Binding of RyR2 "Unzipping" Peptide in Cardiomyocytes Activates RyR2 and Reciprocally Inhibits Calmodulin Binding

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Expanded Materials and Methods

Rat cardiac myocyte isolation

Single ventricular myocytes were isolated from rat hearts as described previously.[1] Briefly, after anesthesia (isoflurane, 5%), hearts were excised and perfused (5min, 37˚C) with the minimal essential medium (MEM, GIBCO Life Technologies) gassed with 95% O_2/5% CO_2 before inclusion of collagenase B (0.5 mg/ml, Boehringer Mannheim) and protease (0.02 mg/ml,Sigma). Triturates were incubated (10min, 37˚C) in the same enzyme solution, washed and kept in 100μM Ca^{2+} MEM solution.

Domain peptide

DPc10 peptides unlabeled and labeled with 5-carboxyfluorescein or HiLyte FluorTM647 were synthesized at AnaSpec (Fremont, CA). The DPc10 sequence is: 2460-GFCPDHKAAMVLFLDRVYGIEV-QDFLLHLLEVGFLP-2495.

Fluorescent labeling of single-cysteine mutants of FKBP12.6 and CaM

A single-cysteine variant of the human FKBP12.6-isoform (T14C-C22A-C76I-FKBP12.6) was labeled using the thiol-specific maleimide derivatives of Alexa Fluor 488 and Alexa Fluor 568 (Invitrogen), as described previously.[2, 3] A single-cysteine CaM (T34C-CaM) was labeled with Alexa Fluor 568 maleimide as described previously.[2, 3]

Laser scanning confocal microscopy

Confocal images was measured using a Biorad Radiance 2100 laser scanning confocal microscope equipped with an Argon ion laser, Green HeNe laser and with a Nikon Fluo x40 oil lens. FRET experiments between CaM and DPc10, and FKBP12.6 and DPc10 were performed using an Olympus FV1000 confocal microscope. All experiments were done at room temperature.

Ca^{2+} sparks in permeabilized cells using confocal microscopy

Myocytes were permeabilized with saponin (50μg/mL) for 60 seconds and placed in internal solution composed of EGTA 0.5 mmol/L; HEPES 10 mmol/L; K-aspartate 120 mmol/L; ATP 5 mmol/L; free MgCl2 1 mmol/L, reduced glutathione 10 mmol/L; and free [Ca^{2+}] 50nmol/L (calculated using MaxChelator), creatine phosphokinase 5 U/mL, phosphocreatine 10 mmol/L, dextran 4% Fluo-4 0.025 mmol/L, pH 7.2. Ca^{2+} sparks were recorded by a laser scanning confocal microscope (Radiance 2000 MP, Bio-Rad, UK) as previously described.[1] Fluo-4 was excited at 488 nm and emission was recorded using 500/530 nm bandpass filter. To assess SR Ca^{2+} content, caffeine (15 mmol/L) was rapidly perfused. Ca^{2+} spark were analyzed as using SparkMaster.[5]

FRET measurements

For the FRET between CaM and DPc10, and FKBP12.6 and DPc10, we used Alexa Fluor 568 attached at the C-lobe of CaM (AF568-110-CaM)[2], Alexa Fluor 488 attached at the N-lobe of CaM (AF488-34-CaM), Alexa Fluor 568-, or Alexa Fluor 488-FKBP12.6[3, 4] (AF488-FKBP12.6, AF568-FKBP12.6) and HiLyte Fluor^{TM}647-DPc10 (HF647-DPc10) as a donor-acceptor pair. AF488-, AF568- and HF647- were excited with separate laser channels of 488 nm, 543 nm and 635 nm, respectively. Emission fluorescence intensity data were obtained at 505-605 nm for AF488-FKBP12.6/AF488-34-CaM, 560-620 nm for AF568-FKBP12.6/AF568-110-CaM and 655-755 nm for HF647-DPc10. We used two experimental approaches, (1) comparing the donor fluorescence intensities before and after equilibration with acceptor (donor quenching) and (2) monitoring the increase in donor fluorescence after acceptor photobleaching (acceptor photo-bleaching), to detect and measure FRET signals in the permeabilized cardiomyocytes.

