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Altered RyR2 regulation by the calmodulin F90L mutation associated with idiopathic ventricular fibrillation and early sudden cardiac death

Michail Nomikos [a,*], Angelos Thanassoulas [b], Konrad Beck [c], Vyronia Vassilakopoulou [a,b], Handan Hu [a], Brian L. Calver [a], Maria Theodoridou [a,b], Junaid Kashir [a], Lynda Blayney [a], Evangelia Livaniou [b], Pierre Rizkallah [a], George Nounesis [b], F. Anthony Lai [a]

[a] Wales Heart Research Institute, Cardiff University School of Medicine, Institute of Molecular and Experimental Medicine, Cardiff CF14 4XN, UK
[b] National Center for Scientific Research "Demokritos", 15310 Aghia Paraskevi, Greece
[c] School of Dentistry, Cardiff University, Cardiff CF14 4XY, UK

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ABSTRACT

Calmodulin (CaM) association with the cardiac muscle ryanodine receptor (RyR2) regulates excitation–contraction coupling. Defective CaM–RyR2 interaction is associated with heart failure. A novel CaM mutation (CaM^{F90L}) was recently identified in a family with idiopathic ventricular fibrillation (IVF) and early onset sudden cardiac death. We report the first biochemical characterization of CaM^{F90L}. F90L confers a deleterious effect on protein stability. Ca^{2+}-binding studies reveal reduced Ca^{2+}-binding affinity and a loss of co-operativity. Moreover, CaM^{F90L} displays reduced RyR2 interaction and defective modulation of [^{3}H]ryanodine binding. Hence, dysregulation of RyR2-mediated Ca^{2+} release via aberrant CaM^{F90L}–RyR2 interaction is a potential mechanism that underlies familial IVF.

Structured summary of protein interactions:
RyR2 physically interacts with **CaM** by pull down (View interaction)

1. Introduction

Calmodulin (CaM) is a small cytoplasmic Ca^{2+}-binding protein that regulates numerous cellular functions. CaM contains four highly conserved Ca^{2+}-binding EF hand motifs that each binds one Ca^{2+} ion. The four EF hand motifs exist in two pairs and are connected by a flexible linker [1]. In cardiac muscle, CaM modulates directly or indirectly the activity of proteins that play a key role in excitation–contraction coupling (ECC), and in particular, those responsible for the release and subsequent sequestration of cytosolic Ca^{2+} into the sarcoplasmic reticulum (SR). The main binding partner of CaM in cardiac cells is the RyR2, which regulates Ca^{2+} release from the SR. CaM binds to RyR2 stoichiometrically (1 CaM per subunit of the homotetrameric RyR2). The CaM binding domain is believed to reside within residues 3583–3603 of the RyR2 [2–4]. It has been shown that CaM inhibits RyR2 channel open probability at nM to mM Ca^{2+} concentrations [4]. Defective CaM–RyR2 binding resulting in impaired CaM inhibition of RyR2 function has been widely implicated in cardiac pathology. Decreased or abolished CaM binding to RyR2 has been associated with defective SR Ca^{2+} release and altered gene expression, leading to cardiac hypertrophy and early death in animal models [5]. Moreover, defective CaM binding to RyR2 has been linked with catecholaminergic polymorphic ventricular tachycardia (CPVT)-associated RyR2 dysfunction [6].

Recent genetic studies have identified five missense CaM mutations associated with severe ventricular arrhythmia and sudden cardiac death susceptibility [7,8]. Two CaM mutations were associated with stress-induced polymorphic ventricular tachycardia reminiscent of CPVT (CPVT-CaMs) [7], whereas the other three mutations led to recurrent cardiac arrest in infancy associated with severe QT prolongation reminiscent of a long QT syndrome

Abbreviations: CaM, calmodulin; RyR2, cardiac ryanodine receptor; ECC, excitation–contraction coupling; IVF, idiopathic ventricular fibrillation; SCD, sudden cardiac death; CPVT, catecholaminergic polymorphic ventricular tachycardia; CD, circular dichroism; LQTS, long QT syndrome

* Corresponding author. Address: Wales Heart Research Institute, Cardiff University School of Medicine, Heath Park, Cardiff CF14 4XN, UK. Fax: +44 29 20743500.
E-mail address: mixosn@yahoo.com (M. Nomikos).

