Supplemental Materials for:

Makoto Ono, et al. Dissociation of calmodulin from cardiac ryanodine receptor causes aberrant Ca^{2+} release in heart failure

EXPANDED MATERIALS AND METHODS

1. Preparation of SR vesicles

Left ventricles were homogenized in a solution containing 30 mmol/L Tris–malate, 0.3 mol/L sucrose, protease inhibitor cocktail (complete EDTA-free, Roche), pH 7.0 (Solution I). The homogenate was centrifuged at 5,500 g for 15 minutes and the resultant supernatant fraction was filtered through four layers of cheesecloth before centrifugation at 12, 000 g for 25 minutes. The supernatant was again filtered through cheesecloth and centrifuged at 143,000 g for 35 minutes. The pellet was resuspended in a solution containing 0.6 mol/L KCl, 30 mmol/L Tris–malate, 0.3 mol/L sucrose, protease inhibitor cocktail (complete EDTA-free, Roche), pH 7.0. This suspension was centrifuged at 143,000 g for 50 minutes. The pellet was suspended in Solution I and centrifuged at 143,000 g. The resultant pellet represents the microsomal fraction that is enriched in SR vesicles, and it was homogenized in a solution containing 0.1 mol/L KCl, 20 mmol/L Tris–malate, 0.3 mol/L sucrose, protease inhibitor cocktail (complete EDTA-free, Roche), pH 7.0, to a final concentration of about 10–20 mg protein/mL. This fraction was rapidly frozen with liquid nitrogen and stored at –80°C.

2. Site-directed fluorescent labeling of DPc10 binding site in the RyR2 (1, 2)

Peptide-SAED conjugate was formed by incubating 0.2 mmol/L peptide with 0.2 mmol/L SAED in a 20 mmol/L HEPES (pH 7.5) solution for 60 min at 22 °C in the dark. The reaction was quenched by 20 mmol/L Tris-HCl, pH 7.5. Unreacted SAED was removed using ion exchange column (GE HiTrap Q XL). The peptide-SAED conjugate (5 μmol/L in a final concentration) was mixed with 1 mg/mL SR protein in the sample solution in the dark and photolyzed with UV light in a Pyrex tube at 4 °C for 2 minutes. Beta-mercaptoethanol was added (100 mmol/L in a final concentration) to cleave the disulfide bond of SAED and to remove the carrier (DPc10). After incubation on ice for 1 hour, the mixture was centrifuged at 100,000g for 15 minutes, and the sedimented vesicles were resuspended in the sample solution to a final protein concentration of 5-10 mg/mL.

3. Fluorescence quenching assay of the methylcoumarin-3-acetamido (MCA) probe attached to the binding site of DPc10 (1, 2)

The zipping/unzipping mode of regulatory domains within the RyR2 was evaluated by fluorescence quenching assay of the MCA probe attached to the binding site of DPc10. To make a large-size quencher, QSY 7 carboxylic acid was conjugated with BSA by incubating 5 mmol/L QSY 7 carboxylic acid with 0.5 mmol/L BSA in 20 mmol/L HEPES (pH 7.5) for 60 minutes at 22^{o}C in the dark. Unreacted QSY 7 carboxylic acid was removed by means of Sephadex G50 gel filtration. Fluorescence quenching by the BSA-QSY was performed by measuring steady-state fluorescence of the labeled MCA (excitation at 368 nm, emission at 455 nm) in the presence or absence of various compounds. The data were analyzed using the Stern-Volmer plot.

4. Preparation of isolated cardiomyocytes (1)

A wedge of LV free wall, perfused by a branch of the left circumflex coronary artery, was dissected free of the heart and perfused with a collagenase-containing buffer solution. LV myocardium was minced with scissors in the fresh collagenase-containing buffer solution. Then, rod-shaped adult canine cardiomyocytes were prepared by retrograde perfusion of quickly excised hearts with 95%O_2/5%CO_2 -bubbled Minimal Essential Medium (Sigma) supplemented with 50 μmol/L Ca^{2+}, 0.5 mg/mL collagenase B, 0.5 mg/mL collagenase D and 0.02 mg/mL protease type XIV. The concentration of Ca^{2+} was then gradually increased to a final concentration of 100 μmol//L by changing the incubation medium (50 μmol/L and 100 μmol/L). The isolated canine cardiomyocytes were transferred to laminin-coated glass culture dishes, and incubated for 12 hours at 37°C in 5%CO_2/95%O_2 atmosphere.

5. Analysis of Ca^{2+} sparks in saponin-permeabilized cardiomyocytes with laser scanning confocal microscopy (3)

Ventricular myocytes were superfused with relaxing solution containing EGTA 0.1 mmol/L, ATP 5 mmol/L, HEPES 10 mmol/L, K- aspartate 150 mmol/L, $MgCl_2$ 0.25 mmol/L, and reduce-glutathione 10 mmol/L, at 23°C. The sarcolemma was permeabilized with saponin (100 μg/mL) for 30-40 seconds. After permeabilization, myocytes were placed in internal solution composed of: EGTA 1 mmol/L; HEPES 10 mmol/L; K-aspartate 120 mmol/L; ATP 3 mmol/L; free [Mg^{2+}] 1 mmol/L; reduced glutathione 10 mmol/L; free [Ca^{2+}] 75 nmol/L (calculated using MaxChelator (http://www.stanford.edu/~cpatton/webmaxcS.htm)); creatine phosphokinase 5 U/ml; phosphocreatine 10 mmol/L; dextran (Mr: 40,000) 4%; Fluo-3 20 μmol/L; pH 7.2.