For the donor quenching method, FRET is indicated by a decrease in the donor fluorescence at wavelengths 505-605 nm (AF488-FKBP12.6) or 560-620 nm (AF568-FKBP12.6). The FRET efficiency (E) was calculated according to:

$$E = 1 - F_{DA}/F_D,$$

Where F_D and F_{DA} are the fluorescence intensities of the donor-only and donor-acceptor samples, respectively.

Complete acceptor (HF647) photobleaching was achieved by repeated scans of a defined area of the myocyte with the 635 nm laser at maximum power, for 60sec. E was calculated according to:

$$E = [(I_{donor\text{-}post} - I_{donor\text{-}pre})/I_{donor\text{-}post}] \times 100\%,$$

where $I_{donor\text{-}post}$ and $I_{donor\text{-}pre}$ are donor fluorescence intensities before and after acceptor photobleach. Donor-acceptor distances, R, were calculated from the equation:

$$R = R_0 (E^{-1}-1)^{1/6},$$

where the Förster distance, R_0 is defined as the distance at which E=0.5. R_0 is calculated from

$$R_0 = 9780(J\kappa^2 n^{-4} \phi_D)^{1/6},$$

where n is the refractive index of protein in aqueous solution (1.4), κ is the orientation factor (set to 2/3, corresponding to random orientation), ϕ_D is the fluorescence quantum yield of the donor (0.92 for AF488, and 0.69 for AF568[5]). J is the normalized spectral-overlap integral of donor emission $F_D(\lambda)$ and acceptor absorbance $\varepsilon(\lambda)$, and is calculated from

$$J = (\int F_D(\lambda)\, \varepsilon(\lambda)\, \lambda^4\, d\lambda)/\int F_D(\lambda)\, d\lambda,$$

by numerical integration using a Microcal Origin template. For the AF488-HF647 and AF568-AF647 donor-acceptor pairs we used ε_{HF647}(652nm) = 250,000 (mol^{-1} cm^{-1}) to calculate R_0 values of 54 A and 75 A, respectively.

Statistics

Data were expressed as mean ± SEM, and significance was evaluated using student's t test or one-way ANOVA. A *P*-value below 0.05 was considered statistically significant.

References

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2. Cornea RL, Nitu FR, Gruber S, Kohler K, Satzer M, Thomas DD, Fruen BR. FRET-Based Mapping of Calmodulin Bound to the RyR1 Ca2+ Release Channel. *Proc Natl Acad Sci U S A.* 2009;106:6128-6133.
3. Cornea RL, Nitu FR, Samsó M, Thomas DD, Fruen BR. Mapping the Ryanodine Receptor FK506-Binding Protein Subunit Using Fluorescence Resonance Energy Transfer. *J Bio Chem.* 2010;285:19219-19226.
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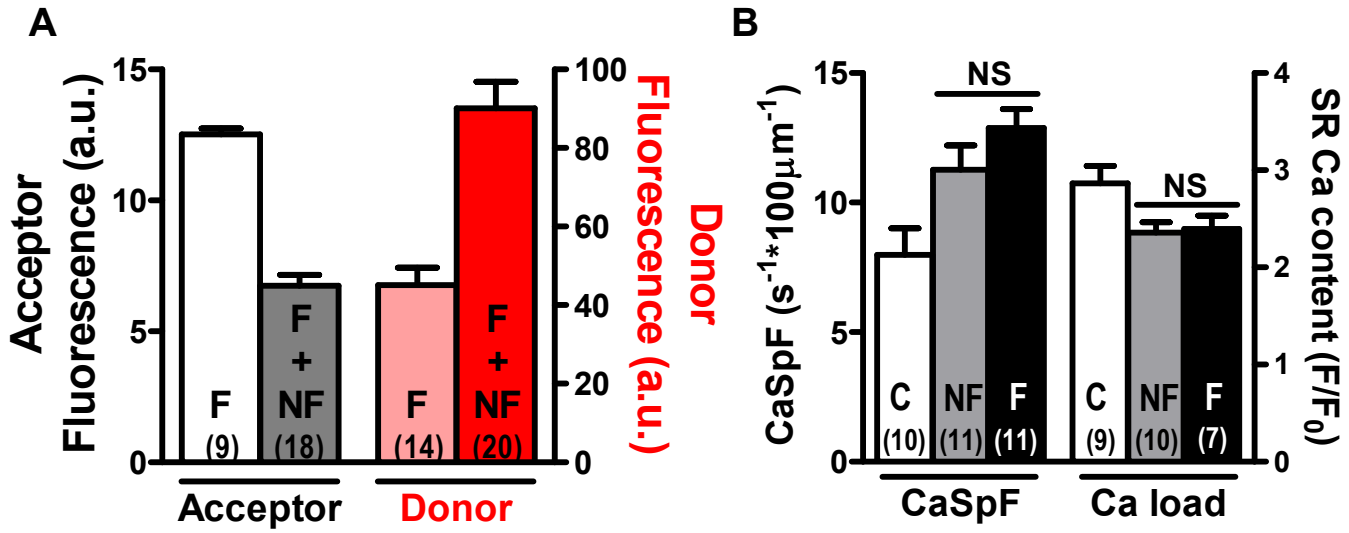
5. Picht E, Zima AV, Blatter LA, Bers DM. SparkMaster: Automated Calcium Spark Analysis with ImageJ. *Am J Physiol Cell Physiol.* 2007;293:C1073–C1081.
6. Jonson I, Spence MTZ. Molecular probes handbook, a guide to fluorescent probes and labeling technologies. *Life Technologies*. 2010

