Supplementary Material

Materials and Methods

Plasmid construction

Human CaM (GenBank® accesion number AAD45181.1) in pHSIE plasmid[1, 2] was subjected to oligonucleotide-mediated, site-directed mutagenesis (QuikChange II; Stratagene) to generate the CaM^{E105A} mutant. Successful mutagenesis was confirmed by dideoxynucleotide sequencing (Applied Biosystems Big-Dye Ver 3.1 chemistry and model 3730 automated capillary DNA sequencer by DNA Sequencing & Services™). CaM^{WT} and CaM^{E105A} mutant were then amplified by PCR from pHSIE plasmid using Phusion polymerase (Thermo Fisher Scientific) and the appropriate primers to incorporate 5'-KpnI and 3'-NotI sites and then cloned into the pCR3 expression vector. The primers used for the amplification of the CaM constructs were: 5'-GGAAGGTACCATGGCTGATCAGCTGACCGAAG-3' (forward) and 5'-GCAAGCGGCCGCTCATTTTGCAGTCATCATCTGTAC-3' (reverse).

Zebrafish care and husbandry

Zebrafish (*Danio rerio*) wild-type line embryos were used in all experiments. Zebrafish were maintained in a recirculating aquaculture system (Pentair Aquatic Ecosystems). The setup of adult fish mating was conducted according to IACUC approved breeding protocol (#: QU-IACUC 039/2017), "*Zebrafish Maintenance and Breeding Practice at BRC Zebrafish Facility*". For the zebrafish experiments, fertilized eggs were collected immediately in N-phenylthiourea (PTU) media and embryos of the same spawns were used for microinjection to ensure validity of data and statistical analysis. All experimental protocols involving zebrafish were performed in accordance with international legislation and national guidelines required by Qatar University for the use of laboratory animal and according to the Policy on Zebrafish Research established by Department of Research in the Ministry of Public Health, Qatar.

cRNA synthesis and cRNA microinjection experiments in zebrafish embryos

For complementary RNA (cRNA) microinjection experiments in zebrafish embryos, pCR3-CaM constructs were linearized and the respective CaM cRNA was synthesized by the mMessage Machine T7 kit (Thermo Fisher Scientific) and then was polyadenylated using the poly(A) tailing kit (Thermo Fisher Scientific), as per manufacturer's instructions.

Zebrafish embryos were injected at 1-2 cell stages with approximately 2 nl of 60 ng/µl cRNA under Zeiss Stemi 2000-C stereomicroscope. Comparable CaM^{WT} and CaM^{E105A} protein expression in the zebrafish embryos was confirmed by western blotting using an anti-CaM antibody (1:5,000 dilution; Source Bioscience). For our experiments an uninjected zebrafish group was also included. Embryos were incubated at 28 °C for further phenotypic examination up to 72 hours post-fertilization (hpf).

Imaging and phenotypic analysis

At 24 hpf, embryos were dechorionated using 1 mg/ml of pronase and evaluated every 24 hours for lethality, developmental or morphological deformities. Zebrafish different groups were mounted in 3% methyl cellulose and visualized under Zeiss Stereo Lumar. V12 microscope to detect any abnormal effect (snaps captured at 25X by DMK 22BUC03 Y800 camera). For cardiac examination, video recordings of total time of 6 seconds, at 15 frames per second, were performed for individual zebrafish larvae at magnification of 100X. The individual heart rate, was calculated by DanioScope software (Noldus, The Netherlands) as heart beats per minute and confirmed by counting beats manually using VLC media player. All examined larvae were euthanized by exposure to tricaine methanesulfonate overdose and collected for later western blot analysis.

