Supplementary Information

Expanded Materials and Methods

Adult Rabbit Myocyte Isolation

5000 U/mL Heparin was administered to 2-3 kg male New Zealand White Rabbit (1.5-3 months old) 5-30 min prior to anesthesia administration. 10 mg/mL Propofol was intravenously injected at a rate of 0.8 mL/min. After the onset of eye nystagmus, the rabbit was intubated using the v-gel system (Docsinnovent, London UK) and anesthesia was established with isofluorane. Upon areflexia, the heart was excised and rinsed in ice cold MEM (135 mM NaCl, 0.33 mM $NaH_2PO_4 \cdot H_2O$, 5.31 mM KCl, 1 mM MgCl, 2 mM $C_3H_3O_3 \cdot Na$, 10 mM HEPES, 10 mM Na-HEPES, 5.5 mM Glucose, 0.04% Insulin, 0.5% Penicillin/Streptomycin, pH 7.4). The aorta was cannulated and hung on a Langendorff perfusion sytem. A wash solution (MEM with 4 U/mL Heparin) was perfused at a rate of 25-65 mL/min until all blood had cleared. Enzyme solution was then perfused until the heart was digested (~20-26 min). The composition of the enzyme solution varied but was comprised of protease and collagenase. The ventricles were removed, cut into small pieces, and placed in stopping solution (2% BSA and 25 μM CaCl in MEM). Ventricle pieces were placed into tubes and inverted multiple times until the ventricle tissue was dissociated. The cell mixture was filtered with 240 micron nylon mesh and gravity settled.

Heart Failure and Age Matched Myocyte Isolation

Heart failure was induced in New Zealand White rabbits by combined aortic insufficiency and stenosis as previously described.[1] HF progression was monitored by echocardiography. Myocytes were isolated when left ventricular end-systolic dimension exceeded 1.55 cm. At this time point hearts had increased weight, dilated and showed reduced fractional shortening. Some animals also exhibited ascites fluid and evidence of lung edema. For myocyte isolation, the procedure was slightly modified. After cardiac excision and rinsing in nominally Ca-free MEM, the right atrium was removed and the aorta opened to visualize the left coronary ostium for cannulation with a 5F Judkins right catheter (Performa; Merit Medical Systems). Perfusion of the left ventricle and atrium was established, before removal of the right ventricle free wall and application of a purse-string suture to secure the catheter in position. The remainder of the procedure was essentially as above. Cells isolated from the same location and in the same manner from healthy age-matched rabbits were used for controls.

Immunocytochemistry

Rabbit cells were resuspended with normal Tyrode (135 mM NaCl, 5.4 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, 10 mM HEPES, pH 7.4) and plated on laminin coated coverslips for 30-45 min. Cells either remained resting, were treated with BAPTA-AM (50 μM for 30 min followed by a 30 min deesterification period), or were electric field stimulated with 5 ms pulses of 20V for 10 min. Cells were fixed with 4% paraformaldehyde for 10 min, washed 3 times with PBS, and permeablized with 0.2% triton for 10 min. A blocking solution (5% BSA, 1% Penicillin/Streptomycin, 5% goat serum in PBS) was applied for 25 min, followed by overnight incubation with primary antibody diluted in dilution buffer (3% BSA, 0.75% goat serum in PBS) at 4C.

Coverslips were washed 3 times with PBS and incubated with the secondary antibodies: goat anti-rabbit alexaFluor 488 (ThermoFisher, catalog number: A11034, lot number: 1751340) and goat anti-mouse alexaFluor 546 (ThermoFisher, catalog number: A11030, lot number: 51813A). The secondary antibodies were diluted 1:200 in dilution buffer for 2 hours at room temperature (RT). Coverslips were mounted on slides using prolong gold mounting media (ThermoFisher, catalog number: P36935). Alexa Fluor 647-Phalloidin (Thermo Fisher, catalog number: A22287) was used according to its commercial protocol after antibody staining to stain for F-Actin. Expansion microscopy was performed as previously described.[2] Briefly, cells were stained as described above with primary and secondary antibodies before crosslinking with Acryloyl-X SE (6-((acryloyl)amino) hexanoic acid, succinimimidyl ester (Thermo Fisher) overnight at RT. Coverslips were covered with monomer solution (2M NaCl, 8.625% sodium acrylate, 2.5% acrylamide, 0.15% N,N'-methylenebisacrylamide, 0.2% TEMED, and 0.2% APS in PBS) and incubated at 4C for 1 min, then 37C for 2 hours. Cells were digested with Proteinase K (New England Biolabs) in digestion buffer (50 mM Tris, 1 mM EDTA, 0.5% Triton X-100, 1 M NaCl, pH 8) overnight at RT. Gels were placed in an excess volume of deionized water. Water was replaced every 15 min for a total of 60 min.

Primary antibodies: CaMKIIδ (custom, 1:2000, available to investigators upon request)[3], Alpha-Actinin (Sarcomeric) (Sigma-Aldrich, catalog number: A7732, dilution 1:500), Alpha-Tubulin (Cell Signaling Technology, catalog number: 3873S, lot number: 11, 1:200), ATP Synthase Complex V (Thermo Fisher Scientific previously Novex, catalog number: 459240, lot number: K1948, 1:200), RyR (Thermo Fisher Scientific, clone: C3-33, catalog number: MA3-916, lot number: PC196808, 1:200), PLB (Badrilla, PLN, mAB A1, catalog number: A010-14, lot number: 642026,1:200).

Western blotting

Freshly isolated myocytes either remained resting or were field stimulated at 0.5 Hz with 5msec pulses at 20 V for 10 min in normal Tyrode solution. Cells were promptly pelleted before removal of the supernatant and flash-freezing. Myocytes were then lysed in ice-cold buffer containing in mM: 150 NaCl, 10 Tris-HCl (pH 7.4), 1 EGTA, 1 EDTA, 1% Triton X-100, 50 NaPyrophosphate and protease and phosphatase inhibitors (EMD Millipore (set III and V respectively). Cell lysates were flash-frozen and stored at -80°C. Protein content in cell lysates was determined using a BCA assay Cat#23225, Thermo Fisher Scientific Inc. USA). Proteins were then size-fractionated on 4-20% SDS-PAGE before transferring to a 0.2 micron nitrocellulose membrane. Immunoblots were blocked with 5% milk in Tris-buffered saline (TBS). The blots were then incubated overnight at 4°C with primary antibody: CaMKIIδ (custom, 1:15000)[3], RyR (Thermo Fisher Scientific, clone: C3-33, catalog number: MA3-916, lot number: PC196808, 1:1000), PLB (Badrilla, PLN, mAB A1, catalog number: A010-14, lot number: 642026,1:5000), RyR pS2814 (a kind gift from Dr. Xander Wehrens), PLB pT17 (Badrilla, catalog number A010-13, lot number 642024; dilution 1:5000) and CaMKII pT286 (Thermofisher clone 22B1, catalog number MA1-047, dilution 1:1000). This was followed by several washes with TBS-T and secondary antibody incubation for 2 hours at room temperature (926-32210 IRDye 800CW Goat anti-Mouse IgG (H + L) and 926-68071 IRDye® 680RD Goat anti-Rabbit IgG (H+L) (Li-Cor, Lincoln, NE USA)). The blots were again washed in TBST before imaging on the Li-Cor's Odyssey.