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(LQTS-CaMs) [8]. Functional characterization of these mutations revealed that the CPVT-CaM mutations caused higher RyR2 single channel open probability and showed enhanced binding affinity to RyR2. In contrast, LQTS-CaMs did not promote Ca^{2+} waves and exhibited either normal regulation of RyR2 single channels or lower RyR2 binding affinity [9].

Another recent study has reported a CaM mutation (Phe90Leu; CaM^{F90L}) as the causative genetic defect in a family of Moroccan descent presenting with idiopathic ventricular fibrillation (IVF) in childhood and adolescence [10]. IVF is a rare cardiac rhythm disorder, characterized by VF in the absence of electrocardiographic or structural heart abnormalities. IVF represents a major diagnostic challenge as it is difficult to track genetically because the affected status of an individual only becomes apparent following an arrhythmic event [10]. CaM^{F90L} has not been characterized and the mechanism describing how it causes IVF is unknown.

Here we report that the CaM^{F90L} mutation fundamentally alters the protein's biophysical and biochemical properties. Thermal circular dichroism (CD) studies reveal that in the presence of Ca^{2+}, CaM^{F90L} displays considerably lower stability than CaM^{WT}, indicating a loss in co-operativity of unfolding. This result is consistent with chemical denaturation experiments showing that the CaM^{F90L} mutation causes a destabilization of the C-domain, resulting in a significantly reduced C-domain Ca^{2+} affinity and a total loss of co-operativity between the Ca^{2+}-binding sites in this domain. Native RyR2 co-immunoprecipitation with recombinant CaM^{WT} and CaM^{F90L} revealed that the F90L mutation impairs CaM–RyR2 interaction. This is compatible with [^{3}H]ryanodine binding studies which suggest that CaM^{F90L} loses its ability to inhibit the RyR2 channel at a specific range of Ca^{2+} concentrations. These observations imply that CaM^{F90L} may cause IVF due to a defective interaction with RyR2 leading to dysregulated SR Ca^{2+} release.

2. Material and methods

A detailed description of methods is provided in the Supplementary Materials section.

3. Results and discussion

CaM^{WT} and CaM^{F90L} were cloned into the pHSIE plasmid vector which expresses a fusion protein containing an N-terminal 6×His-tag, followed by a SUMO2-tag, the protease intein and an intein cleavage site. This enables the auto-cleavage of the fusion tags during the one-step purification protocol detailed in Material and Methods (see Supplementary Materials) [11]. Fig. 1A shows the affinity-purified recombinant CaM proteins analyzed by SDS–PAGE and anti-CaM immunoblot analysis with the ~17.4 kDa predicted molecular mass for CaM^{WT} and CaM^{F90L} observed in both the gel (left-hand panel) and immunoblot (right-hand panel).

CD spectra of both CaM^{WT} and CaM^{F90L} recorded at low temperature are nearly identical and in very good agreement with previously published spectra [12] confirming proper protein folding (Fig. 1B). Ca^{2+} binding results in an increase in the CD amplitude indicating an increase in the α-helical content and a decrease in the ratio of $\Delta\varepsilon_{208nm}/\Delta\varepsilon_{221nm}$ (Fig. 1B). In the absence of Ca^{2+}, both proteins show very similar thermal denaturation behavior with melting temperatures (T_m) of 53.8 ± 0.2 and 58.0 ± 0.2 °C, and van't Hoff enthalpies (ΔH_{vH}) of −112 ± 1.8 and −126 ± 2.9 kJ/mol for the CaM^{WT} and CaM^{F90L}, respectively (Fig. 1B). These values agree well with those reported for CaM^{WT} previously [13]. Addition of Ca^{2+} has a stabilizing effect resulting in an increased T_m, estimated at 105 ± 0.2 °C and ΔH_{vH} = −105 ± 1.6 kJ/mol for CaM^{WT}. In contrast, CaM^{F90L} showed considerably lower thermostability with T_m = 87.3 ± 1.5 °C and ΔH_{vH} = −33 ± 0.4 kJ/mol, indicating a loss in the co-operativity of unfolding.

