression from the CALM2 in mouse heart of 32% (Supplementary material S1h).

3.2. Knock-in of D130G in CALM2 causes decreased beating frequency and an altered Camui activation profile

To investigate the cardiomyocyte-like phenotype of the CALM2<sup>D130G/D130G</sup> P19CL6 cells we differentiated both wt and D130G CaM harbouring cells to produce beating clusters (Fig. 3a). Differentiation into a cardiomyocyte-like phenotype was verified by using the differentiation marker SOX2 and the cardiomyocyte-specific marker GATA4 (Supplementary Fig. S4). The growth rate and morphology of the mutated cells did not appear to differ from wt, however, the contraction rate of individual cell clusters of the CALM2<sup>D130G/D130G</sup> cells was significantly reduced (40.4 versus 24.3 beats per minute) indicating that D130G CaM has a profound influence on cardiomyocyte contractility. Pharmacological inhibition of Camui in P19CL6 cells by the KN93 inhibitor completely abolished beating indicating that indeed Camui is necessary for cardiomyocyte contraction, although this observation cannot be directly linked to the principal mode of action of arrhythmogenic CaM mutations.

We then expressed the Camui-sensor Camui in the P19CL6 wt and CALM2<sup>D130G/D130G</sup> cells to investigate how D130G CaM affects the function of Camui in a live cell setup. These experiments were done in non-differentiated cells as the transfection protocols used were not compatible with sustaining a phenotype with beating clusters in the differentiated P19CL6 cells. To test whether preventing phosphorylation of TT305/306AA would restore Camui activity in a live cell setup we transfected wt and CALM2<sup>D130G/D130G</sup> P19CL6 cells with the TT305/306AA (α) or wt Camui constructs and compared their activation profile following Ca<sup>2+</sup> ionophore ionomycin treatment. Analogous to the <sup>in vitro</sup> experiment (Fig. 1b) the reduced ability of Camui activation in the CALM2<sup>D130G/D130G</sup> live cells could be rescued by expressing the TT305/306AA phosphorylation incompetent version of the Camui (Fig. 3b).

3. Discussion

In this study, we have elaborated on the molecular mechanisms causing the arrhythmia-linked CaM mutation D130G to interfere with the function of Camui. Using a proteomics approach, we identified a changed phosphorylation pattern and could both <sup>in vitro</sup> and live cell methods reverse the D130G induced defect using phosphosite mutants. Furthermore, we identified nine phosphosites as possible regulators of Camui<sup>2+</sup> sensitivity. Three of these are located in the border region between the kinase domain and the regulatory segment, and six in the kinase-hub linker.