Immunosignals were quantified with Image J. All experiments were performed in duplicate on lysates from n=5 rabbit myocyte isolations.

Fluorescence Recovery after Photobleaching (FRAP)

Rabbit myocytes were cultured on laminin-coated coverslips with either PC-1 media supplemented with 1% Pen-Strep, or Media 199 supplemented with 0.1% BSA, ITS liquid media supplement (Sigma), chemically defined lipid concentrate (Thermo Fisher Scientific), and 1% Pen-Strep. Non-attached cells were washed away after 1 hour, and cells were infected with recombinant adenoviruses containing CaMKIIδB-GFP, GFP-CaMKIIδC, or GFP-CaMKIIδC T287D. All CaMKII constructs were based off the rat CaMKIIδ sequence. An HA tag was inserted after the first nine nucleotides of the sequence. CaMKII sequences were inserted into pEGFP-N1 or pEGFP-C3 vectors. These fusion sequences were then put in the pShuttle backbone for use in the AdEasy system (Addgene). WT CaMKIIδB-GFP and GFP-CaMKIIδC constructs were previously generated.[4] The T287D mutant was generated using the QuikChange(R) II XL kit (Agilent) and the following primers: catgatgcacaggcaggaggatgtagactgcttgaagaaa (forward), tttcttcaagcagtctacatcctcctgcctgtgcatcatg (reverse).

Myocytes were used for FRAP experiments within 26 hours of infection. For cytosolic FRAP measurements performed at room temperature, a circular bleaching region (BR) with a diameter of 7.3 μm was used to bleach the fluorescence signal down to 30-40% of the initial value. The regions were bleached with 100% 488 nm laser for 0.4-0.9 s. For nuclear FRAP measurements, a circular BR entirely encompassing the entire nucleus and nuclear envelope was bleached with 100% 488 nm and 50% 405 nm laser for 3 s. Prior to bleaching, three images were acquired, followed by image acquisition every 1.14 s after bleaching. Under BAPTA conditions, cells were treated exactly as the cells used in immunocytochemistry experiments. Under pacing conditions, cells were electric field stimulated with 5 ms pulses of 20V for 5 min after which pacing was stopped and FRAP measurements began. Cells were treated with 250 μm Colchicine (Sigma) (dissolved in water) for 30 min.

Photoactivation Experiments

Photoactivatable tagRFP tagged CaMKII (CaMKII-PA-tRFP) was generated by removing the GFP tag and inserting pPA-tagRFP. Prior to insertion of pPA-tagRFP insertion, a PacI site was removed using the QuikChange(R) II XL kit (Agilent) and the following primers: actggggcacaagcttaattagagcggccgctc (forward), gagcggccgctctaattaagcttgtgccccagt (reverse).

Myocytes were transduced with CaMKIIδC-PA-tRFP for 42-48 hours (longer culturing time was necessary for sufficient expression) before performing experiments at room temperature. A Nikon A1 confocal and 40X objective were used to obtain data in **Fig 2 B-E**. Regions of Interest (ROIs) were activated with 100% 405 nm laser for 30 seconds, and images were acquired every 10 s. An Olympus FV-1000 and 60x objective were used to obtain the data in **Fig 2 A and F**. ROIs were activated with 100% 405 nm laser for 6 s, and images were acquired every 1.14 s.

Image Analysis

All fluorescence values were background subtracted. The CaMKII/actinin profile analysis was automated using Python version 3.5 (http://www.python.org) and the Spyder interactive development environment (http://www.pypi.python.org/pypi/spyder). The actinin channel was skeletonized and used to define regions of interest (ROI). Each skeletonized Z-line was divided into 1.2 μm sections (ROI width), and the ROI was extended 1.2 μm on either side of the Z-line (total ROI length of 2.4 μm). The mean longitudinal CaMKII profile was generated for each ROI. Profiles were sorted into one of four groups based on the proximity of the CaMKII maxima and minima to the Z-line. Among the profiles sorted as Type 1 or Type 2, a further analysis was performed to determine the presence of a smaller CaMKII peak near the m-line. A midline CaMKII peak was counted if there was a greater than 10% increase in CaMKII fluorescence signal within 150 nm of the lowest α-actinin signal (corresponding to the half way point between Z-lines).

The nuclear localization of CaMKII was measured using Image J. DAPI was used to define the nuclear region. A representative cytosolic region at least 3 times the nuclear area was also defined, and the mean fluorescence intensity was measured for both regions.

Colocalization of CaMKII with other proteins was measured using the Image J plugin JaCOP (http://rsb.info.nih.gov/ij/ plugins/track/jacop.html).[5] The nuclear region of each cell was excluded from colocalization analysis.

ImageJ was also used to analyze FRAP and photoactivation images. For FRAP experiments, a circular measurement ROI the same size as the bleaching ROI was used to measure the fluorescence of the bleached region over time. A region on the opposite end of the cell from the bleach ROI was also measured in a subset of cells and showed that the effects of repeated imaging resulted in no decrease in general fluorescence over the entire time course (**Fig S1.**).
Using ImageJ, Photoactivation (PA) measurements were made using a transverse 0.41 x 0.41 μm ROI at defined positions relative to the PA region. Measurements were taken at the center and edge of the PA region. The edge was defined as the region furthest from the PA center that had greatest fluorescence intensity immediately after PA (as opposed to more distal regions that experienced peak fluorescence at later time points). From the edge four successive ROIs were placed so that their center was 0.82, 1.64, 2.46, and 3.28 μm from the center of the edge ROI. Each ROI had 0.41 μm of non-overlapping space between them. The threshold for a cell to be included in analysis required each of the six ROIs to reach 1.5 times the initial (pre-activation) fluorescence value.

The colormap representing the time to peak of each pixel (**Fig 2C)** was made using Python version 3.6. The threshold for inclusion of each pixel in the time to peak determination was 1.5 times the pre-activation fluorescence.