Fluo-3 was excited by 488 nm laser lines, and fluorescence emission was acquired at wavelengths of 505-530 nm.

Ca^{2+} sparks were measured in saponin-permeabilized cardiomyocytes with a laser scanning confocal microscope (LSM-510, Carl Zeiss). equipped with an argon ion laser coupled to an inverted microscope (Axiovert 100, Carl Zeiss) with a Zeiss x40 oil-immersion Plan-Neofluor objective (numerical aperture, 1.3; excitation at 488 nm; emission > 505 nm) (4). The images of Ca^{2+} sparks were acquired in the linescan mode of the confocal microscope at a rate of 1.92 ms per scan, with the 3000 scan lines (5.76sec) oriented along the longitudinal axis of the cell. We sampled and analyzed Ca^{2+} sparks from 20-40 cells. Data were analyzed with SparkMaster, an automated analysis program which allows rapid and reliable spark analysis (5). The analysis involved general image parameters (number of detected sparks, spark frequency) as well as individual spark parameters (Amplitude, FWHM: full width at half maximum, FDHM: full duration at half maximum).

6. Cross-linking of CaM-SANPAH to the RyR2

For CaM binding on the RyR2, we first tried to perform the conventional pull-down assay using the anti-RyR2 antibody, followed by detection of the bound CaM using the anti-CaM antibody. However, there was marked non-specific CaM binding on Sepharose A or G even without the anti-RyR2 antibody, which precluded the reliable assessment of the specific CaM binding on the RyR2. Therefore, we assessed the CaM binding to the RyR2 using a photoreactive crosslinker, sulfosuccinimidyl-6-[4'-azido-2'-nitrophenylamino]hexanoate(Sulfo-SANPAH, PIERCE). First, CaM-SANPAH conjugate was formed by incubating 0.2 mmol/L CaM

with 0.8 mmol/L SANPAH in a 20 mmol/L HEPES (pH 7.5) solution for 60 minutes at 22 °C in the dark. The reaction was quenched by 20 mmol/L Tris-HCl, pH 7.5. Free Sulfo-SANPAH was removed using Sephadex G25 column. The CaM-SANPAH conjugate (16 nmol/L-1μmol/L in a final concentration) was mixed with 0.5mg/mL SR protein in the sample solution containing 150 mmol/L NaCl, 50 mmol/L MOPS, 1 mmol/L EGTA/calcium buffer (0.3 μmol/L free [Ca^{2+}]) pH6.8, in the dark and photolyzed with UV light in a Pyrex tube at 4 °C for 30 seconds. Then, the mixture was immunoblotted with a monoclonal anti-CaM antibody (MILLIPORE, CA) to detect the RyR2-bound CaM.

7. Direct binding of exogenous CaM to the RyR2 in saponin-permeabilized cardiomyocytes.

The exogenous CaM, fluorescently labeled with Alexa Fluor 488 (Molecular Probes, OR), was added to the saponin-permeabilized normal and failing cardiomyocytes under the same condition as the above-mentioned Ca^{2+} spark measurements. Then, quantification of the distribution of localized CaM was achieved through densitometric measurement of CaM-Alexa fluorescence. Briefly, the fluorescently labeled cardiomyocytes were laser-scanned with the confocal microscope system (LSM-510, Carl Zeiss). (numerical aperture, 1.3; excitation at 488 nm; emission 505-530 nm). The sarcomere-related periodical increase in the Alexa488 fluorescence intensity from baseline was integrated with respect to the longitudinally selected distance (~25 μm) and then divided the value by the distance. Thus obtained signal-averaged Alexa488 fluorescence intensity was expressed as the ratio to the maximum value obtained in the presence of CaM-Alexa (1 μmol/L). The CaM-Alexa

labeled cardiomyocytes were also applied to the immunofluorescent staining by a monoclonal anti-RyR antibody (C3-33, Sigma), as follows. The cardiomyocytes were fixed with 4% paraformaldehyde in PBS for 10 minutes, washed three times with PBS, and permeabilized in 0.5% Triton X-100 and 1% BSA for 15 minutes. Then, the cardiomyocytes were incubated overnight at 4°C with the anti-RyR antibody (C3-33, Sigma) in 1% BSA and 0.5 % Triton X-100, followed by labeling with an Alexa633-conjugated rabbit anti-mouse secondary antibody (Molecular Probes, OR). The cardiomyocytes were washed three times with PBS. Images for co-localization of CaM-Alexa and RyR were acquired at 488nm excitation wavelength using a BP 505-530 nm band-pass detection filter for green channel (CaM-Alexa) and 633 nm excitation wavelength in conjunction with a LP 650 nm long pass filter for red channel (anti-RyR antibody). Secondary antibody labeling alone showed no detectable fluorescence pattern.