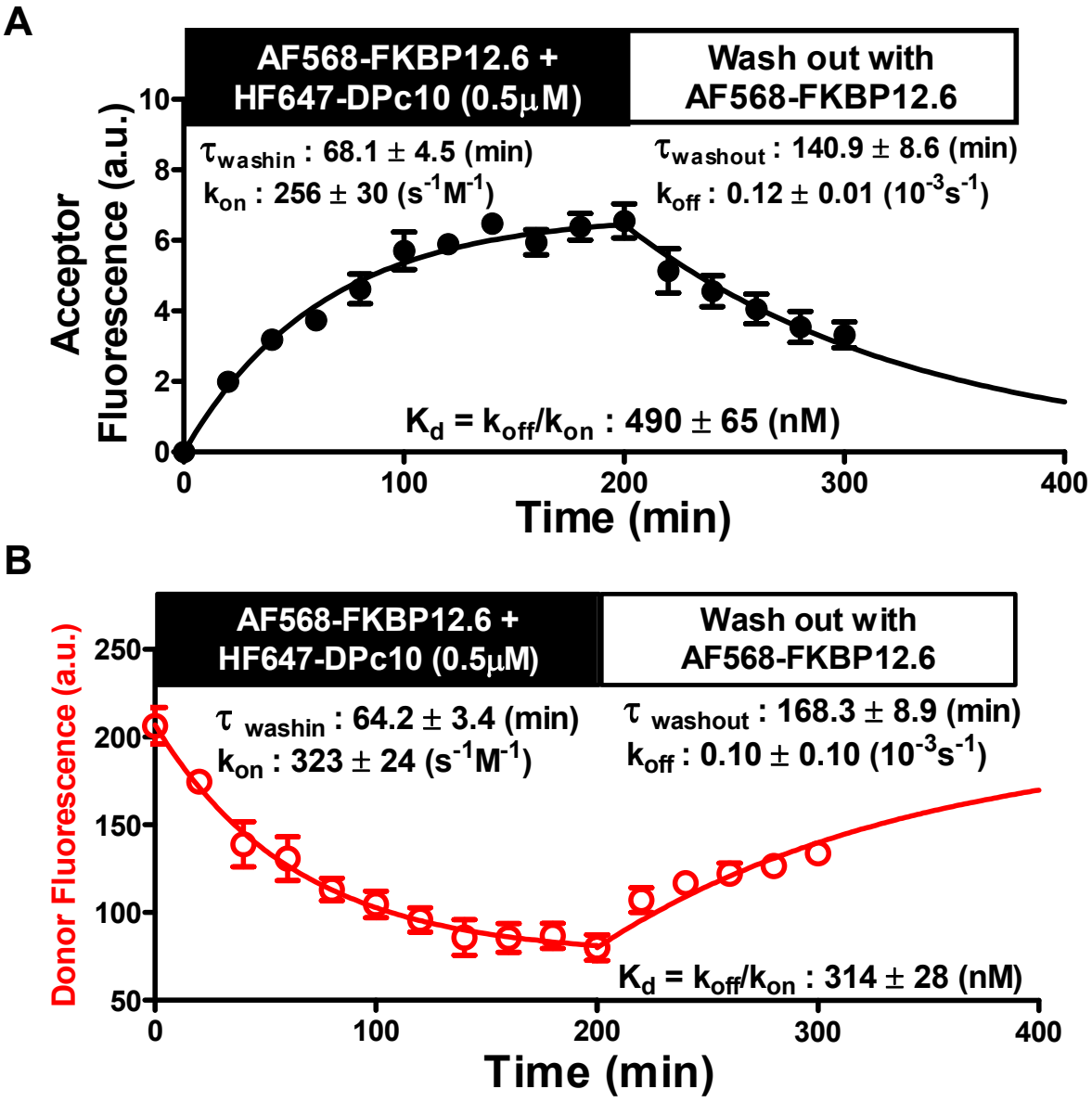
Table I. Ca^{2+} spark characteristics in permeabilized cardiomyocytes