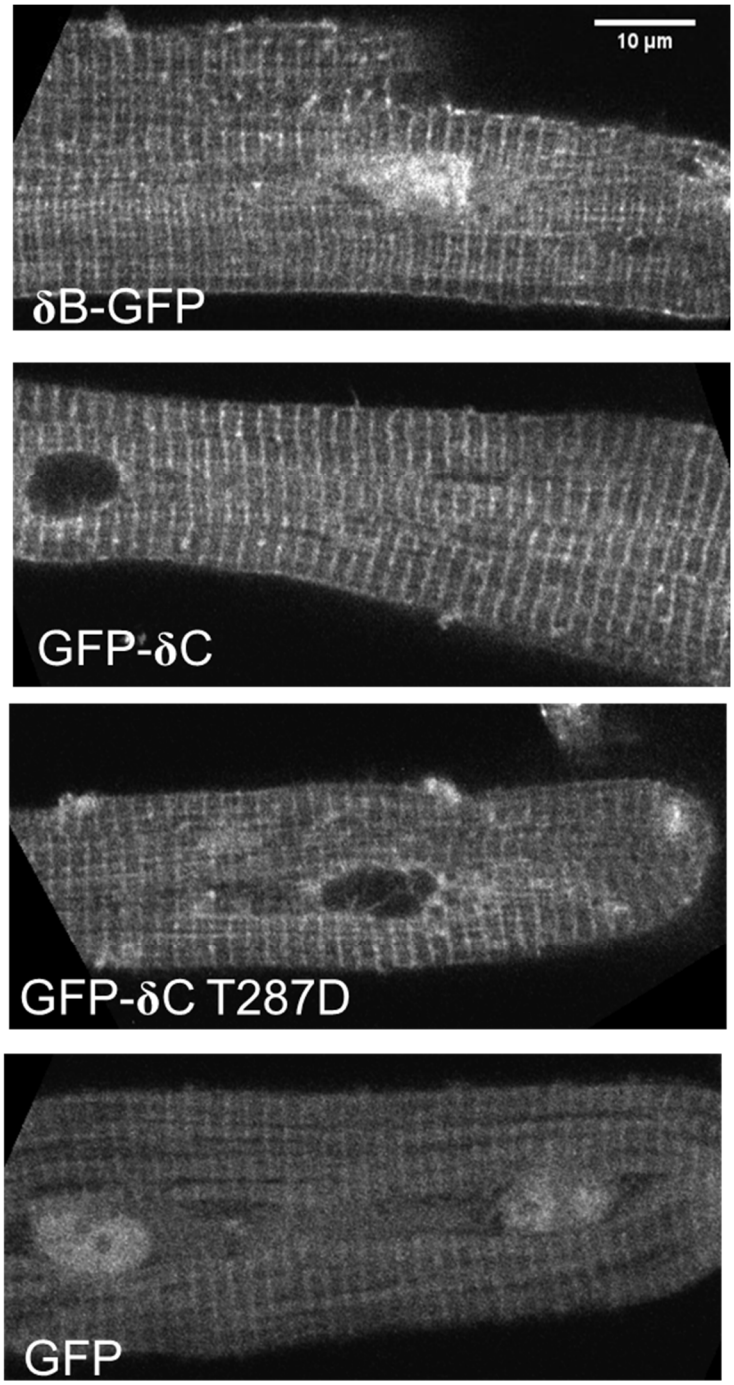
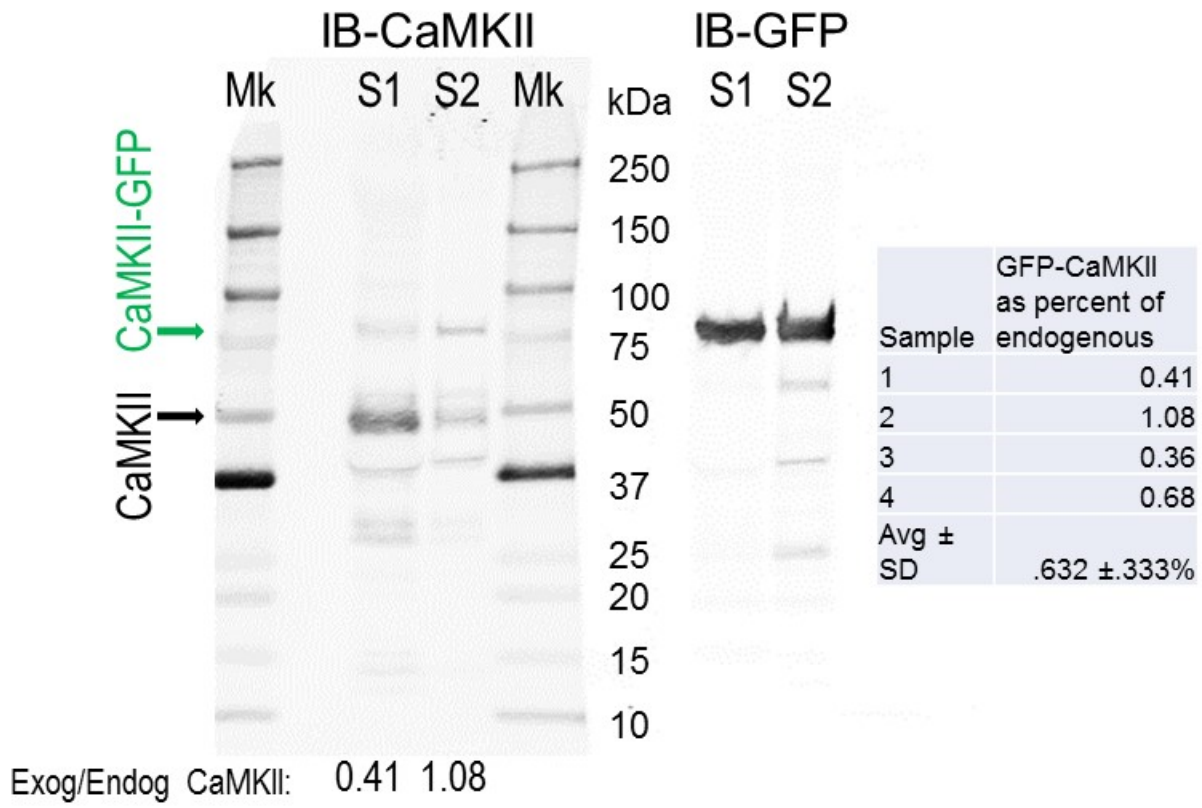


Fig. S1. Example images of CaMKIIδB-GFP, GFP-CaMKIIδC, GFP-CaMKIIδC T287D, and GFP expressing cardiomyocytes prior to FRAP recording.



Sample	GFP-CaMKII as percent of endogenous
1	0.41
2	1.08
3	0.36
4	0.68
Avg ± SD	.632 ±.333%

Fig. S2. Immunoblotting of lysate from GFP-tagged CaMKII expressing myocytes. Blots were probed for CaMKIIδ (left) and GFP (right). Endogenous CaMKII ran at ~50kDa (black arrow), while tagged CaMKII ran at ~80kDa (green arrow). Lane 1 & 4 are MW markers (Mk). Averaged data is from 4 samples taken from 3 rabbits.