8. Determination of the binding of endogenous CaM to the RyR2 in intact cardiomyocytes.

The isolated cardiomyocytes were plated on glass-based dishes, fixed with 4% paraformaldehyde in PBS for 10 min, washed three times with PBS, and permeabilized in 0.5% Triton X-100 and 1% BSA for 15 min. These cardiomyocytes were incubated overnight at 4°C with a monoclonal mouse anti-CaM antibody (MILLIPORE, CA) and polyclonal rabbit anti-RyR antibody (C-teminal Ab4963, Sigma; cf. ref 10) in 1% BSA and 0.5 % Triton X-100, followed by each labeling with an Alexa488-conjugated goat anti-rabbit secondary antibody (Molecular Probes, OR) and Alexa633-conjugated rabbit anti-mouse secondary antibody (Molecular Probes, OR). Cardiomyocytes were then

washed three times with PBS. For co-localization of anti-CaM antibody (Alexa633; red) and anti-RyR antibody (Alexa488; green), images were acquired at 633 nm and 488 nm excitation in conjunction with LP 650 filter and BP505-530 nm, respectively. Both secondary antibody labeling showed no detectable fluorescence pattern and did not cross-react with each primary antibody. For co-localization analysis, the fluorescent images were acquired sequentially in order to prevent spectral cross-talk.

References

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2. Yamamoto T, Ikemoto N. Spectroscopic monitoring of local conformational changes during the intramolecular domain-domain interaction of the ryanodine receptor. *Biochemistry.* 2002; **41**: 1492–1501.
3. Guo T, Zhang T, Mestril R, Bers DM. Ca2+/Calmodulin-dependent protein kinase II phosphorylation of ryanodine receptor does affect calcium sparks in mouse ventricular myocytes. *Circ. Res.* 2006; **99**:398-406.
4. Guo T, Zhang T, Mestril R, Bers DM. Ca2+/Calmodulin-dependent protein kinase II phosphorylation of ryanodine receptor does affect calcium sparks in mouse ventricular myocytes. *Circ. Res.* 2006; **99**:398-406.
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Supplementary figure legends

Supplementary Figure 1

Cross-linking of CaM-SANPAH to the RyR2. Note that the antibody against CaM detected only RyR2 among many other proteins in the SR.

Supplementary Figure 2

[Ca^{2+}] dependency of CaM-SANPAH cross-linking to the RyR2 in normal SR. The CaM-SANPAH conjugate (128 nmol/L) was mixed with 0.5mg/mL SR protein in the sample solution containing 150 mmol/L NaCl, 50 mmol/L MOPS, 1 mmol/L EGTA/calcium buffer (0.03 - 1 μmol/L free [Ca^{2+}], pH6.8 in the dark and photolyzed with UV light in a Pyrex tube at 4 °C for 30 seconds. Then, the mixture was immunoblotted with a monoclonal anti-CaM antibody (MILLIPORE, CA) to detect the RyR2-bound CaM. The immunoblot density of CaM cross-linked to the RyR2 was measured and expressed as the ratio to maximum value obtained at 1μmol/L free [Ca^{2+}]. Data represent means±SD of 3 SR preparations.

Supplementary Figure 3

[Ca^{2+}] dependency of CaM-Alexa binding in normal cardiomyocytes. The exogenous CaM, fluorescently labeled with Alexa Fluor 488 (Molecular Probes, OR), was added to the saponin-permeabilized normal cardiomyocytes, with internal solution composed of:

EGTA 1 mmol/L; HEPES 10 mmol/L; K-aspartate 120 mmol/L; ATP 3 mmol/L; free [Mg^{2+}] 1 mmol/L; reduced glutathione 10 mmol/L; free [Ca^{2+}] 0.03 – 0.3 μmol/L free [Ca^{2+}]; buffered by 1 mmol/L EGTA/calcium buffer; creatine phosphokinase 5 U/ml; phosphocreatine 10 mmol/L; dextran (Mr: 40,000) 4%; pH 7.2. The CaM-Alexa fluorescence was measured and expressed as the ratio to maximum value obtained at 1μmol/L free [Ca^{2+}]. Data represent means±SD of 20-25 cells from 3 hearts.

Supplementary Figure 4

Effect of endogenous CaM on the exogenous CaM-Alexa binding in saponin-permeabilized normal cardiomyocytes. **A.** Representative images (top) and summarized data (bottom) of CaM detected by anti-CaM antibody, under condition with or without pre-incubation by 5 mmol/L EGTA (free [Ca^{2+}]= 0 μmol/L) and/or exogenous CaM (200 nmol/L). After incubating cells with 5 mmol/L EGTA, the solution was washed away and then replaced with a solution containing 75 nmol/L free [Ca^{2+}]; buffered by 1 mmol/L EGTA just before fixation by 4% paraformaldehyde and the following the immunofluorescent staining. The fluorescence signal of the endogenous CaM was measured and expressed as the ratio to control. Note that pre-incubation by 5 mmol EGTA largely depleted CaM. Data represent means±SD of 18-20 cells from 2 hearts. **B.** Delivery of various concentrations of the CaM-Alexa (left) and the summarized data (right) under condition of free [Ca^{2+}]=75 nmol/L, with or without pre-incubation by a solution with 0 μmol/L free [Ca^{2+}]; EGTA 5 mmol/L; HEPES 10 mmol/L; K-aspartate 120 mmol/L; ATP 3 mmol/L; free [Mg^{2+}] 1 mmol/L; reduced glutathione 10 mmol/L; creatine phosphokinase 5 U/ml; phosphocreatine 10