Inhibition of Camui has been in the focus as a way to improve heart arrhythmia in recent years indicating that interfering with this multifunctional enzyme would be advantageous when treating cardiac arrhythmia (reviewed in [30]). Therefore, one would not expect that a major effect of the CaM mutations to cause arrhythmia would be to inhibit this enzyme. Many pathways lead to Camui hyperactivation. This may occur during unbalanced Ca<sup>2+</sup> handling, upon beta adrenergic stimulation [31] or by oxidation, which may lead to arrhythmia and other heart pathologies. Promising results using Camui inhibitors have been obtained from experiments in cell cultures and in vivo with small animals reviewed in [30]. However, Camui is an essential player in normal heart physiology and cannot be down-regulated to a major degree [32]. Therefore, it is plausible that interfering with basic Camui functions including CaM dependent stimulation may be detrimental and result in cardiac pathologies. In a recent study, we showed that <sup>in vitro</sup>
Fig. 2. Autophosphorylation sites in CaMKII. a. Schematic representation of the domains of CaMKII, where amino acid positions refer to CAMKII ɑ. The lower part is a sequence logo plot representing the conservation according to (http://weblogo.berkeley.edu/logo.cgi) of the amino acids through the relative size of the letters. The phosphosites are shown in color (serine in yellow and threonine in green), and the autophosphorylation sites functionally analysed in this paper are indicated by numbers, the activation autophosphorylation site T286 is shown in red. b. Ratio of phosphorylation of CaMKII ɑ residues S272, S279 and T276/S279 at 15 min relative to 2.5 min after stimulation with either wt CaM or D130G CaM. Values from two independent experiments are indicated with different colors. P-values represents comparison of all phosphorylation sites grouped together for D130G CaM and wt CaM (* paired t-test, one tail, p-value < 0.05). c. Ratio of phosphorylation, after stimulation with wt CaM or D130G CaM, comparing 15 min and 2.5 min phosphorylation values from the 329-ESTESSNTTIEDEDVK-344 peptide of CaMKII ɑ carrying single or multiple (indicated in the figure) phosphorylation sites (different experiments are indicated with different colors). Statistics comparing D130G CaM to wt CaM when grouping all phosphorylation sites together (* homoscedastic one-tailed t-test p-values < 0.05) are shown). d. Overview of the results in Figs. 1a, 2b and c. The effect of D130G CaM compared to wt CaM on the phosphorylation of the three highly conserved CaMKII regions is shown. The T306 ɑ and T307 ɑ depicted is the average of the four values shown in Fig. 1a. The numbering of sites 272–279 refers to the CaMKII ɑ, and 330–337 to the CaMKII ɑ2 (see Supplementary Table S1 for CaMKII isoforms/spliceforms). e. Activation kinetics of different Camui constructs following addition of wt CaM and the indicated [Ca^{2+}]. Camui constructs were made with non-phosphorylatable Ala in place of Thr and Ser or Glu/Asp to mimic constitutively active phosphosites. Error bars represents SEM of the average from three independent experiments each with three technical replicas. Theoretical effect on the Camui (CaMKII) FRET signal by CaM/Ca^{2+} binding is illustrated in the inserted cartoon. Raw data points are shown in Supplementary material Fig. S3. Data for the last time-point were analysed using Wilcoxon rank-sum test, and p values shown on figure.
Fig. 3. D130G knock-in leads to decreased beat ratio and reduced Camui activation profile. a. Beating frequencies of P19CL6 cell clusters. Three independent experiments were done in parallel with wt and CALM2-D130G/D130G cells. A two tailed independent t-test shows a significant reduction in mean beat frequency when grouping all results (40.4 vs 24.3 beats per minute). Inhibition with the CaMKII inhibitor KN93 did not result in any beating clusters. P value refers to a two tailed t-test. b. Activation kinetics of Camui in wt or CALM2-D130G/D130G p19CL6 cells transfected with either wt or TT305/306AA Camui constructs. The chart shows that for each of the conditions the maximum rate recorded by which the FRET (F<sub>Wt</sub>/F<sub>Rep</sub>) ratio was changing was per second. Five technical replicas are indicated by different symbols, and mean values by red dashes. P value refers to a two tailed t-test.

autophosphorylation of T286, the hallmark of CaMKII activation, was severely diminished in the presence of the arrhythmogenic D130G CaM, when compared to wt CaM and that this mutation decreased the zebrafish heart rhythm [19]. Here, we show that in the presence of D130G CaM, CaMKII activity was markedly changed in vitro as well as in a cellular system.

Using phosphoproteomic approaches we observed that the presence of D130G CaM during in vitro phosphorylation led an ~8-fold increase of T306 (CaMKIIα) and ~20-fold increase of T307 (CaMKIIδ) phosphorylation in the presence of D130G CaM compared to wt CaM (Fig. 1a). This gave us a hint to the source of the dysfunction of CaMKII caused by this mutation and we were able to fully reverse the defect in D130G/CaMKII binding/activation by mutating these sites (Fig. 1b).