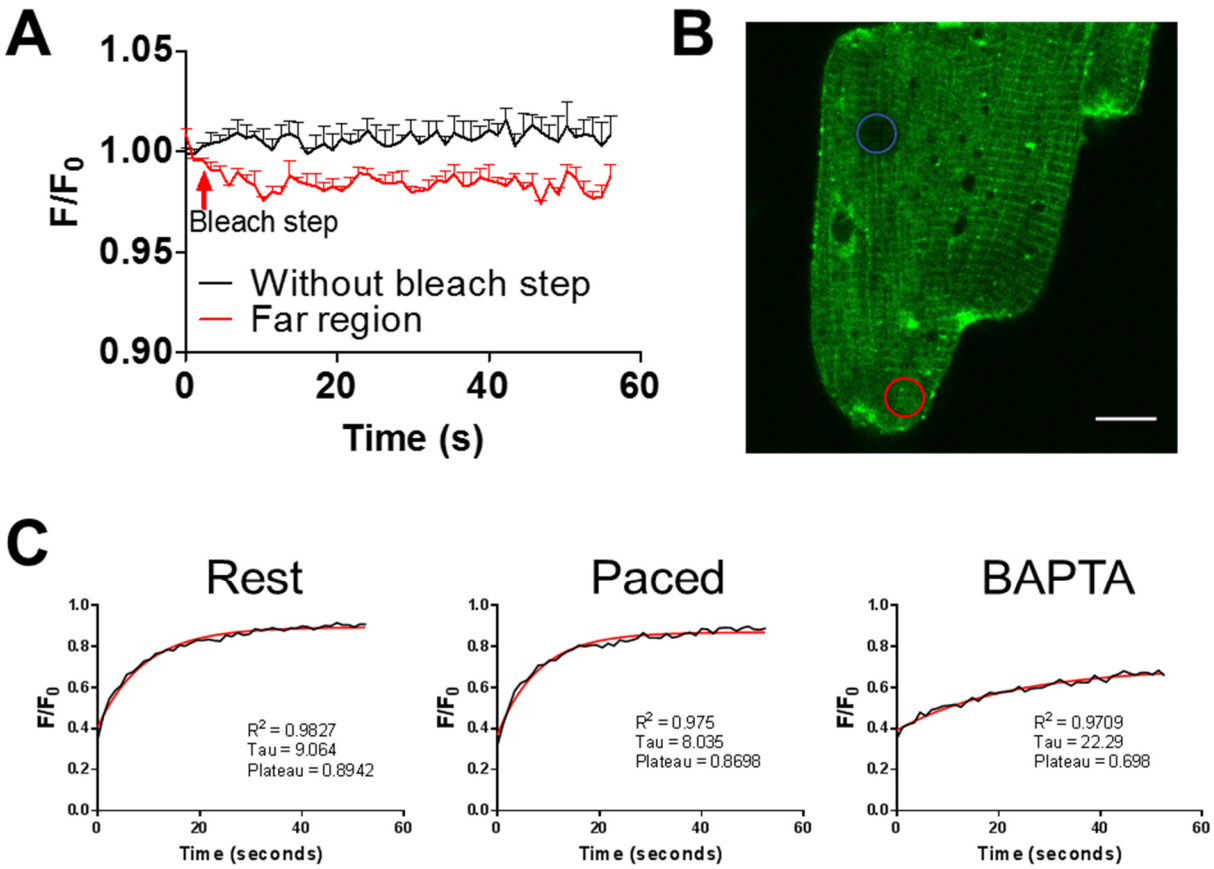


Fig. S3. Verification of FRAP conditions and curve fit. To verify no inadvertent photobleaching had occurred during FRAP experiments, a blank FRAP timecourse was performed by excluding the bleach step. A) Normalized fluorescence was plotted over time for blank FRAP timecourses (black) and for normal FRAP timecourses in regions far from the bleach ROI (red). B) A far region (red) had negligible (~1-2%) reduction in signal compared to the bleach ROI (blue) measured in Figure 1. Scale bar: 10 μm. C) Examples of individual FRAP recovery curves (black) with their single exponential curve fit (red) in Rest, Paced, and BAPTA treated myocytes expressing CaMKIIδC-GFP.

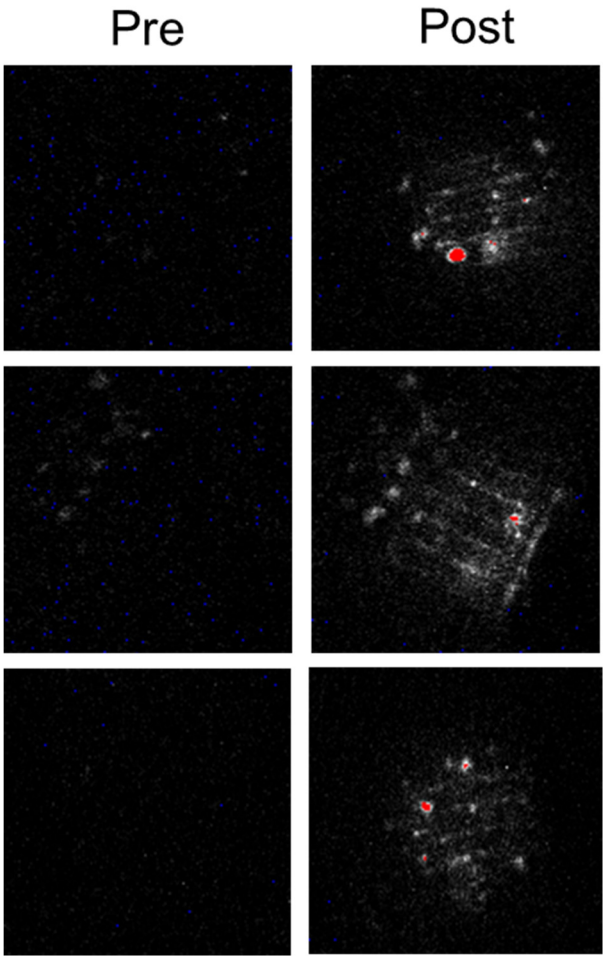


Fig. S4. Example images of PA-tRFP-CaMKIIδC expressing myocytes pre- and post-photoactivation. ROIs were drawn around the identifiable striations, cytosol, and clusters for analysis in Fig 2F. The striation or cytosolic region closest to the middle of the photoactivated region was used. Images from 3 different myocytes (also different from the one shown in Fig 2).