mmol/L; dextran (Mr: 40,000) 4%; pH 7.2. The CaM-Alexa fluorescence was measured and expressed as the ratio to maximum value. Data represent means±SD of 12-20 cells from 2 hearts.

Supplementary Figure 5

Summarized data for Ca^{2+} spark characteristics. FWHM: full width at half maximum, FDHM: full duration at half maximum. Data represent means±SD of sparks from 26-43 cells from 4-6 hearts.

Supplementary Figure 6

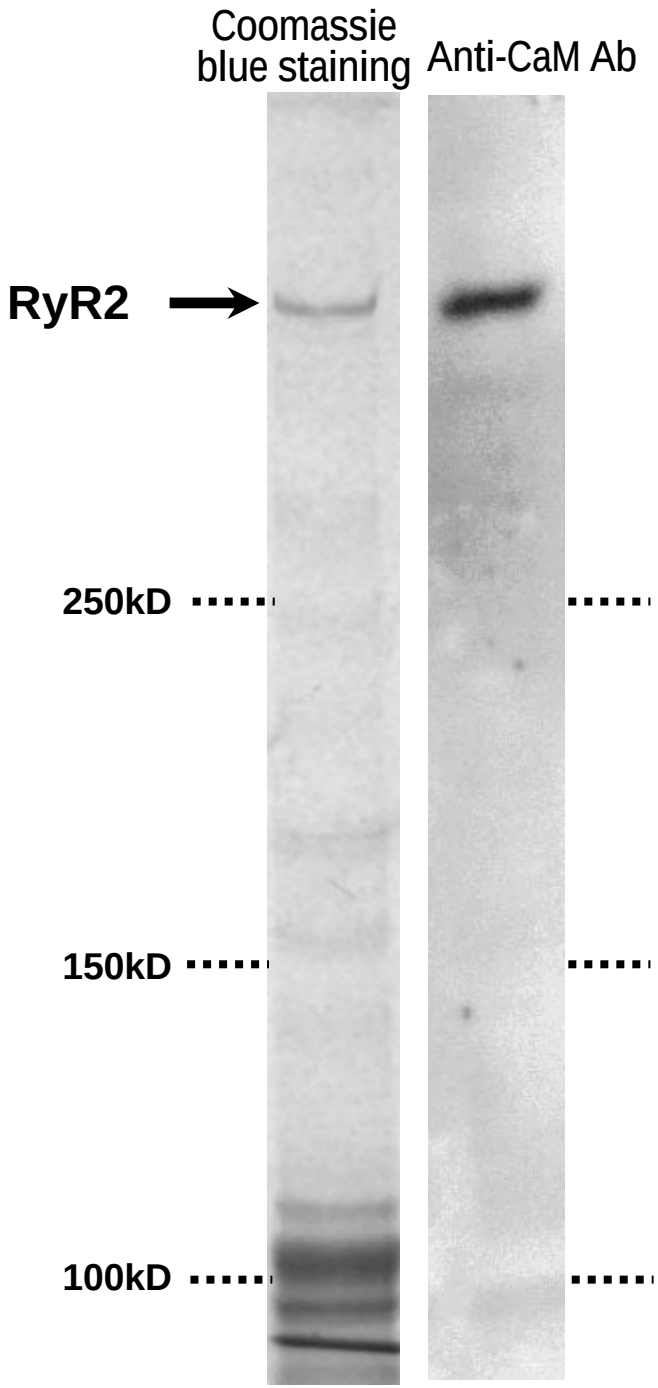
Site-directed MCA fluorescence labeling in the RyR2 by using the central domain peptide, DPc10 as a carrier. No MCA fluorescence was seen in the presence of an excess concentration of unlabeled DPc10 (10 mmol/L).