The structure of CaM in a complex with CaMKII is known and indicates that CaM sandwiches the CaM binding site turning it into a helix [33]. It has been shown that the N-terminal lobe of CaM binds first to CaMKII during its activation possibly explaining why D130G CaM can bind to CaMKII as this mutation is not expected to affect the N-terminal lobe [34] and as shown by the crystallographic analysis only a minimal structural effect on the N-lobe could be observed [35]. Work by Evans and Shea described the contribution of the two lobes to CaMKII binding, in particular the importance of each of the four EF hands and the individual Ca<sup>2+</sup> coordinating residues [35]. Importantly, eliminating Ca<sup>2+</sup> binding to CaM in EF hand 3 and 4 by two point mutations of crucial Ca<sup>2+</sup> coordinating residues reduced the affinity of CaM to a CaMKII CaM binding peptide at saturating Ca<sup>2+</sup> concentration to a major degree. Given the defect in Ca<sup>2+</sup> binding of EF hand 4 of D130GCaM this mutation is expected to affect the Kd for the D130G CaM/CaMKII interaction as well. This is supported by the recently published structure of D130G CaM [29] indicating that this mutation leads to uncoupling of the Ca<sup>2+</sup>-binding cooperativity and a dramatic misfolding of the EF3,4 lobe, eliminating high affinity Ca<sup>2+</sup> binding. The large change in the CaM structure is reflected by our observation of a changed electrophoretic mobility and affinity to an antibody epitope in EF hand 4. Wang et al. [29] showed that a part of the CaM binding site binding CaM to CaV1.2 IQ was not in contact with D130GCaM in contrast to wt CaM. CaM wraps around the CaV1.2 IQ binding domain in the same way as to CaMKII even though the binding motifs are different. We propose that the N-terminal lobe of D130G CaM competes with wt CaM in binding to CaMKII, but, is incapable of activating CaMKII as shown by its inability to allow T286 phosphorylation [19]. This may explain the dominant negative effect of D130G CaM we have previously observed [19]. Based on our findings we hypothesise that Ca<sup>2+</sup>-D130G CaM when bound to CaMKII would not protect TT305/306 (α) from being phosphorylated causing inability of CaM to bind. However, we cannot exclude that a faster off-rate of D130G CaM compared to wt CaM plays a role in the observed changes of the phosphorylation of the inhibitory phosphosite. Structural analysis of the D130G CaM in complex with CaMKII combined with binding studies are expected to reveal insights into how the compromised C-terminal structure of D130G CaM affects the affinity of the N-terminal lobe of this protein compared to the wt CaM.

The linker region of CaMKII connecting the kinase hub regulatory segment with the hub domain is intrinsically disordered and most divergent in length and sequence in the four different CaMKII isoforms with totally 40 splice forms (reviewed in [36]). However, the linker domain is conserved among the same isoforms in different species. It has been shown that the length of the linker region plays a prominent role in the balance between activating (Thr286α) and inactivating (Thr305/306) CaMKII functions [39], and Wang et al. [40] found that besides dephosphorylating TT305/306 (α), as mentioned above, also T337 can be dephosphorylated by the phosphatase and tensin homolog PTEN and may contribute in modulating the function of CaMKIIα. However, it remains unclear how phosphorylation of the linker phosphosites is involved in the fine-tuning of the CaMKII holoenzyme. In addition to the changed patterns of the TT305/306 (α) phosphorylation, we observed changes in the degree of phosphorylation of a number of phosphosites with previously undescribed function including S272/T276/S279 near the boundary of the kinase/regulatory domains and S330/T331/S333/S334/T336/T337 in the linker region. Whereas the former sites were phosphorylated to a lower degree in the D130G CaM compared to the wt CaM the latter showed the inverse characteristic (summarized in Fig. 2d). Interpretation of these findings is difficult in the absence of structural information. However, these changes could be explained by the differences in binding affinities between wt and D130G CaM to CaMKII that would allow lower degree of phosphorylation of the phosphosites near the activation sites and higher degree of phosphorylation of the linker phosphosites in the presence of
D130G CaM depending on accessibility.