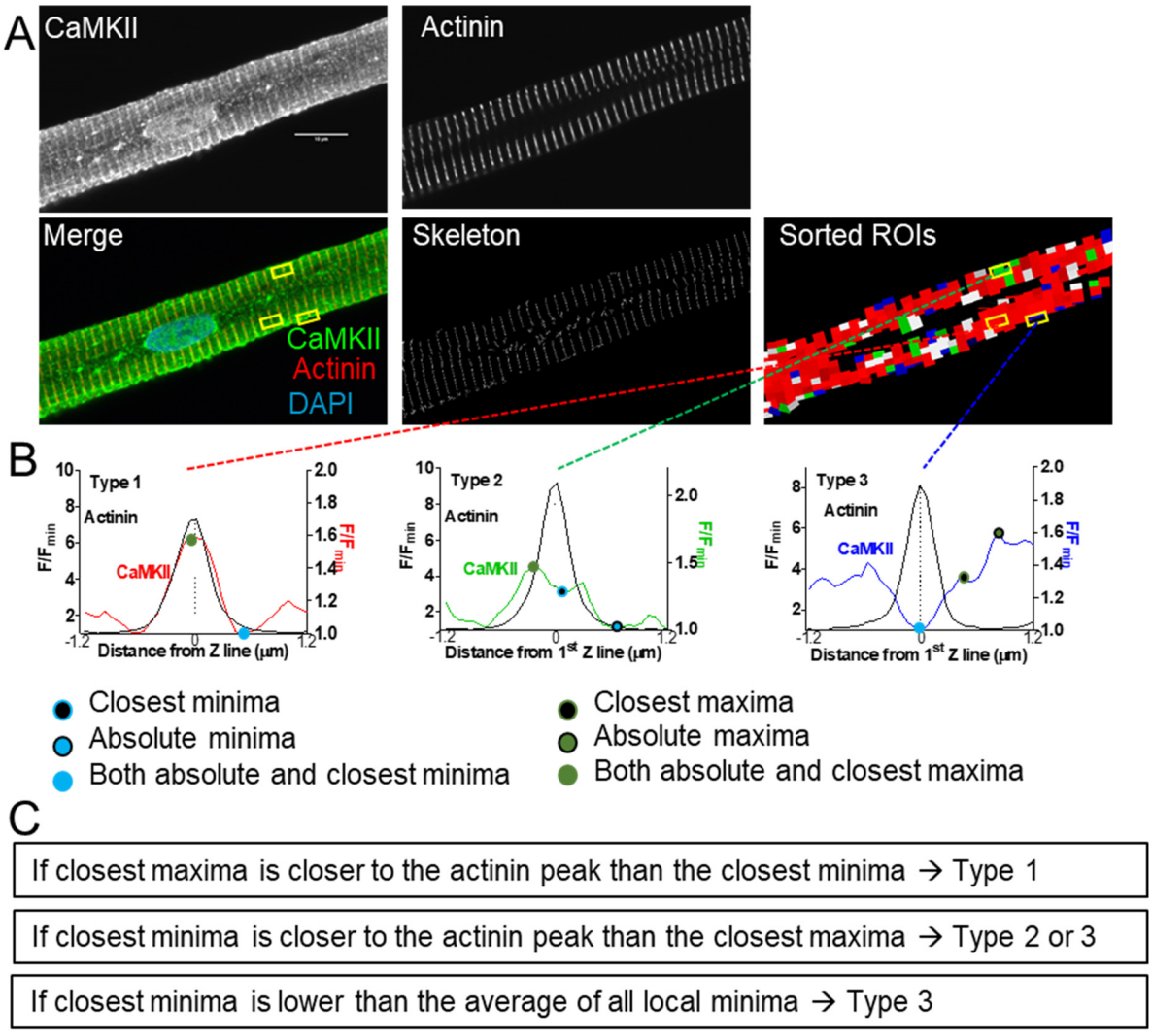


Fig S5. A) Using images of rabbit myocytes coimmunostained for CaMKII and actinin, a skeleton was created using the actinin signal. The skeleton was divided in to 1.2 μm segments and used to define 1.2 x 2.4 μm regions of interest (ROI) across which the mean CaMKII and actinin profiles were measured. The mean profiles for each ROI were generated by averaging longitudinal line profiles across the width of the ROI. Each ROI CaMKII profile was categorized as Type 1 (red), Type 2 (green), Type 3 (blue), or flat (white). B) Type determination was based on identification and the relative proximity of local and absolute minima and maxima. The profiles generated from the yellow boxes shown were obtained after analysis for display purposes. The automated analysis only included sorting of the profiles, not graphing of each individually. C) Profiles were sorted based on the distance of identified minima and maxima to the actinin peak. Scale bar: 10 μm

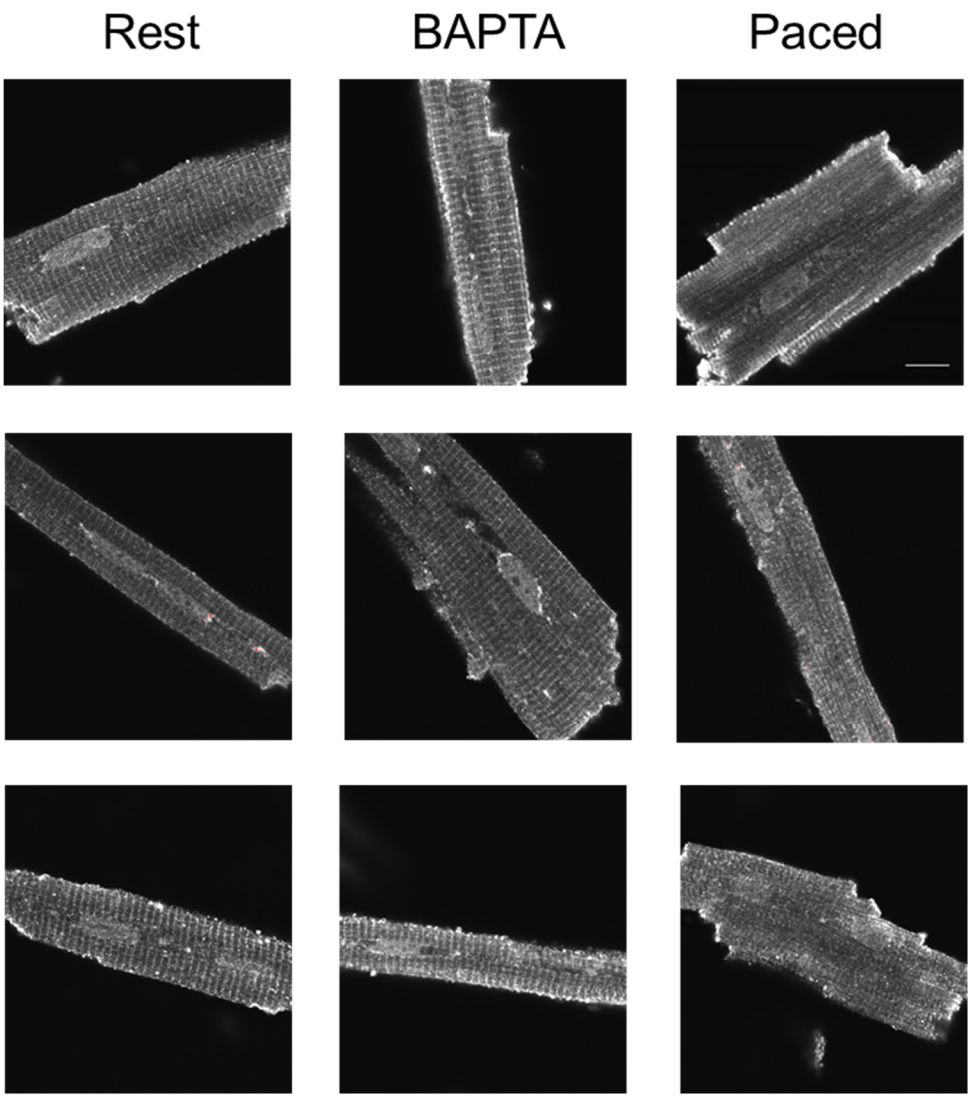


Fig S6. Three randomly chosen example images of rest, BAPTA-AM treated, and 0.5 Hz paced rabbit cells immunostained for CaMKIIδ. CaMKII images, along with their actinin counterpart images were analyzed using the method in Supplementary Figure III to obtain the data in Figure 2. Scale bar: 10 μm