	No. of Sparks	Peak (F/F_0)	FWHM (μm)	FDHM (ms)
Control	1761	0.499 ± 0.004	1.28 ± 0.01	55.7 ± 0.67
+DPc10	1467	0.475 ± 0.004*	1.34 ± 0.01*	58.5 ± 1.00*
+FKBP12.6/DPc10	1921	0.439 ± 0.002*	1.21 ± 0.01*	60.5 ± 0.73*
+CaM/DPc10	2195	0.467 ± 0.003*	1.23 ± 0.01*	56.9 ± 0.74

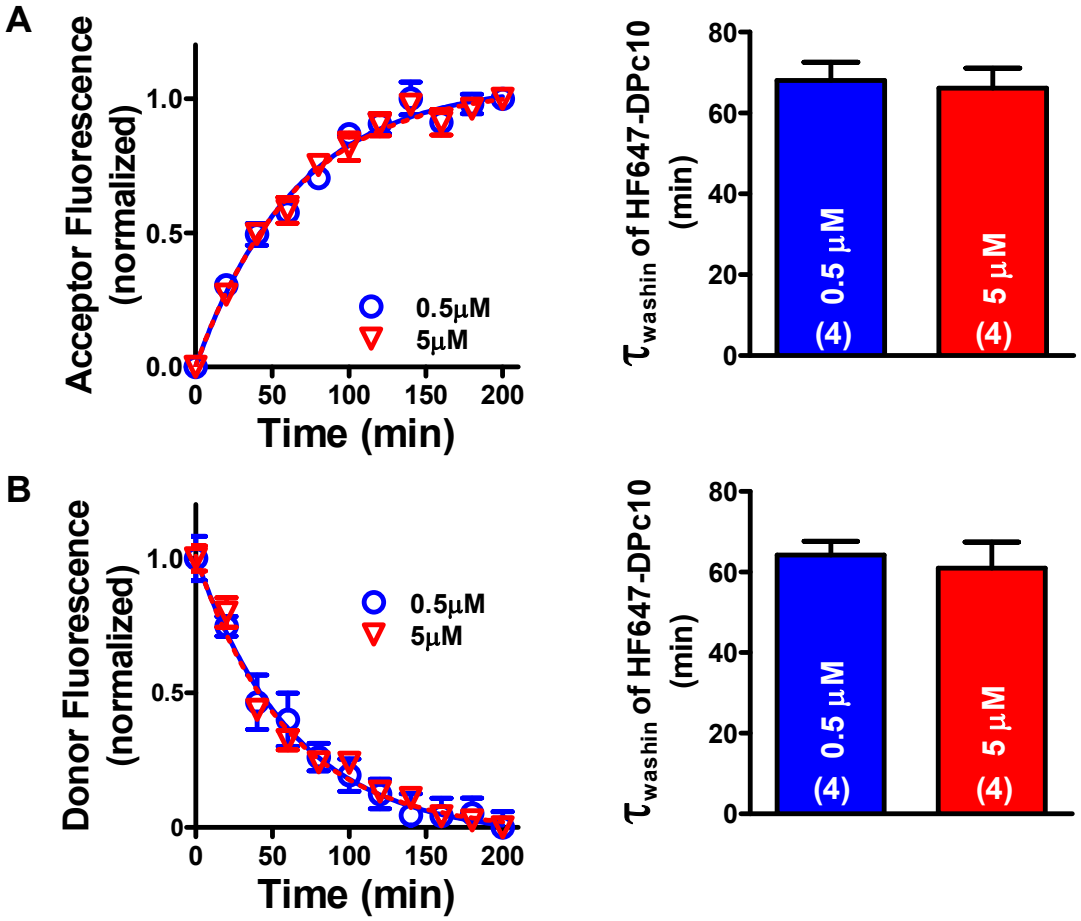
Supplemental Figures



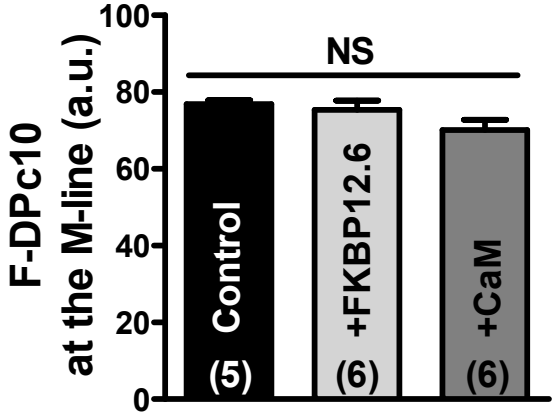
Online Figure I. A, Detection by FRET, as EAF or quenching of donor fluorescence, of the competitive inhibition of F-DPc10 binding to RyR2 by NF-DPc10 in permeabilized myocytes. Cells were incubated with F-DPc10 (F, 1 μmol/L) or with equal concentrations (1 μmol/L) of F-DPc10 and NF-DPc10 (F+NF). **B**, Ca spark frequency and SR Ca content for control (C) myocytes, and after addition of NF-DPc10 (NF, 5μmol/L) or F-DPc10 (F, 5μmol/L). Data are reported as mean ± SE (n values on bars).



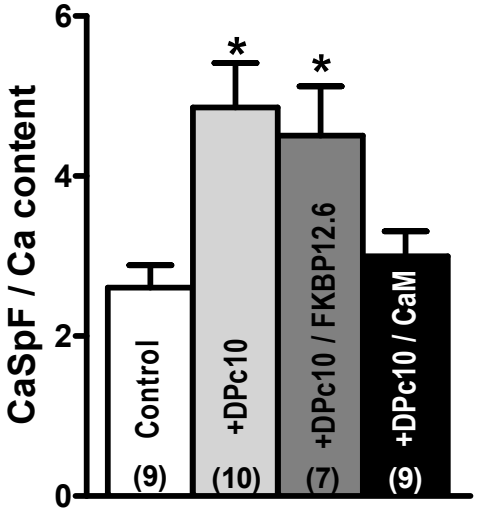
Online Figure II. Kinetics of HF-DPc10 binding, measured by FRET between F-FKBP12.6 and HF-DPc10. Wash-in and wash-out time course of FRET after addition of HF-DPc10 (0.5 μmol/L), detected as EAF (**A**) or as donor quenching (**B**). Data are reported as mean ± SE (n=4).