Supplementary Figure 7

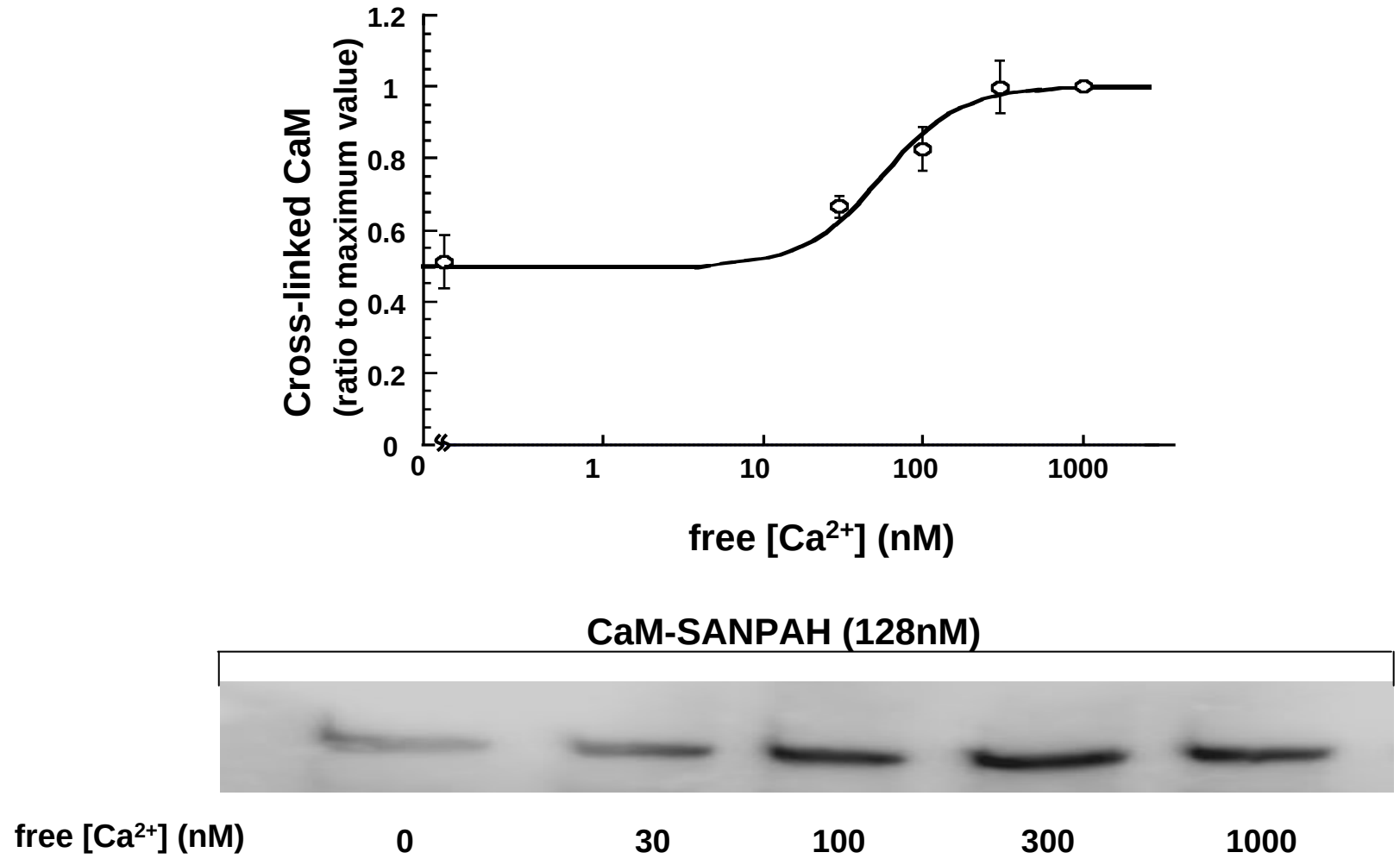
Effect of FK506 (30 μmol/L), dantrolene (1 μmol/L), and/or CaM (1 μmol/L) on the association of FKBP12.6 to the RyR2 in normal and failing SR vesicles. Representative Western blotting of FKBP12.6 and the summarized data (bottom). Data represent means±SD of 4 SR preparations. SR vesicles (0.4 mg/mL) were preincubated for 10 min in RIPA Buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 1 mmol/L Na_3VO_4, 1 mmol/L NaF, 0.25 % Triton X-100, at PH 7.4, and pulled down by

Sepharose A in the presence of the anti-RyR2 antibody (C-terminal, Sigma). Then, the co-immunoprecipitated FKBP12.6 with the RyR2 was detected by the anti-FKBP12 (C-19) antibody (Santa Cruz Biotechnology, CA).