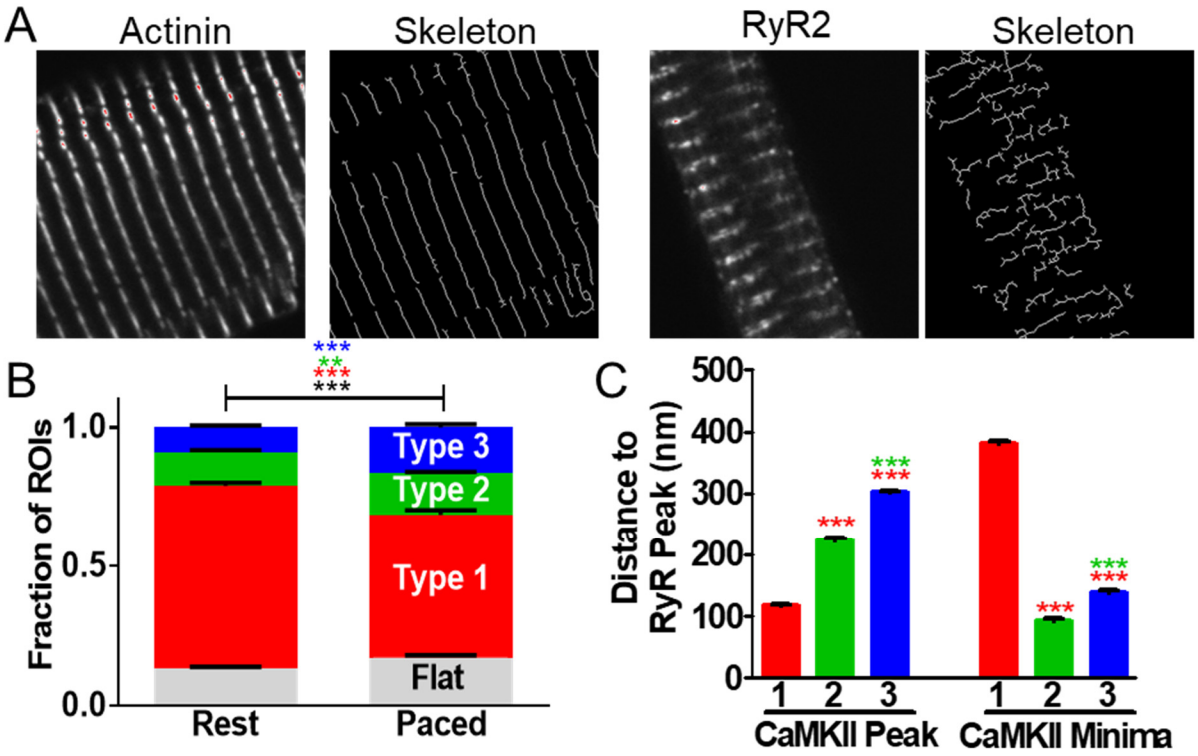


Fig S7. A) Skeletonization of the RyR2 signal results in more longitudinal projections than the actinin signal. Sorting of the CaMKII profiles was repeated with respect to the RyR2 signal. B) The fraction of ROIs of each profile type was determined for rest, BAPTA, and paced cells. D) The distances between the RyR2 peak and the nearest CaMKII peak, and the distance between the RyR2 peak and the nearest CaMKII minima were measured from the mean profile of each ROI for Type 1, 2, and 3 profiles. P values were obtained using a one-way Anova and Tukey's multiple comparison test (***: P value <0.001). Color of the asterisks indicates the comparison being made among the conditions (black: Flat, red: Type 1, green: Type 2, blue: Type 3) Error bars reflect SEM. Cell/animal number per condition: 30/3.