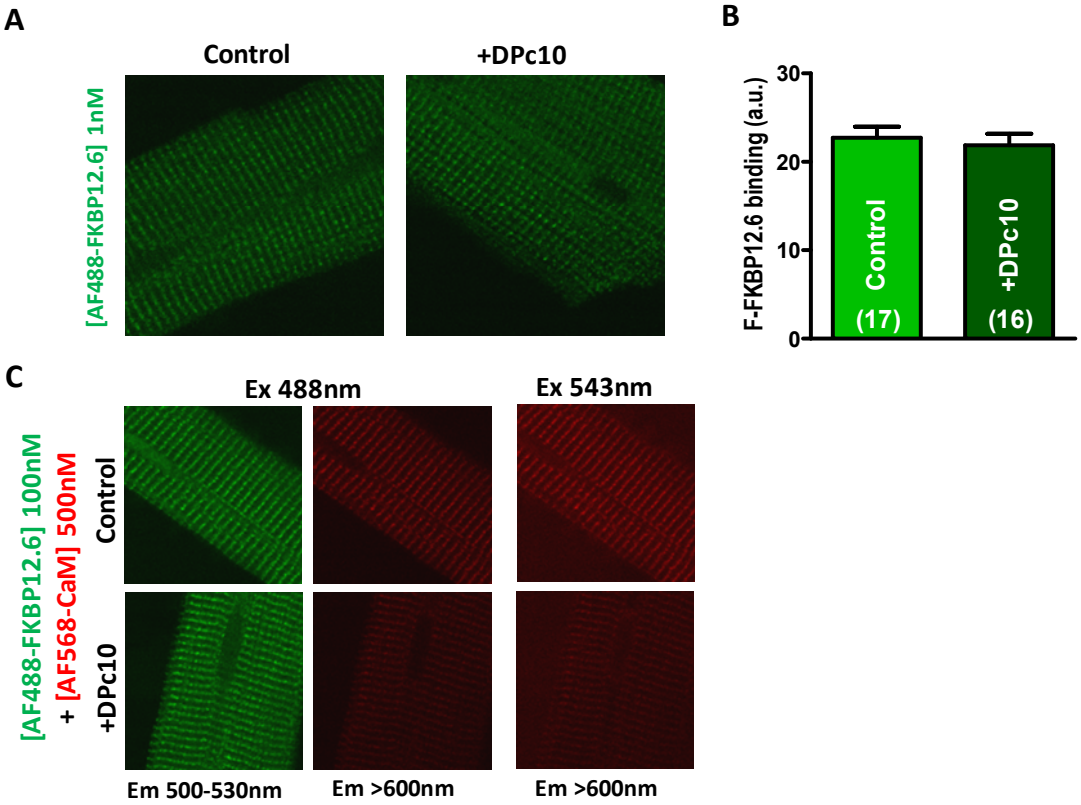
Online Figure III. Effect of DPc10 concentration on the wash-in rate, measured using FRET between F-FKBP12.6 and HF-DPc10. FRET was detected as EAF (**A**) or as donor-fluorescence quenching (**B**), at 0.5 µM (circles) and 5 µM (triangles) HF-DPc10. Data are reported as mean ± SE (n values on bars).