To further investigate the biophysical impact of the F90L mutation we studied the chemically-induced unfolding of both CaM^{WT} and CaM^{F90L} by monitoring their tyrosine fluorescence emission intensity (λ_{ex} = 277) at 320 nm. Since the fluorescence emissions from the N-domain is practically zero at this excitation wavelength [14], the collected data reflect only the thermodynamic stability of the C-domain. Fig. 1C shows fluorescence data for the GuHCl-induced denaturation of both protein samples. Table 1 (Supplementary Material section) summarizes the results of a two-state model nonlinear least-squares fit to the experimental data. These data indicate that the F90L mutation has a negative effect on the protein stability as it destabilizes the C-domain by almost 60% ($\Delta\Delta G$ = 3.0 kJ/mol) at 25 °C.

To understand the biochemical impact of the F90L mutation on the Ca^{2+}-binding properties of CaM^{WT}, we performed in vitro Ca^{2+} binding studies by monitoring the intrinsic fluorescence. VanScyoc et al. [14,15] previously demonstrated that Ca^{2+}-dependent changes in Phe fluorescence specifically indicate ion occupancy within the N-domain, as Phe residues in the C-domain are non-emissive. In a similar fashion, they proved that Tyr emission from the C-domain does not interfere with Phe fluorescence signals from the N-domain, allowing selective insight to the Ca^{2+}-binding properties of each of the four sites within CaM. Using 277 nm excitation/320 nm emission to monitor Tyr fluorescence, we determined that both CaM^{WT} and CaM^{F90L} exhibited an increase in fluorescence intensity as a function of free Ca^{2+} concentration (Fig. 2A,I). The situation is reverted when using 250 nm excitation/280 nm emission for selective Phe fluorescence. In this case, the fluorescence intensity decreases with increasing free Ca^{2+} concentration (Fig. 2A,II). Averaged binding data from three independent titrations following nonlinear least-squares fitting are summarized in Table 2 (Supplementary Material section). For CaM^{WT}, C-domain binding sites have an affinity for Ca^{2+} that is ~3-fold higher than that of the N-domain binding sites (apparent K_d of 2.9 ± 0.1 and 7.2 ± 0.2 μM, respectively, Table 2, Supplementary Material section). The free energy change accompanying binding of two Ca^{2+} ions at the C-domain binding sites was found to be −63.18 ± 0.42 kJ/mol with a co-operative free energy of −5.52 ± 0.92 kJ/mol, while the corresponding values for the N-domain binding sites were −58.58 ± 0.42 and −5.94 ± 0.92 kJ/mol, respectively. These calculations are in good agreement with earlier studies under the same experimental conditions [8,13]. For CaM^{F90L}, the N-domain binding sites appear to have higher Ca^{2+} affinity than their C-domain counterparts (13.1 ± 0.3 and 24.1 ± 1.5 μM respectively), as a result of the close proximity of the Phe90 residue to the C-domain active sites. Examining the C-domain Ca^{2+} binding energetics of CaM^{WT} and the CaM^{F90L} mutant, it is evident that the main difference lies with the free energy change associated with the capture of a second Ca^{2+} while the first site is already occupied ($\Delta G^{site\ 2}$ = −63.18 ± 0.42 kJ/mol *vs.* −52.72 ± 0.42 kJ/mol for CaM^{WT} vs. CaM^{F90L}) and the co-operative free energy of the two binding sites (ΔG^{coop} = −5.52 ± 0.92 kJ/mol for CaM^{WT} *vs.* −0.42 ± 0.21 kJ/mol for CaM^{F90L}). Working under the assumption of equivalency for the two Ca^{2+} binding sites, these data taken together suggest an almost complete loss of co-operativity for CaM^{F90L} at the C-domain. The F90L mutation most likely disrupts the relative position of the two binding sites, thus interfering with the ability of the first occupied site to induce conformational changes that facilitate binding to the second site. Co-operative binding is very important for CaM, as it produces a narrow Ca^{2+} concentration range for the protein to switch from the apo- to the holo- form, whereas having active protein over a broad Ca^{2+} concentration range makes it less efficient as a biological Ca^{2+} sensor. The combined effect of decreased domain stability and loss