We found that the Ca$^{2+}$ sensitivity of the CaMKII sensor was changed by inactivating these phosphosites by alanine mutations. This feature could be reversed close to wt levels by phosphomimetic mutants indicating that the CaMKII linker region phosphosites are phosphorylated under the conditions used when assaying the cellular lysates (Fig. 2e). These data can be difficult to interpret since the structural effects these mutations may infer on the Camui are not known, but this adds for the first time a possible functional relevance to these sites. In support of the potential importance of these sites we also show that they are evolutionarily conserved. In our previous study we showed that even though D130G CaM binds to CaMKII, as analysed by FRET based CaM binding to the enzyme, its activity is markedly reduced compared to wt CaM. Full stimulation requires T286 phosphorylation, which could further extend the structure as proposed by Bhattacharyya et al. [41] or may even result in subunit exchange as proposed by Stratton et al. [42]. Since the activation of CaMKII and specifically the transautophosphorylation of T286 in subunit exchange as proposed by Stratton et al. [42]. Since the activation of CaMKII and specifically the transautophosphorylation of T286 is dependent on the movement of the catalytic lobe of CaMKII [43] and furthermore, that this movement and in extension the activity of CaMKII is dependent on the length [44] and possibly also flexibility of the linker region, we propose that the observed change in Ca$^{2+}$ sensitivity may reflect changes in steric freedom of the catalytic CaMKII domain respective to the hub domain. In order to further investigate this possibility, it would be necessary to establish potential binding pockets on either the catalytic and/or hub domains where the linker region may dock in its nonphosphorylated versus phosphorylated state. It is worth mentioning that phosphorylation of phosphosites in the linker region may lead to the induction of alpha helices in disordered regions leading to linker length reductions shown for NHE1 [45] and RYR2 [46].

We show that the D130G mutation affects the beating pattern of cardiomyocyte-like cells by replacing wt CALM2 with D130G CALM2 in the mouse P19CL6 cell line, which were differentiated into cardiomyocyte-like cells. This model cannot directly be compared to the situation of diseased human cardiomyocytes as we replaced both CALM2 alleles with the mutant version whereas only one allele was found to harbour this mutation in a patient causing arrhythmia. However, it shows that a strong effect on cardiomyocyte physiology was caused by the D130G mutation, which was present only as a minor part (approx. 30%) of the total CaM concentration. These findings are in line with our previous observations in zebrafish pointing to a defect in heart rhythm caused by the D130G CaM mutation [19]. In addition, we could also use this cellular model system to confirm what we found by in vitro experiments that interference with the CaMKII function by the presence of the D130G CaM mutation may be connected with hyperphosphorylation of the CaMKII inhibitory phosphorylation sites. However, these experiments do not prove that misfunction of CaMKII, caused by the presence of D130G CaM mediates, the observed effect on the beating phenotype of cardiomyocyte-like P19 cells. To support and extend our results, further studies including CaM versions not causing alterations of cardiomyocyte beating as controls, and importantly also CaMKII mutants with altered phosphorylation sites expressed in differentiated cells would have to be carried out in the future.

We do not claim that inhibiting or eliminating CaMKII activity is the direct or main cause of LQTS as actually inhibiting this enzyme can improve the LQTS condition. However, it could potentially contribute to heart arrhythmia as it is essential for the heart function. It is well documented that D130G CaM affects several targets including the RyR [47] and the L-type Ca$^{2+}$ channels [14], the latter shown by CRISPR interference-mediated suppression of D130G expression to be responsible for alterations of the action potential duration in induced human pluripotent stem cells harbouring this mutation [48]. Therefore, these two channels orchestrating the Ca$^{2+}$ handling or other ion channels may be the main players leading to arrhythmia in the presence of D130G CaM.

4. Materials and methods

4.1. DNA constructs, oligos for CRISPR/Cas9 and CaM expression analysis

The gRNA sequence was designed to induce a double strand break in the CALM2 gene, and point mutations were incorporated using a single stranded 80 nucleotide donor for homology directed repair (HDR) (Supplementary material Fig. S2.). Unwanted Cas9 activity at the target site after donor based HDR was prevented by silent mutations in the donor sequence at the gRNA binding site. The desired D130G codon exchange was made by an A to G mutation at nucleotide position 389 in the Asp codon of the coding region. An AluI restriction site was introduced by an A to T silent mutation creating an AGCT sequence upstream of the Asp codon enabling RFLP selection. Successful knock-in was verified by sequencing of CALM2 cDNA (not shown) as well as protein analysis exploiting the change in electrophoretic mobility of D130G CaM to separate the mutated protein from wt CaM.