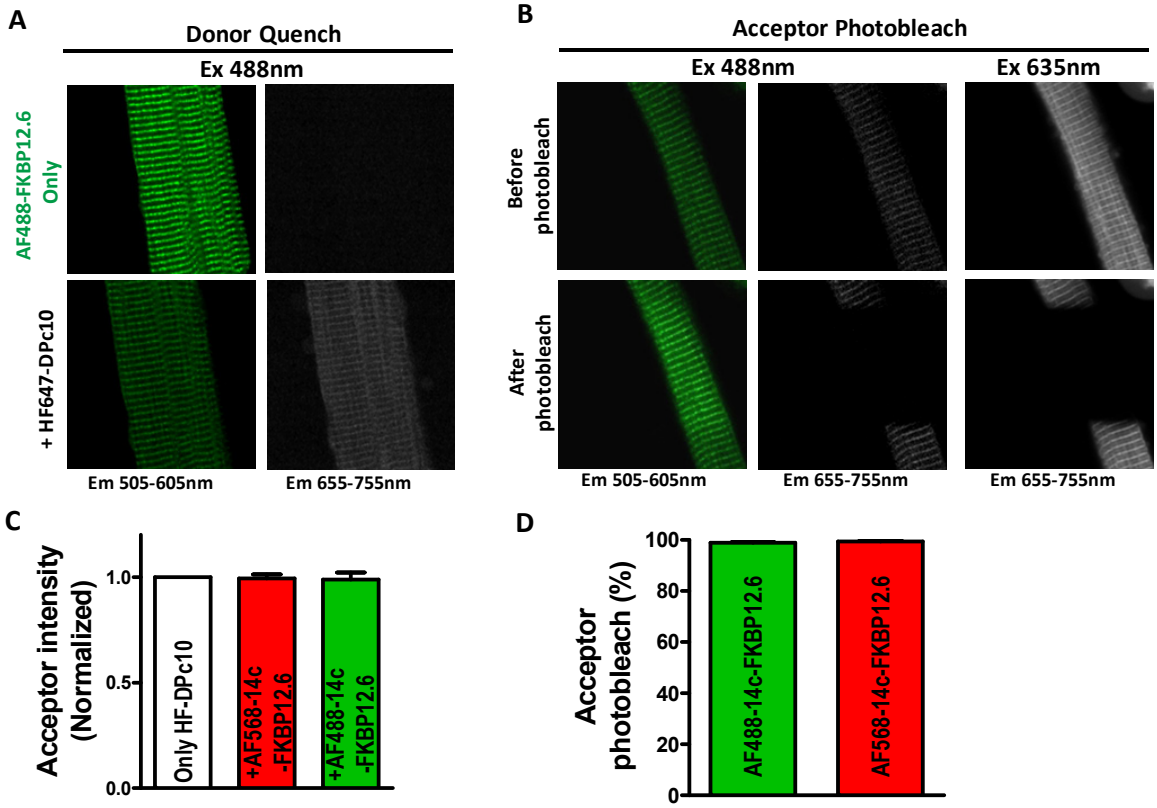
Online Figure IV. Influence of FKBP12.6 or CaM on F-DPc10 fluorescence at the M-line. Addition of FKBP12.6 or CaM does not significantly change the M-line fluorescence intensity of F-DPc10. Data are reported as mean ± SE (n values on bars).