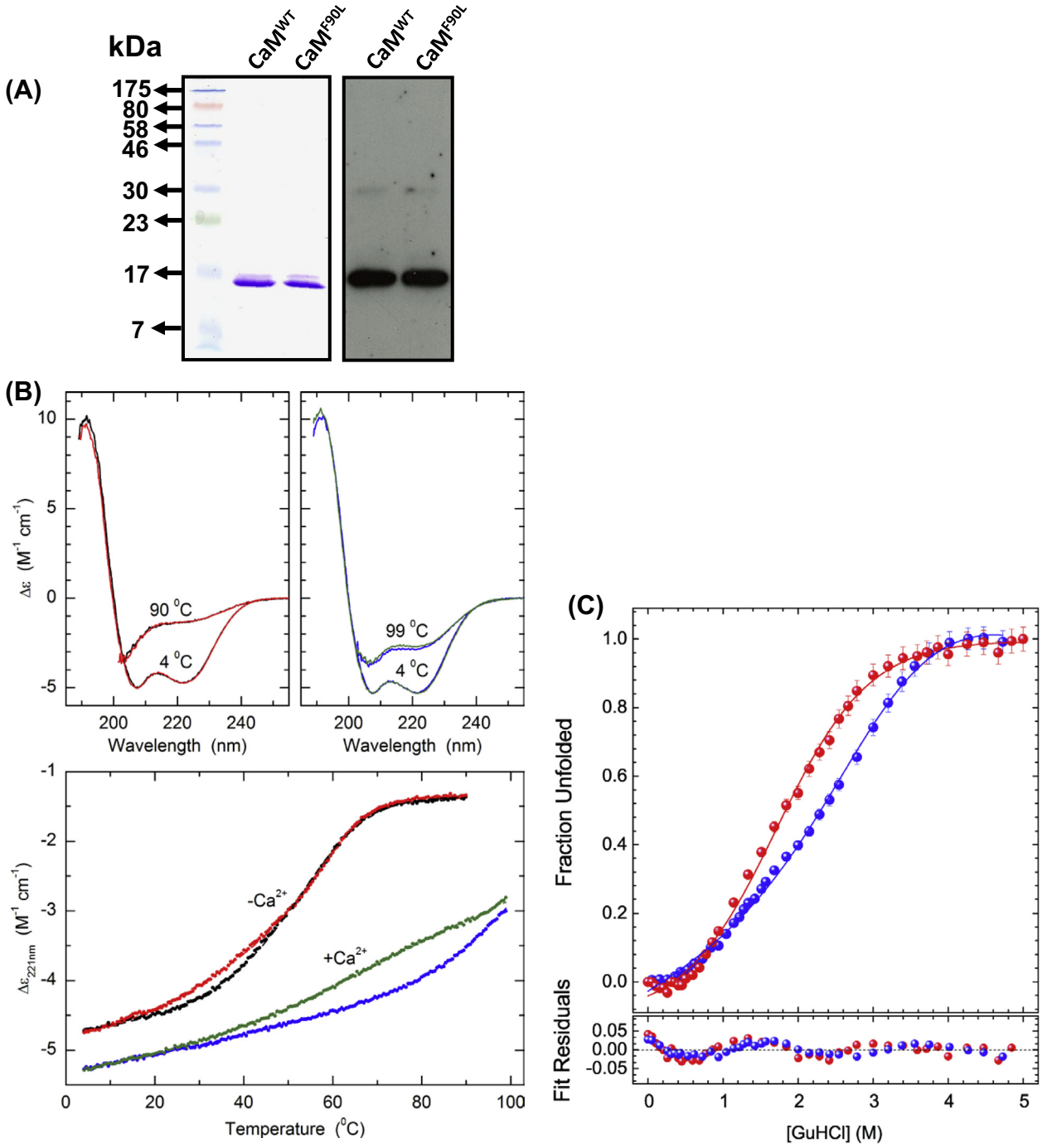

Fig. 1. (A) Expression and purification of recombinant CaM^{WT} and CaM^{F90L} proteins. Affinity-purified proteins (1 μg) were analyzed by 15% SDS–PAGE followed by either Coomassie Brilliant Blue staining (left panel) or immunoblot analysis using an anti-CaM rabbit monoclonal antibody (1:7500 dilution); (right panel). (B) CD spectra of CaM^{WT} (black, blue) and the CaM^{F90L} mutant (red, green) are shown in the absence (upper left panel) and presence (upper right panel) of Ca^{2+} recorded at low and high temperature, as indicated. Thermal denaturation curves measured at 221 nm are shown in the lower panel. (C) Chemical denaturation profiles, induced by GuHCl under equilibrium conditions at 25 °C, of CaM^{WT} (●) and CaM^{F90L} mutant (●) in apo-buffer. Solid lines represent the nonlinear least-squares fit of a two-state unfolding model to each set of experimental data (upper panel). Scatter plot of residuals generated by the curve-fitting procedure (lower panel).

of co-operativity between binding sites in the C-domain could have a profound impact on the biological functions of the CaM^{F90L} mutant.

RyR2 is the major binding partner for CaM along the Z-line in cardiomyocytes, and aberrant CaM dissociation from RyR2 can cause heart failure. In heart failure, RyR2 shows decreased interaction with CaM but unaltered FKBP12.6 affinity [4]. To compare the relative RyR2-binding affinities of recombinant CaM^{WT} and CaM^{F90L} we used a co-immunoprecipitation assay as detailed in Material and Methods (see Supplementary Material section). Native RyR2 from pig cardiac SR was immunoprecipitated with an RyR2-isoform-specific antibody (Ab^{1093}) in the presence of either recombinant CaM^{WT} or CaM^{F90L}. Association of CaM^{WT} and CaM^{F90L} with RyR2 was analyzed by SDS–PAGE and immunoblot detection using an anti-CaM antibody. Densitometric analysis showed that CaM^{F90L} had a ~50% reduced RyR2-binding compared to CaM^{WT} in the absence of Ca^{2+} and ~30% reduced RyR2-binding affinity in the presence of either 10 or 100 μM free [Ca^{2+}] (Fig. 2B). The level of endogenous CaM