Short guide oligos (upper and lower) were designed using the Zhang Lab CRISPR design tool (http://crispr.mit.edu), and cloned into the Addgene plasmid pSpCas9(BB)-2A-Puro (PX459) (Cat. #48139) for Cas9 and gRNA expression in transfected cells.

**Upper 5′-CAGCGAGGTCAAGTTAAGTACGA Lower 5′-AAACACTCGTAGTTACCTGACCCTC**

Sequence of ssODN HDR template (HPLC purified): 5′GATGATCAGGGAAGCTGCATTGACGGGGATGGGCAAGTTAATTGATGAGTCACAAGGGCAGTCTCACTCGCTCAAGTTGACGGGGATGGGCAAGTTAATTGATGAGTCACAAGGGCAGTCTCACTCGCTCA. Western blotting was done using the following Antibodies:

- #1- CaM recognising N-terminal part of CaM: Sigma Aldrich Cat# 05-173, #2-CaM recognising C-terminal part of CaM: Abcam Cat# ab124742 (clone EPR5028)
- GATA4: Santa Cruz-1237, SOX2: RD systems Mab 2018.

Mutant Camui constructs were made by PCR technique using the Camui vector containing full length CaMKIIa kindly provided by Yasunori Hayashi [49] as template. Expression and purification of wt and mutant CaM was done as described in [19].

4.2. Differentiation of P19CL6 cells to beating phenotype

The P19CL6 cell line was obtained from Søren T. Christensen, Department of Biology, University of Copenhagen. It is of mouse origin and isolated from embryonal carcinoma tissue. The originator is Habara, Akemi and registered with Murofushi, Kimiko, Japan (ref #24063467). Cells were grown in MEM alpha medium (Gibco, Cat. #22561) supplemented with 10% PBS (Biochrom AG) and 1% penicillin/streptomycin (P/S). For cardiac differentiation cells were seeded at a density of 10^3/ cm² in 6-well plates in their normal medium supplemented with 1% DMSO with a starting volume of 2 ml. Medium was changed every second day with increasing volumes as such, day 2: 2 ml, day 4: 4 ml, day 6: 5 ml, day 8: 7 ml. Wt and D130G harbouring cells were grown in parallel in two separate plates. At day 8 plates were removed from the incubator in succession, and short videos of beating clusters for each cell type were recorded for analysis.

4.3. Electroporation

15 x 10^6 cells were suspended in 400 µl PBS and transferred to a 0.4 mm cuvette on ice. 20 µg plasmid and 20 µg ssODN were added to the
cuvette and gently mixed. The cuvette was kept on ice for 5 min before electroporation with the Gene Pulser II (BIORAD) system using settings of 260 V, 1050 μF, 0 Ω resistance (resulting in a pulse duration of ~33 ms). The cuvette was kept on ice for 10 min before being transferred to a T75 flask containing 15 ml growth medium without antibiotics. The cells were incubated for 24 h in 5% CO₂ humidified atmosphere before Puromycin dihydrochloride (SIGMA) at a final concentration of 2.2 μg/ml was added. Puromycin selection was performed for 24 h, after which the medium was replaced.

4.4. Cell cloning and screening of single clones

Cells were grown to 80% confluency before being seeded at 0.3 cells/well in 96 well plates. DNA was extracted by boiling cell pellets in 50 mM NaOH for 20 min and then neutralized by one volume 1 M Tris pH 7.5. Digestion with AluI (Thermo Scientific Cat. #ER0011) was then performed on DNA from PCR reactions with primers flanking the area of HDR. Sanger sequencing was used to confirm the presence of the D130G replacement in clones with the desired AluI restriction fragment pattern. Correct expression of the gene was confirmed by sequencing the reverse transcribed cDNA using primers up- and downstream of the amplified region.