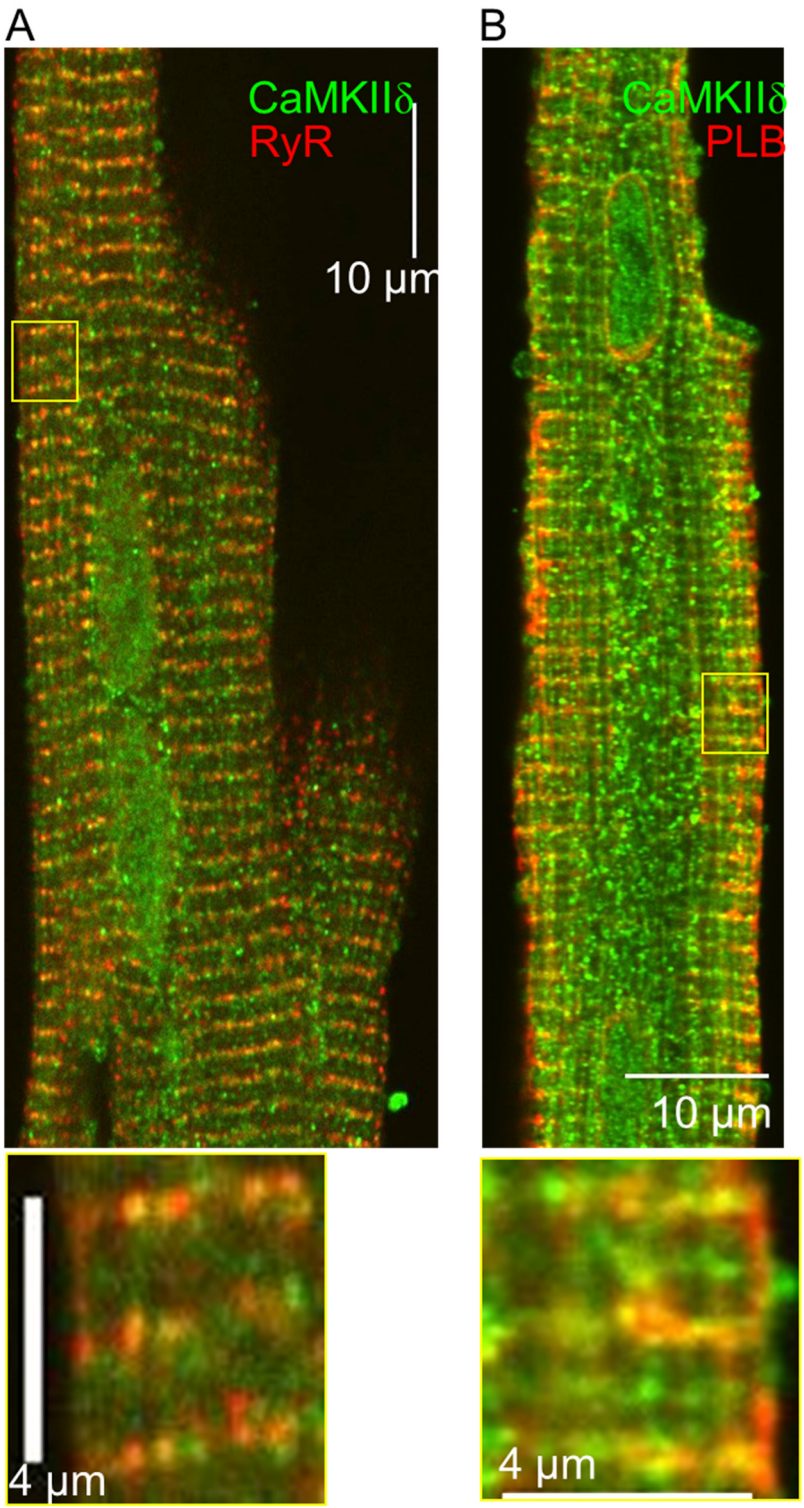


Fig S8. Full image examples of A) CaMKII (green) and RyR (red) and B) CaMKII (green) and PLB (red) immunostained rabbit cardiomyocytes (resting) used for the colocalization analysis in Figure 4.

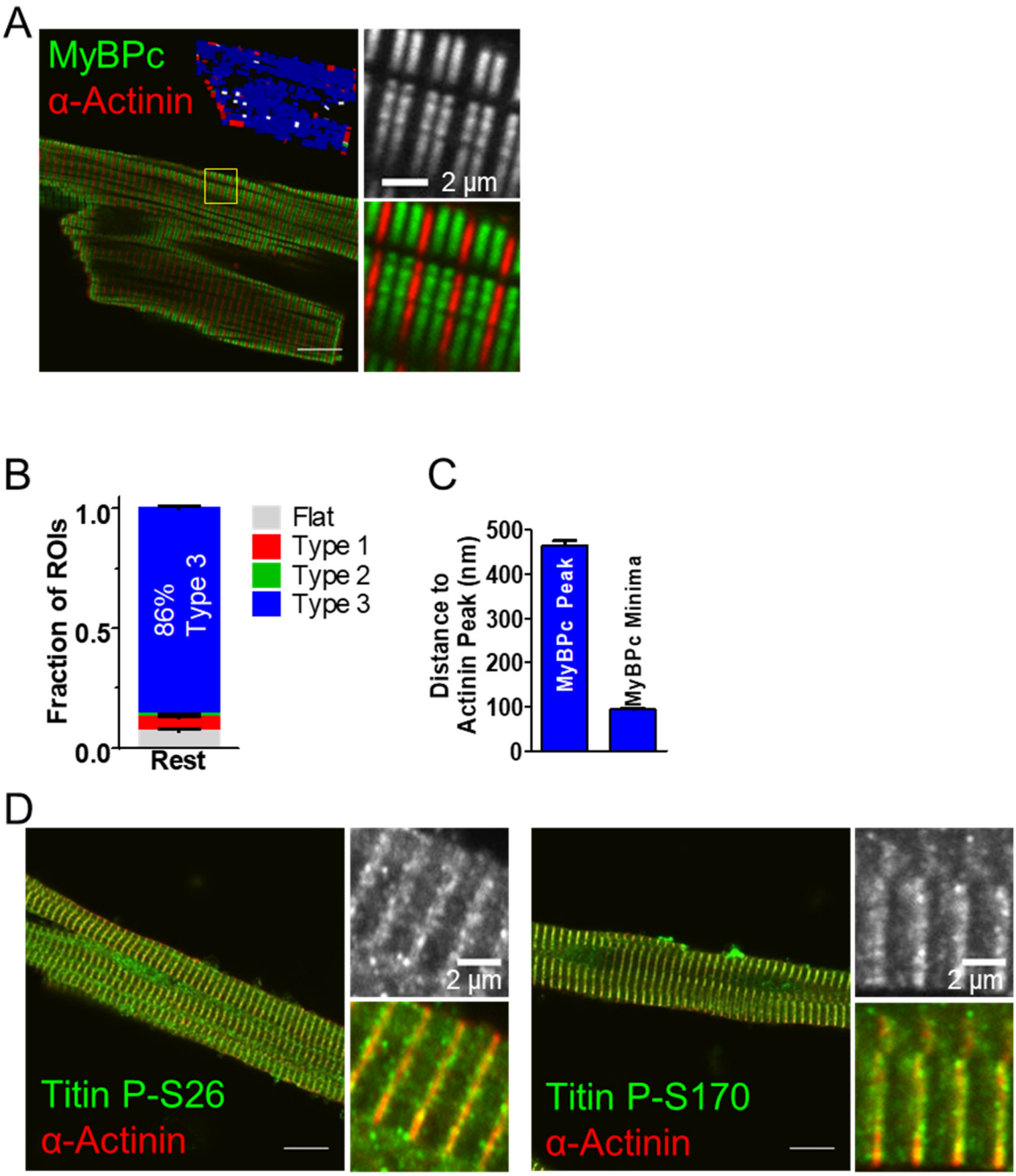


Fig S9. A) Myocytes immunostained for Myosin Binding Protein C (MyBPc) (green: left and lower right, grey: top right) and α-actinin (red) were analyzed as shown previously with CaMKII. ROIs were generated and sorted into Type 1 (red), Type 2 (green), and Type 3 (blue) (upper inset of left image). B) The fraction of ROIs of each profile type was determined for resting myocytes. C) Among the Type 3 classified ROIs, the distance between the actinin peak and the nearest MyBPc peak, and the distance between the actinin peak and the nearest MyBPc minima were measured. D) Myocytes immunostained for α-Actinin (red) and phospho-Titin S26 (green, left) or phospho-Titin S170 (green, right). Cell/animal number: 30/3. Scale bar = 10 μm unless otherwise indicated.