Protein expression and purification

For 6xHis-SUMO2-intein-CaM-fusion protein expression, *Escherichia coli* [BL21-CodonPlus(DE3)-RILP; Stratagene], transformed with the appropriate pHSIE-CaM plasmid, was cultured at 37 °C until the A_{600} nm reached 0.6. Protein expression was induced for 18 h at 16°C with 0.2 mM IPTG (isopropyl β-D-thiogalactopyranoside), (ForMedium). Bacterial cell pellets were harvested by centrifugation at 6,000 g for 15 minutes at 4°C. Recombinant CaM proteins were then purified as previously described.[2]

Circular Dichroism (CD) spectroscopy and thermal stability analysis

Folding and stability of CaM^{WT} and CaM^{E105A} recombinant proteins were assessed by CD spectroscopy as previously described.[1] Briefly, spectra and melting curves were recorded on an Aviv 215 spectrometer (Aviv Biomedical Inc., Lakewood, NJ) using 0.1-cm quartz cuvettes. Recombinant proteins (c ~ 5µM) were dissolved in 100 mM KCl, 10 mM HEPES pH 7.4, including either 1 mM $CaCl_2$ or 1mM EDTA. Concentrations were determined using molar extinction coefficients $\varepsilon_{M,277nm}$ of 3,000 and 3,300 $M^{-1}cm^{-1}$ in the presence and absence of Ca^{2+}, respectively.[3] Melting curves recorded at 221 nm in 0.5 °C intervals from 4 to 90 and 99 °C in the absence and presence of Ca^{2+}, respectively, were analyzed assuming a three-state transition from the native (N) to denatured (D) state *via* an intermediate one (I) resulting in melting temperatures T_m and van't Hoff enthalpies of unfolding ΔH_{vH}.[4] As in the presence of Ca^{2+} no complete denaturation could be achieved, for curve fitting the values of the CD signal in the denatured states observed in the absence of Ca^{2+} were used. The coefficients of determination for fitted curves *vs*. measurements were $r^2 > 0.99$.

Steady-state Fluorescent Intensity Measurements

Fluo-5N, pentapotassium salt fluorescent calcium indicator was purchased from Thermo Fischer Scientific. Buffer reagents HEPES, KCl, EGTA, NTA and $CaCl_2$ were purchased from Sigma-Aldrich at reagent-grade purity.

Fluorescence measurements were made at room temperature using a Quantamaster-400 fluorometer (Photon Technology International, Inc.) equipped with a 75W xenon short arc lamp (Ushio). All samples were dialyzed against a calcium buffering system consisting of 50 mM HEPES (pH 7.4), 100 mM KCl, 0.05 mM EGTA and 5 mM NTA to minimize the effect of small protein concentration differences among samples.

Fluorescence emission spectra were collected from samples containing 6 μM of protein and 4 nM of the Fluo-5N calcium indicator at various calcium concentrations ([Total Ca^{2+}] = 0 - 15 mM) using a 0.5 nm wavelength step size and 5 nm bandpasses. Binding to CaM N-Domain binding sites was monitored using a 250 nm excitation wavelength and recording phenylalanine fluorescence emission changes at 280 nm, while binding to CaM C-Domain binding sites was monitored using a 277 nm excitation wavelength and recording tyrosine fluorescence emission changes at 320 nm. The free calcium concentration ([Free Ca^{2+}]) of each sample was determined using a 467 nm excitation wavelength and tracking Fluo-5N fluorescence emission changes at 510 nm. These changes can then be used to calculate [Free Ca^{2+}] from the equation:

$$[Free\ Ca^{2+}] = K_d \frac{F^{Max} - F}{F - F^{Min}}$$

where F^{Min} and F^{Max} is the fluorescence intensity in the absence and excess (full saturation) of calcium respectively and F is the intensity of the sample. The dissociation constant K_d of Fluo-5N was found to be 92 ± 3 μM in the buffer used in this study, determined in a separate experiment. All spectra were corrected by subtracting their corresponding buffer blanks and all experiments were repeated at least four times.

The resulting normalized fluorescence intensity vs [Free Ca^{2+}] data were plotted and fitted to a model-independent two-site Adair function which allowed non-equal intrinsic affinities and cooperativity of the two binding sites:

$$Y = \frac{K_1[Free\ Ca^{2+}] + 2K_2[Free\ Ca^{2+}]^2}{2(1 + K_1[Free\ Ca^{2+}] + K_2[Free\ Ca^{2+}]^2)}$$

where $K_1 = k_1 + k_2$ is the sum of the intrinsic microscopic equilibrium constants of each binding site, $K_2 = k_1 k_2 k_c$ is the equilibrium constant for binding to both sites and Y is the fractional occupancy of the binding sites.[5]

The intradomain cooperativity constant k_c cannot be determined analytically from these data alone, however a lower limit can be estimated from the apparent cooperativity constant K_c using equation.[6, 7]

$$k_c^{min} = K_c = \frac{4K_2}{K_1}$$

The apparent dissociation constants are reported as the average value for the pair of sites derived from the square root of K_2. Gibbs free energy changes (ΔG_i) were calculated using the well-known equation:

$$\Delta G_i = -RTlnK_i$$

were K_i is the appropriate equilibrium constant.