4.5. In vitro Camui assay

Structural changes of CaMKII induced by binding of CaM were measured by FRET between CFP (donor) and YFP (acceptor) fused to each end of the CaMKIIα protein as described in [18]. The N- and C-termini of CaMKIIα are in close proximity in the inactive CaMKII allowing FRET, which is diminished when CaM is bound. CFP and YFP fluorescence from HeLa cell lysates in 384 well plates were measured using a Tecan infinite F200 Pro (Thermo Fisher Scientific) plate reader. Measurements were done in the presence of 40 μM ATP. Camui was activated by addition of 1μM CaM (wt or D130G). Ca²⁺ concentrations (after addition of CaCl₂) and Camui kinetics measurements were calculated as described in [18].

4.6. Live Camui assay

P19CL6 cells were grown as described above and seeded in Cell-Carrier Ultra 96 well plates (Perkin Elmer) the day before assaying in the presence of Camui DNA plasmid variants/L-PEI complexes. The assays were done in an OPERA high content screening system equipped with a live cell chamber and a dispenser unit. Cells were kept at 5% CO₂ and 37 °C during the experiments and ionomycin was added in a single jet of 20 μl. A Camui construct with GFP (donor) and RFP (acceptor) fluorophores was expressed in adherent P19CL6 cells and visualized by a single laser at 488 nm and the emission light was picked up after dichroic filtering (568 short pass) by using two cameras and bandpass filters 520/35 (GFP) and 600/40 (RFP). Automated image analysis was performed using the Columbus server tools.

4.7. Stimulation of CaMKIIδ/α autophosphorylation

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise stated. Protein concentration was determined by amino acid composition analysis using a Biochrom 30 amino acid analyser (Biochrom, Cambridge, UK). CaMKδ/α autophosphorylation was induced by adding commercially acquired recombinant human CaMKIIδ/α, generated in the baculovirus expression system with 85–90% purity according to the company’s certification (1.5 μg in 0.5 mM EDTA; Life technologies, Carlsbad, CA, USA) 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 (EMD Millipore, Calbiochem, Fisher Scientific) and 10 μg of wt CaM or D130G CaM respectively. Stimulation of CaMKIIδ/α was performed for 2.5 min or 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 (mass spectrometry-grade, FUJIFILM Wako Chemicals, Neuess, Germany) to the mix, followed by 1 h incubation at room temperature (RT). Lys-C generated peptides were alkylated in 20 mM iodoacetamide for 30 min at RT in the dark. Subsequently, samples were digested with trypsin (sequence-grade; Promega, Madison, WI, USA) at an enzyme to substrate ratio of approximately 1:50 for 12 h at 37 °C. The samples were acidified to 5% formic acid and desalted using Poros Oligo R3 HP (PerSeptive Biosystems, Framingham, MA, USA) column packed in a P200 stage tip with C18 3 M plug (3 M Bioanalytical Technologies, St. Paul, MN, USA). The purified peptide samples were dried by lyophilization and labelled using isobaric tagging labelling (iTRAQ 4-plex; Applied Biosystems, Foster City, CA) as described by the manufacturer (iTRAQ-114, wt CaM 2.5 min; iTRAQ-115, wt CaM 15 min; iTRAQ-116, D130GCaM- 2.5 min; iTRAQ-117, CaM-D130G 15 min). After labelling, all samples were pooled into one tube and dried by vacuum centrifugation to 50 μl for phosphopeptide enrichment by titanium dioxide (TiO₂) beads (GL Sciences Inc., Tokyo, Japan). The TiO₂ enrichment of phosphopeptides was performed essentially as previously described [50,51]. Prior nano liquid chromatography-tandem mass spectrometry (nanoLC-MS/MS) analysis, the phosphorylated peptide samples were resuspended in 0.1% TFA and desalted using Poros Oligo R3 HP column packed in a P200 stage tip with C18 3 M plug. Purified phosphopeptide samples were dried by lyophilization and stored at -80 °C until further analysis.