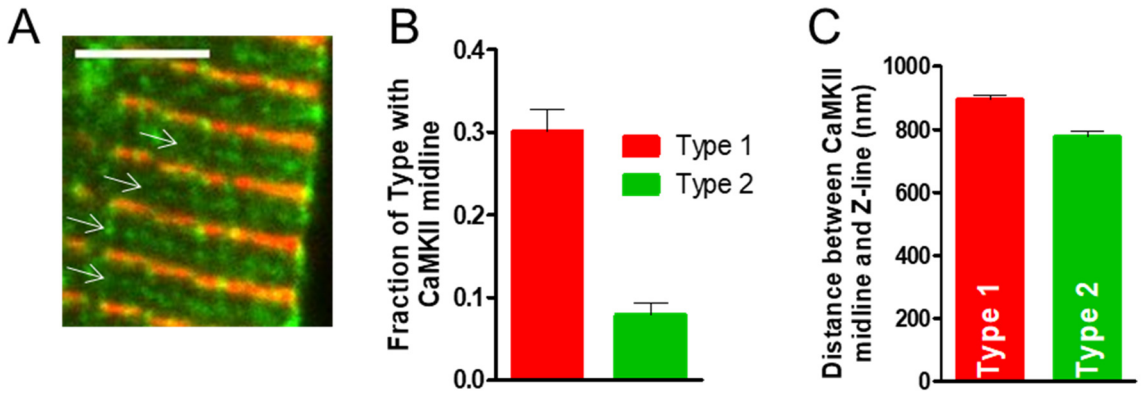


Fig S10. A) An example image of a region with minor CaMKII (green) peaks (indicated by white arrows) at the midpoint between α-actinin (red) peaks. B) The same ROI profiles generated for profile type analysis (Supplementary Fig III) were analyzed for the presence of a 10% increase in signal within 150 nm of actinin minima. The fraction of ROIs with a midline peak present was determined for both Type 1 and Type 2 ROIs. C) The distance between the CaMKII midline peak and the α-actinin peak were determined for Type 1 and Type 2 ROIs. Cell/animal number: 60/5

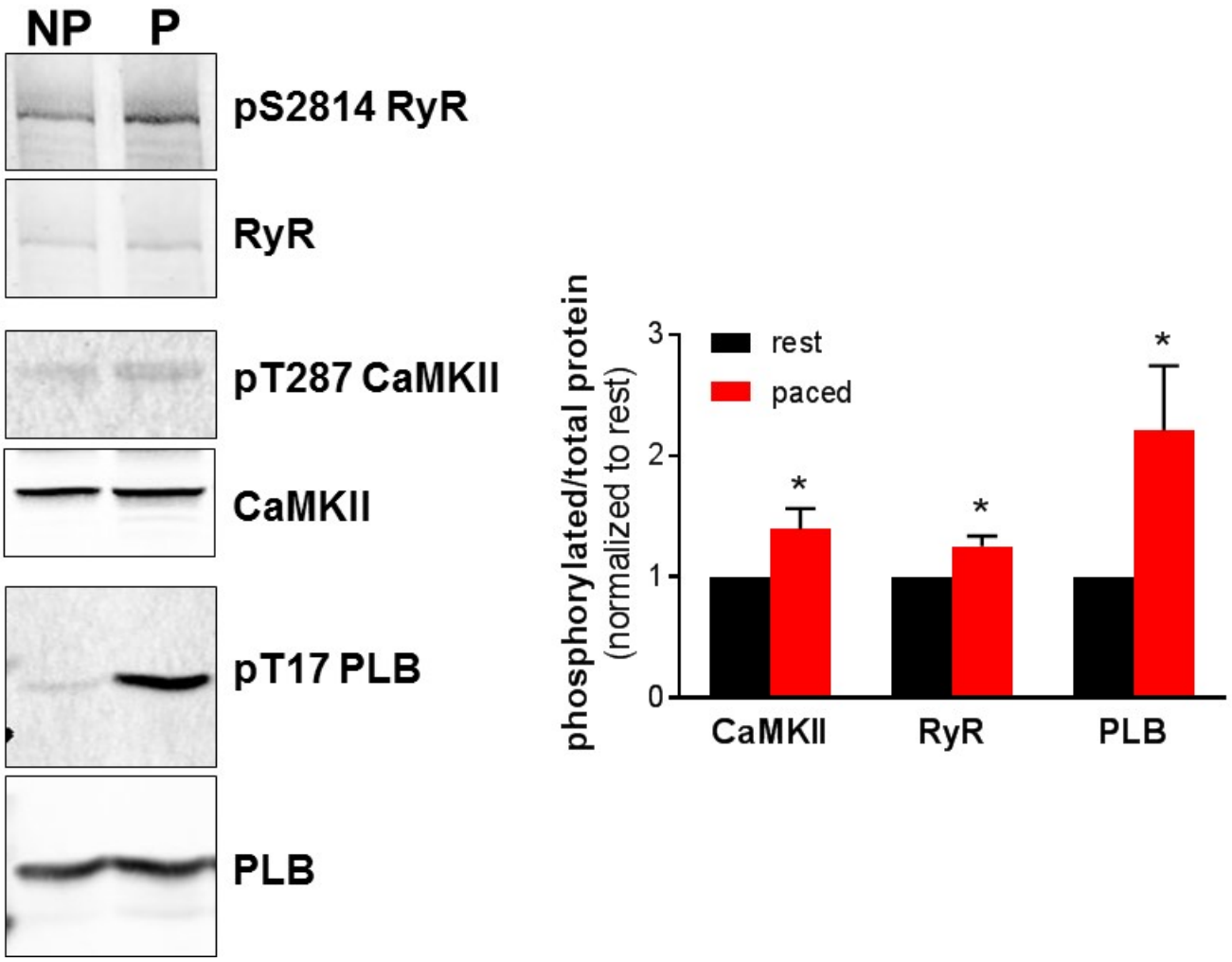


Fig S11. Representative western blots of CaMKII, RyR and PLB phosphorylation levels in myocyte lysates prepared from quiescent cells vs. cells paced for 5 min at 0.5 Hz. Blots were probed with pS2814, pT287 and pT17 antibodies and counterstained with antibodies detecting respectively total RyR, CaMKIIδ and PLB. Isolated myocyte lysates from n=5 hearts; paired).

Movie S1. Example FRAP recording of a GFP-CaMKIIδC expressing rabbit cardiomyocyte before (first 3 images) and after photobleaching of a cytosolic ROI. The video is shown in real time with 1.14s between frames.

Movie S2. Example FRAP recording of a GFP-CaMKIIδC expressing rabbit cardiomyocyte before (first 3 images) and after photobleaching of a nuclear ROI. The video is shown in real time with 1.14s between frames.

References

1. Pogwizd, S.M., Qi, M., Yuan, W., Samarel, A.M., and Bers, D.M. (1999). Upregulation of Na(+)/Ca(2+) exchanger expression and function in an arrhythmogenic rabbit model of heart failure. Circulation research *85*, 1009-1019.
2. Tillberg, P.W., Chen, F., Piatkevich, K.D., Zhao, Y., Yu, C.C., English, B.P., Gao, L., Martorell, A., Suk, H.J., Yoshida, F.*, et al.* (2016). Protein-retention expansion microscopy of cells and tissues labeled using standard fluorescent proteins and antibodies. Nature biotechnology *34*, 987-992.
3. Huke, S., and Bers, D.M. (2007). Temporal dissociation of frequency-dependent acceleration of relaxation and protein phosphorylation by CaMKII. J Mol Cell Cardiol *42*, 590-599.
4. Mishra, S., Gray, C.B., Miyamoto, S., Bers, D.M., and Brown, J.H. (2011). Location matters: clarifying the concept of nuclear and cytosolic CaMKII subtypes. Circulation research *109*, 1354-1362.
5. Bolte, S., and Cordelieres, F.P. (2006). A guided tour into subcellular colocalization analysis in light microscopy. Journal of microscopy *224*, 213-232.