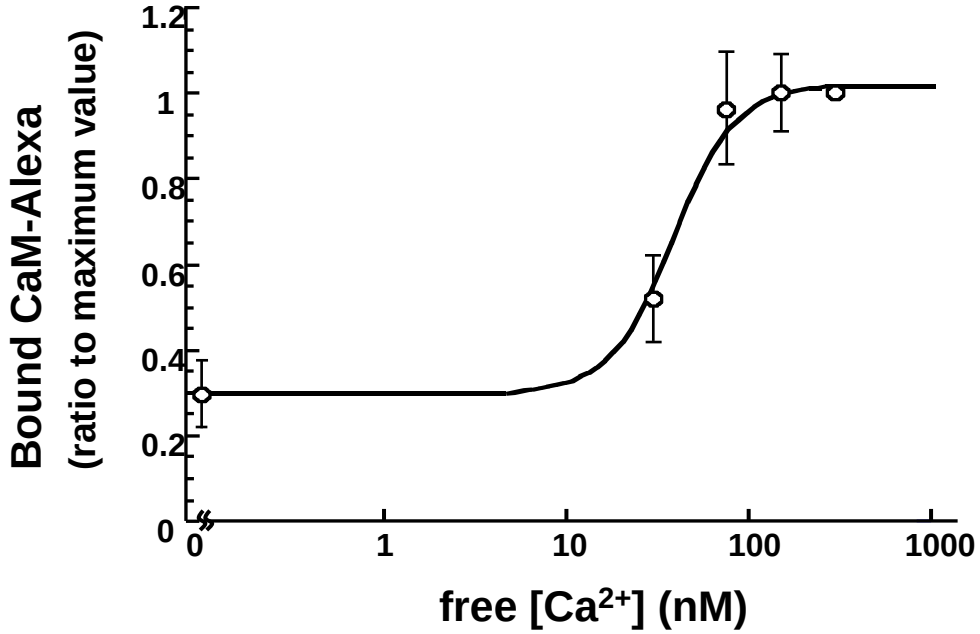
Supplementary Figure 1

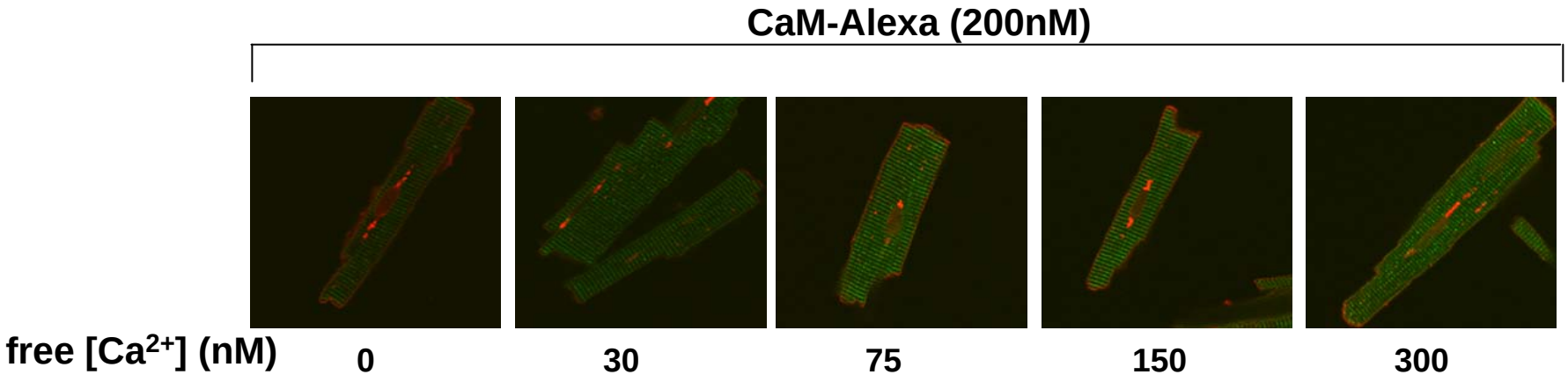


Supplementary Figure 2

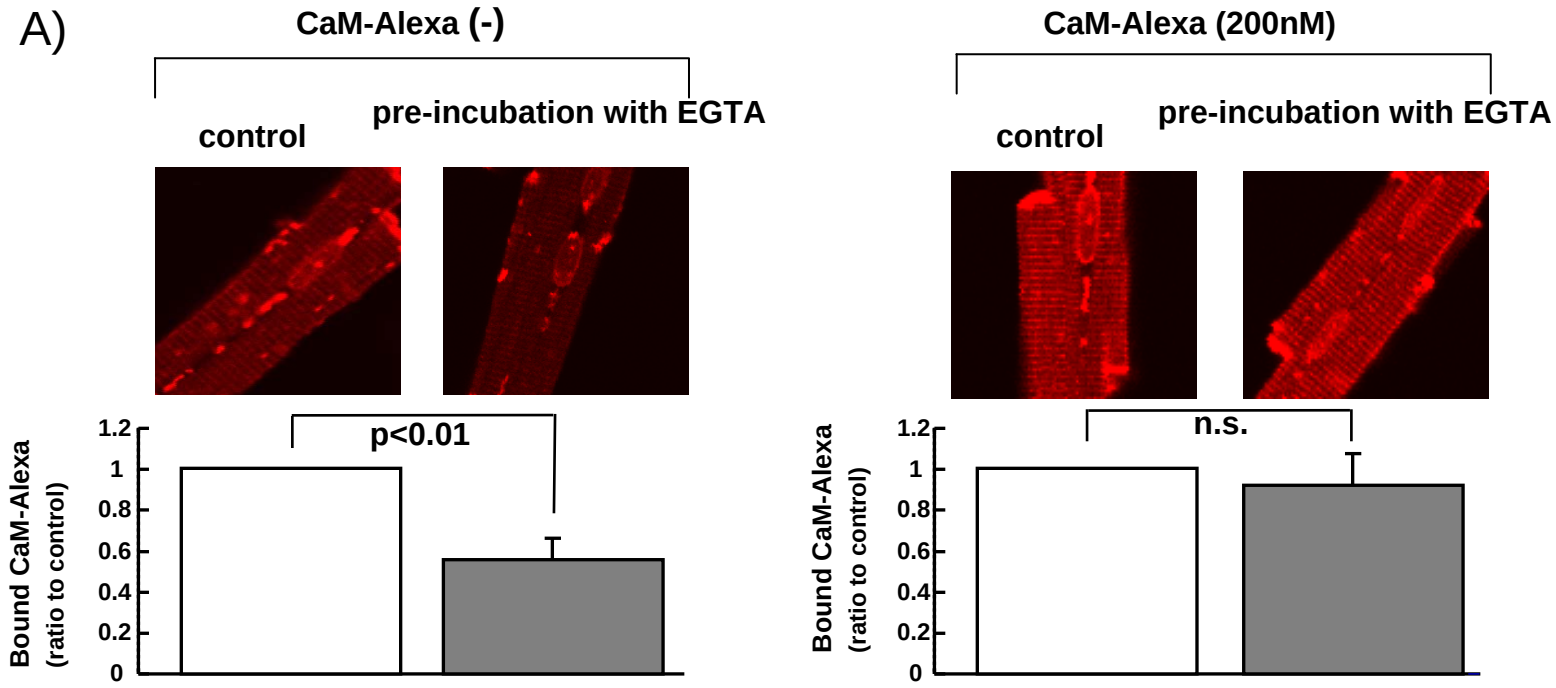