4.8. NanoLC-MS/MS analysis

The peptide mixture was separated using a Dionex Ultimate 3000 RSLC high-performance liquid chromatography (HPLC) system (Thermo Scientific) equipped with a two-column system, consisting of a commercial Acclaim PepMap100 trap column (C18, 5 μm, 100 Å, 300 μm i.d. × 5 mm, Thermo Scientific) and an in-house packed reversed-phase analytical column (75 μm i.d. × 18 cm Reprosil-Pur C18-AQ; 3 μm; Dr. Maisch GmbH, Germany). The injection volume was set to 10 μl. Loading buffer was 0.1% trifluoroacetic acid (Merck Millipore) in water. The HPLC gradient was 0–34% solvent B (A: 0.1% formic acid; B: 95% acetonitrile, 0.1% formic acid) over 10 min and from 34% to 100% solvent B in 5 min at a flow-rate of 250 nl/min. The HPLC was coupled with a Q-Exactive Plus mass spectrometer (ThermoFisher Scientific, Bremen, Germany). Nanoelectrospray was used with a spray voltage of 2.5 kV. The ion transfer tube temperature was 275 °C. The instrument was used in data-dependent mode. Acquisition was performed for MS with a mass resolution of 70,000 (full-width at half-height) with an auto gain control (AGC) target of 1 × 10⁶ and a maximum injection time of 120 ms. MS m/z acquisition window was set at 300–1400. Precursor charge states 1+ and unassigned were excluded. MS/MS acquisition was performed with a resolution of 17,500, a target value of 2 × 10⁶ and a maximum injection time of 100 ms. The 10 most intense ions over a threshold of 10,000 counts were isolated for fragmentation with normalized collision energy (NCE) of 31. The dynamic exclusion time was 3 s.

4.9. MS-data processing and database searching

The raw mass spectrometer files were processed for protein identification using the Proteome Discoverer v1.4 (ThermoFisher Scientific). All peak lists were searched against the UniProt/Swiss-Prot Human CaMKIIα and CaMKIIδ database (version March 2016) using an in-house MASCOT server v2.5 (Matrix Science Ltd., London, UK). Database searching was performed with the following parameters: enzyme, trypsin; maximum missed cleavages, 2; peptide mass tolerance of 10 ppm; fragment m/z tolerance of 0.05 Da; fixed modification, cysteine carbamidomethylation, N-terminal and lysine iTRAQ; variable modifications, methionine oxidation, serine/threonine/tyro
phosphorylation, asparagine and glutamine deamidation. Only peptides with a false discovery rate (FDR) < 1% (Percolator), MascOT rank 1, search engine 1, and a cut-off value of Mascot score ≥ 20 were considered for further analysis. PhosphoRS score was used to determine the phosphosite localization probability. iTRAQ quantification was performed using Proteome Discoverer with reporter ion area integration within a 50 ppm window. In our study we used isotopic labelling techniques iTRAQ (isobaric tags for relative and absolute quantification) [52] which allows for relative quantification of peptides based on ratios of reporter ions in the low m/z region of spectra produced by precursor ion fragmentation. In contrast to label-free approaches, multiplexed isotopically labelled samples can be simultaneously analysed resulting in increased reproducibility and accuracy for quantification of peptides and proteins from different biological states. Statistical analyses were performed on three different biological replicates. The sample size was chosen to provide enough statistical power to apply parametric tests (paired; one- or two-tailed homoscedastic t-test). The t-test was considered as valuable statistical test because binary comparisons were performed, and the number of replicates was limited. Proteins with t-test p value smaller than 0.05 were considered as significantly altered between the two tested conditions. The ratio values between two tested conditions were shown independently for each replicate if phosphopeptide was not identified in all three biological replicates. Data distribution was assumed to be normal, but this was not formally tested.

CRediT authorship contribution statement

MWB planned and supervised the experiments and wrote the article; MM did the CRSPR/Cas9 work, the p191 beating experiments and made the figures; KK did the proteomics work; IP contributed to the work with p19 cells; LL did proteomics work and bioinformatics; TZ did in vitro CaMKII assays; SP contributed to the plasmid constructions and with intellectual input, MRL supervised the proteomics work and JMC planned and supervised the project.

Declaration of competing interest

Authors declare that no competing interests exist.

Acknowledgement

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Funding

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbamcr.2021.119119.

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