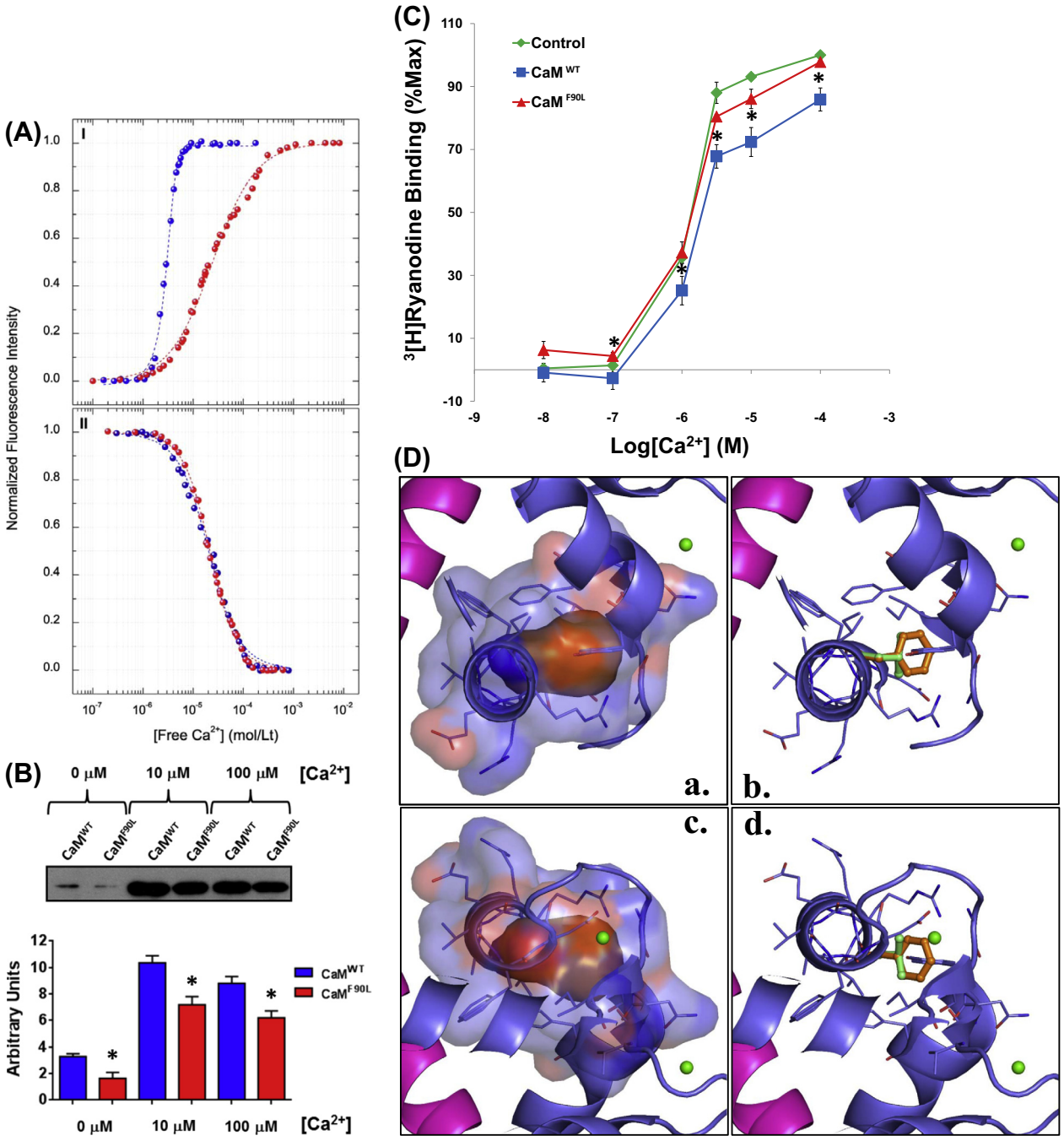

Fig. 2. (A) Ca^{2+} titration profiles of CaM^{WT} (●) and CaM^{F90L} mutant (●) in apo-buffer, monitored at two pairs of wavelength: 277 nm excitation/320 nm emission (I) and 250 nm excitation/280 nm emission (II). Blue and red dashed lines represent the nonlinear least-squares fit of a two-site, model-independent Adair function for the CaM wild-type and F90L mutant data, respectively. (B) Co-immunoprecipitation assays showing the association of CaM^{WT} and CaM^{F90L} mutant with cardiac RyR2. Native RyR2 was immunoprecipitated with Ab^{1093} from CHAPS-solubilised cardiac SR in the presence of 1 μM of exogenous CaM^{WT} or CaM^{F90L} at different Ca^{2+} concentrations (0, 10 & 100 μM). The presence of RyR2-coprecipitated CaM^{WT} and CaM^{F90L} mutant was analyzed by 18% SDS–PAGE followed by immunoblot analysis using an anti-CaM rabbit monoclonal antibody (1:7500 dilution); (upper panel). Densitometry analysis and normalization followed three independent experiments using three different pig cardiac SR preparations (lower panel), $^{*}P < 0.05$ (GraphPad, Prism 5). (C) Effect of CaM^{WT} and CaM^{F90L} on [^{3}H]ryanodine binding to cardiac SR vesicles. The basic binding buffer contained 50 mM HEPES, 25 mM Tris, 500 mM KCl, pH 