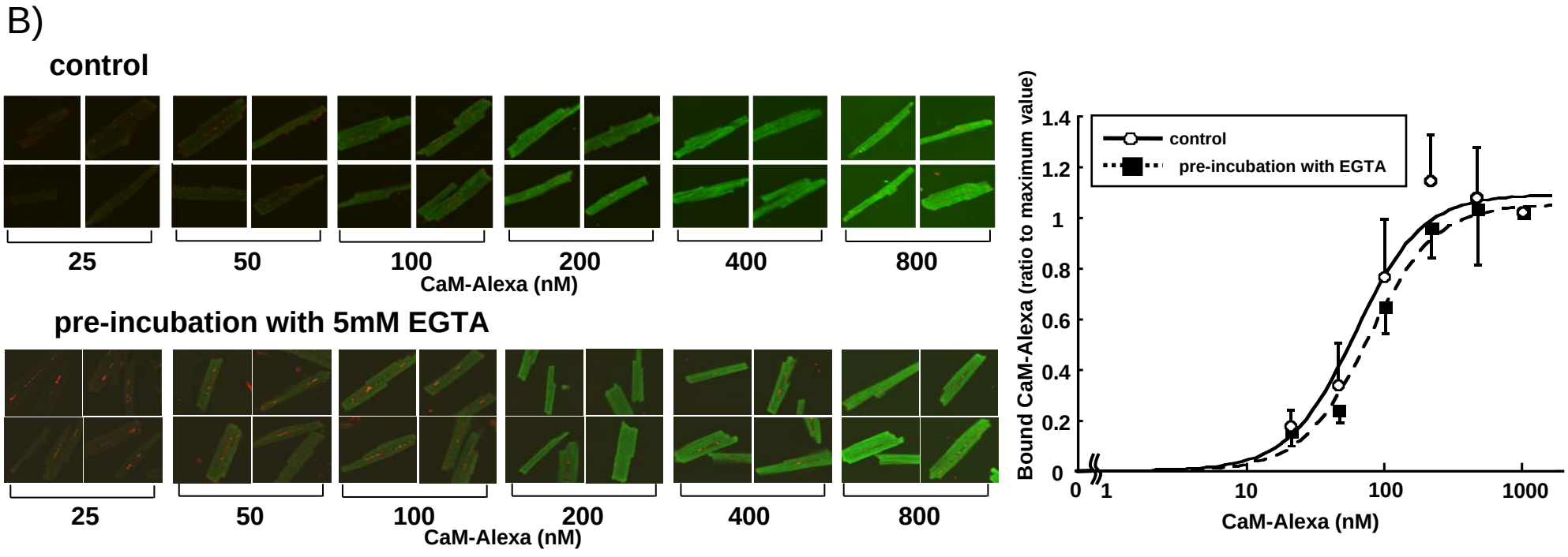
Supplementary
Figure 3



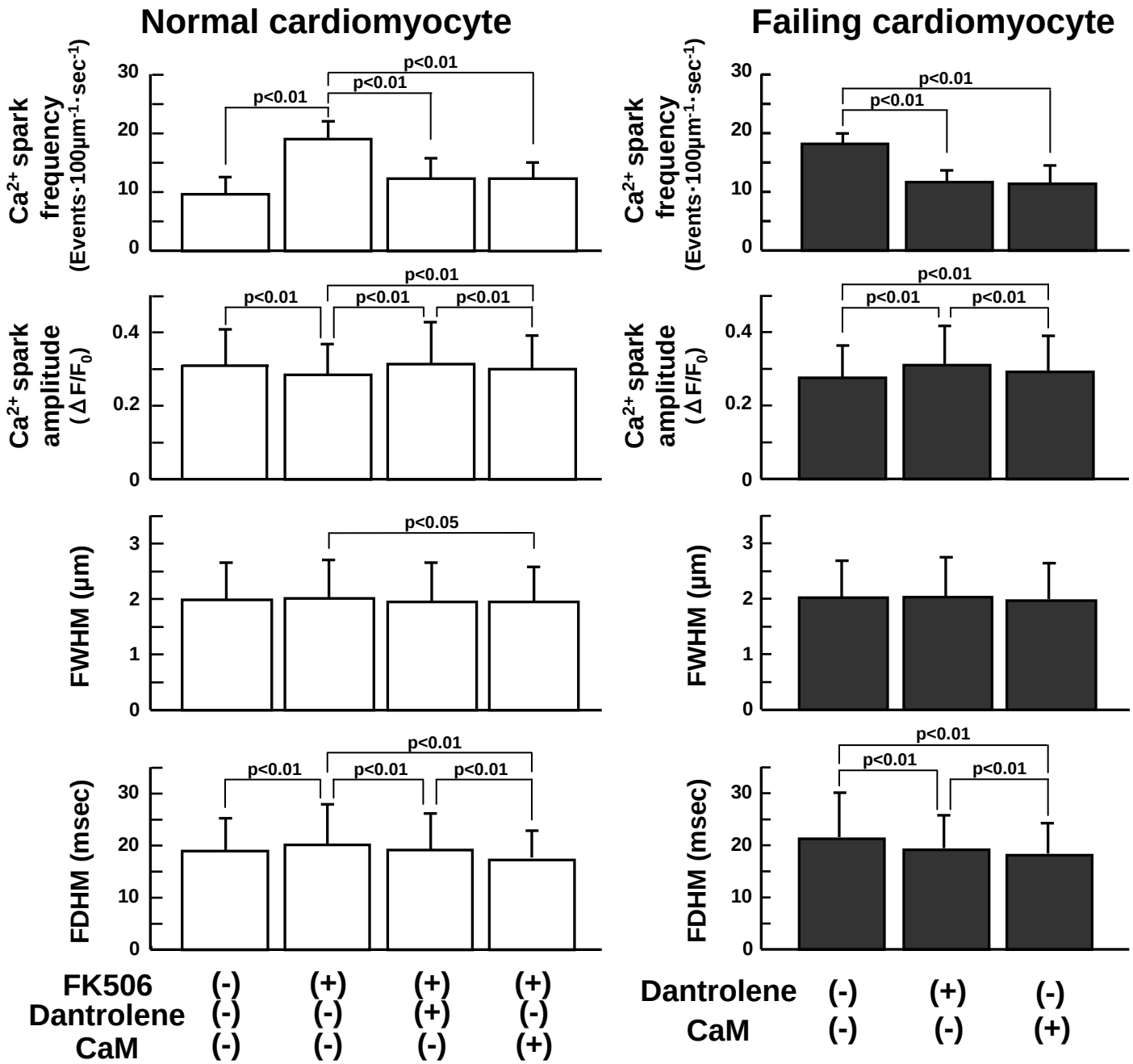


Supplementary
Figure 4