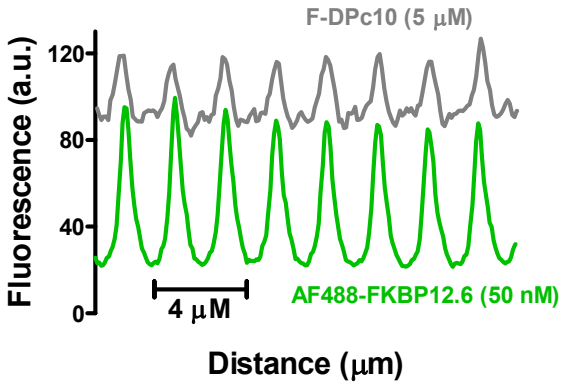
Online Figure V. Ca spark frequency normalized to the SR Ca content for myocytes after addition of DPc10 (5µmol/L), DPc10 (5µmol/L) plus FKBP12.6 (100 nmol/L), DPc10 (5µmol/L) plus CaM (1 µmol/L). Data are reported as mean ± SE (n values on bars).



Online Figure VI. **A** Confocal image of permeabilized myocytes after exposure to 1 nmol/L AF488-FKBP12.6 in the absence or presence of DPc10 5 μmol/L. **B**. Average data for AF488-FKBP12.6 binding to Z-lines with or without DPc10. **C**. Confocal FRET image between AF488-FKBP12.6 100 nmol/L and AF568-CaM 500 nmol/L to detect CaM at the RyR2 and total CaM at the Z-lines with or without DPc10.



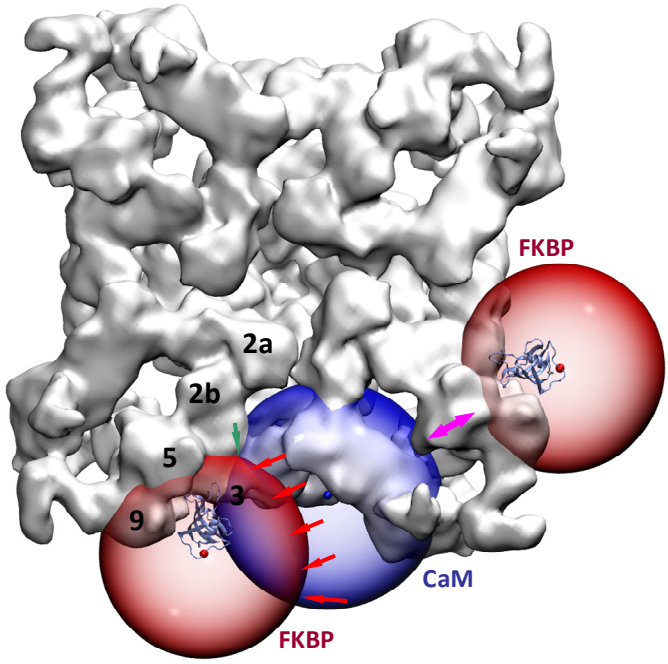
Online Figure VII. **A.** Representative image of AF488-FKBP12.6 quench following addition of HF647-DPc10. **B**. Confocal images showing AF488-FKBP12.6 and HF647-DPc10 fluorescence before and after acceptor photobleaching from permeabilized myocytes. **C**. Summarized data of acceptor (HF647-DPc10) fluorescence intensity with or without equilibrated donors which was normalized to without donor condition. **D**. The extent of the acceptor photobleaching in both cases (AF488-FKBP12.6 and AF568-FKBP12.6).