7.4 with either 1 mM EGTA (less than 0.01 μM Ca^{2+}) or a series of free Ca^{2+} concentrations. At least 2 different cardiac SR vesicle preparations were used. Significant effects (asterisks) were calculated by comparison of each point using an unpaired Student's *t*-test. $n = 4 \pm$ S.E.M., $^{*}P < 0.05$, (GraphPad, Prism 5). (D) (a) Solid space-filling surface of residue F90 (orange) of CaM^{F90L} is shown completely engulfed inside the semi-transparent surface of residues with atoms that are within 4 Å distance of F90 (blue), shown as sticks. Figure is based on 1YR5. The rest of the protein is shown as cartoon. The Ca^{2+} atoms of the EF hand motifs are shown as green spheres. (b) This is the same as for (a) but after removing the surfaces. The side chain of F90 is shown in orange ball-and-stick, and the modelled side-chain of the Leu mutation is in green. (c, d) These panels correspond to (a, b) respectively, but with the view rotated 180° about the vertical axis (i.e. back view).

associated with the cardiac SR microsomes was also assessed by immunoblotting with the anti-CaM antibody. There was no native CaM detected using this approach, whereas blots containing either 10 or 25 ng of recombinant CaM was readily detected by this antibody (Supplementary Fig. 1).