Supplementary Figure 5



Supplementary Figure 6

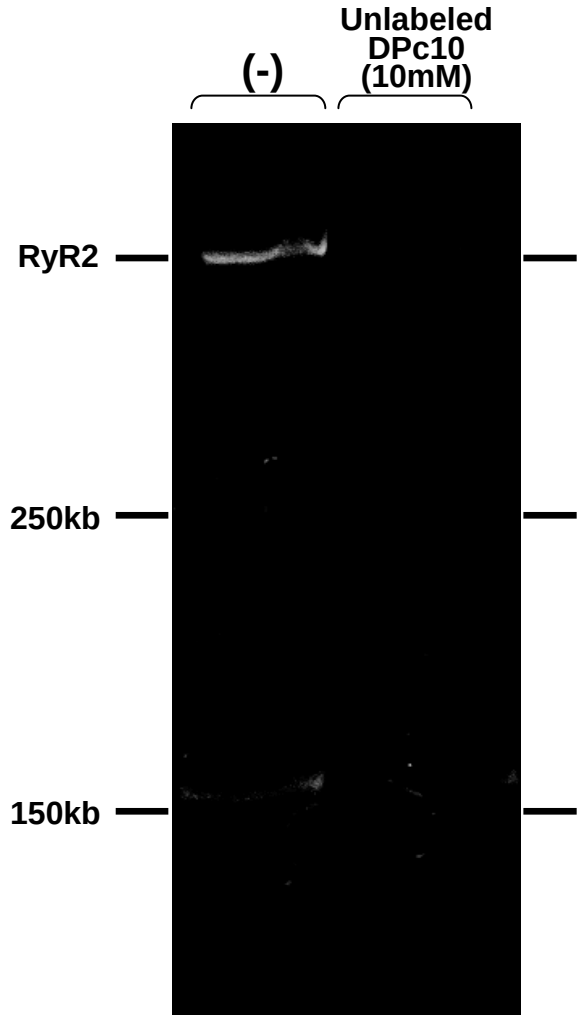


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Figure 7