Molecular Modeling

Molecular model was generated using the ICM-Pro molecular modeling software package version 3.8 (Molsoft LLC, San Diego, CA). The model is based on PDB entry 1CLL. The CaM^{E105A} mutation was introduced in the 1CLL structure and the final structure was acquired following the ICM Biased Probability Monte Carlo (BPMC) method,[8] with flexible side chains for the mutated residue and its neighbouring residues. The rest of the protein structure was considered rigid.

Co-immunoprecipitation assays

Co-immunoprecipitation assays were performed as described previously.[12] Cardiac SR microsomes (300 μg) were solubilized in 200 μl of IP buffer (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.4% CHAPS and protease inhibitors) containing the appropriate free [Ca^{2+}] (achieved by mixing different proportions of 1 mM EGTA and 1 mM Ca^{2+} together, according to the Max Chelator software [http://maxchelator.stanford.edu/]) by overnight incubation at 4°C with continuous mixing. The insoluble material was pelleted at 20,000 *g* for 10 min at 4°C, and the supernatant was removed. Concurrently, the RyR2-specific antibody, Ab^{1093} (4 μl) was captured on 20 μl nProtein-A–Sepharose beads (GE Healthcare) in 200 μl of PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) overnight at 4°C. Beads were recovered at 1500 g for 2 min at 4°C and washed twice with the appropriate IP buffer. Solubilized SR proteins and 1μM of CaM^{WT} or CaM^{E105A} mutant were transferred into Eppendorf tubes with RyR2 antibody Ab^{1093}-protein-A beads, and incubated for 6 h at 4°C with mixing. Beads were recovered at 1,500 g for 2 min at 4°C and washed twice with the appropriate IP buffer for 5 min. Immunoprecipitated proteins were eluted with SDS-PAGE loading buffer, heated at 80°C for 5 min, and analyzed by SDS-PAGE and western blotting using an anti-CaM rabbit monoclonal antibody (1:7,500 dilution; Source Bioscience).

SDS-PAGE and western blotting

Recombinant CaM proteins were separated by SDS–PAGE as previously described.[2] Separated proteins were transferred onto polyvinylidene difluoride membrane (Immobilon-P; Millipore) using a semi-dry transfer system (Trans-Blot SD; Bio-Rad) in buffer (48 mM Tris, 39 mM glycine, 0.0375% SDS, 20% v/v methanol) at 20 V for 1 h. Membranes were incubated overnight at 4°C in Tris-buffered saline, 0.1% Tween 20 containing 5% non-fat milk powder, and probed with anti-CaM rabbit monoclonal antibody (1:7,500 dilution; Source Bioscience).

Detection of horseradish peroxidase-coupled secondary antibody was achieved using enhanced chemiluminescence detection (ECL; Amersham Biosciences).

[^{3}H]Ryanodine binding assays

Ryanodine binding assays were performed as previously described.[1, 2] Ryanodine binding was determined using 200 μg of cardiac SR microsomes per assay (volume 300 μL) incubated with 10 nmol/L ryanodine containing [^{3}H]ryanodine (100 Ci/mmol, Amersham) for 90 min at 37°C. The basic buffer contained 25 mM PIPES, 150 mM KCl, pH 7.1 with either 1 mM EGTA (less than 0.01 μM Ca^{2+}) or a series of free Ca^{2+} concentrations (values expressed as pCa 8–pCa 4 where pCaX = $-\log_{10}[Ca]X$) achieved by mixing different proportions of 1 mM EGTA and 1 mM Ca^{2+} as calculated using Max Chelator. CaM^{WT} or CaM^{E105A} mutant were added to a final concentration of 1 μM.

References

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