The binding of [^{3}H]ryanodine to RyR2 depends on the functional state of the channel, and thus represents a useful tool to analyze the action of modulators of Ca^{2+} release channel function such as CaM. The effect of CaM^{WT} and CaM^{F90L} on [^{3}H]ryanodine binding to RyR2 was studied at a range of Ca^{2+} concentrations from

10 nM to 100 μM. As shown in Fig. 2C, CaM^{F90L} showed a ~65–85% reduced inhibition of ryanodine binding to RyR2 compared to CaM^{WT}. This result is consistent with the co-immunoprecipitation experiments that revealed impaired RyR2–CaM^{F90L} association (Fig. 2B) and suggest that defective CaM–RyR2 interaction leading to dysregulated SR Ca^{2+} release in cardiomyocytes might explain the molecular mechanism for how the F90L mutation leads to IVF and sudden cardiac death (SCD).

The F90L mutation is located within the inter-EF hand linker between the 2nd and 3rd EF hand domains. Fig. 2D shows the F90 surface, which fits inside an envelope formed by the neighbouring residues with at least one atom within 4 Å from F90. The 'front' and 'back' views provide a better perspective of the relative orientation. Also shown are the two views without the surfaces, emphasising the wrap-around disposition of the neighbouring residues. The Leu mutation, also shown in Fig. 2D-b,d highlights the 3 atom sites from the Phe side chain that cannot be filled by Leu. This vacancy may presumably affect the protein folding stability, as was shown by the decreased stability both by thermal and chemical denaturation (Fig. 1B and C). However, from the high stability shown by the CD measurements, complete protein misfolding is not apparent. Instead the decreased protein stability is consistent with local misfolding, as indicated by the hydrophobic packing of the F90 residue. Moreover, it could allow for a smaller gap between the two Ca^{2+} binding sites in the neighbouring EF motifs. A closer distance between the two motifs presents an altered topology for binding, and effects a disparate energy requirement for conformational change from the unbound conformation. These hypotheses for the F90L mutation in CaM would have to be underwritten with a high-resolution 3D crystal structure for CaM^{F90L} in due course.

There are 3 human CaM genes and patients with the F90L mutation are heterozygous, raising the issue of how the reduced affinity of a mutant CaM (1 of 6 copies) could affect RyR2 function. It is possible that a CaM mutant with lower RyR affinity may act in a dominant-negative fashion, as there are believed to be four CaM binding sites per RyR tetramer. Also, because the RyRs can form into 2D membrane clusters of interlinked oligomers, a single defective RyR (with only one mutant CaM bound) potentially could affect the function of a large number of RyRs within the 2D array. In addition, the relative expression of each of the CaM genes is not equal and this differential gene expression may play an important role. These important points require further study.

This is the first structure–function analysis providing an insight into the biochemical mechanism for how the F90L mutation might lead to IVF and SCD. Our results suggest that the F90L mutation in CaM results in altered Ca^{2+} binding which leads to reduced ability to interact with the RyR2 thus consequently producing dysregulated Ca^{2+} release. However, further studies of the effect of this CaM F90L mutant on single channel recordings of the human RyR2 functionally reconstituted into planar lipid bilayers, on cardiomyocyte intracellular Ca^{2+} release assays using fluorimetric Ca^{2+} imaging and the distinct possibility that the mutant CaM may also affect other CaM-regulated cardiac ion channels, Ca^{2+} ATPases and cytoplasmic protein targets such as, calcineurin & CaM-dependent protein kinases (CaMK) [16], all require further investigation.

Conflict of interest

None declared.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2014.07.007.

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