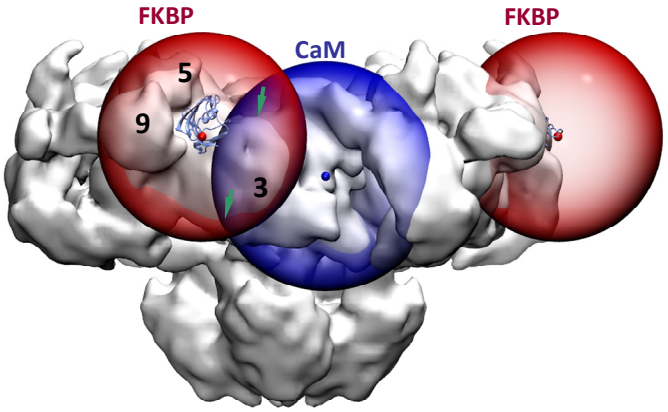


Online Figure VIII. Plot profile of striated sarcomeric pattern shows both F-DPc10 (5 μmol/L) binding and AF488-FKBP12.6 (50 nmol/L) binding at Z-lines and M-lines.

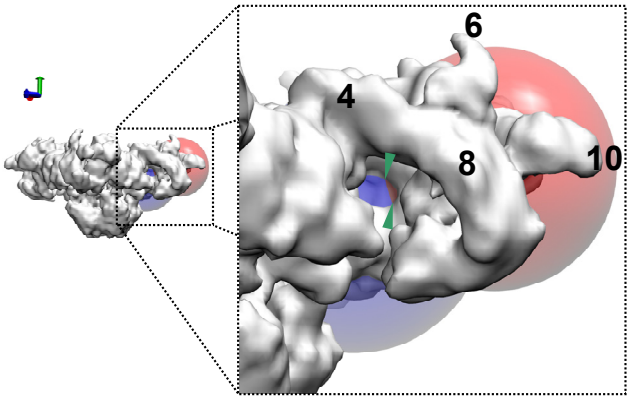
Top view

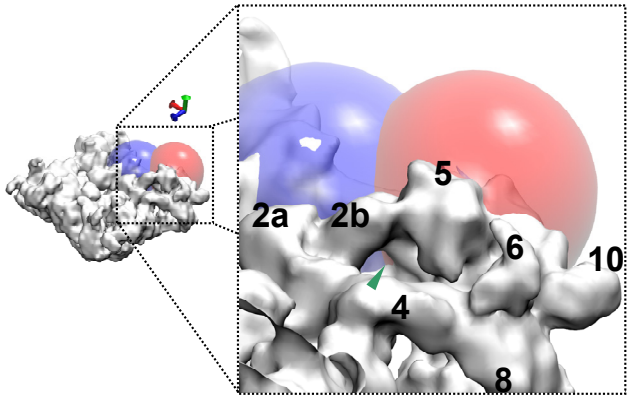
Side view





Online Figure IX. Variant of Figure 7B to better illustrate the location of the DPc10 acceptor within the RyR cryo-EM map, as suggested by FRET measurements from FKBP and CaM donors. The CaM-centered sphere (blue, R = 58 Å) is flanked by two FKBP-centered spheres (red, R = 53 Å) – one on the same face as the shown CaM, and the other one on an adjacent face. Spheres are of radii calculated from FRET, which indicate the distance between the donors at FKBP or CaM and the acceptor at DPc10). The FKBP and CaM spheres on the same face of the RyR clearly intersect along a circle (arrowheads indicating the portion that is not buried within the RyR map) that meets the RyR density map to delineate the locus of the DPc10 acceptor (green arrowhead). However, the FKBP and CaM spheres on adjacent faces of the RyR are separated by more than 20 Å (distance of closest approach indicated by magenta arrow in Top View), suggesting that the DPc10 donor does not locate in domains 5 or 9.





Online Figure X. Sight-lines via galleries formed between the peripheral RyR domains 3, 8, 9, and 10, and the more central domains 2 and 4 allow viewing the HF647-DPc10 locus along the inside face of domain 3.