SUPPLEMENTARY MATERIAL TO:

Heart failure leads to altered β_2-adrenoceptor/cAMP dynamics in the sarcolemmal phospholemman/Na,K ATPase microdomain

Bastug et al. Phospholemman cAMP microdomain

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Supplementary Methods

Immunofluorescence and confocal microscopy. Adult rat ventricular myocytes (ARVMs) were fixed for 20 min with ice-cold absolute ethanol at -20°C (for PDE3 staining) or for 5 min with 4% paraformaldehyde (for all other stainings), washed thrice with PBS and treated with the blocking buffer (20% fetal calf serum and 0.15% Triton X in PBS) for 2 h. Next, cells were washed with PBS and co-stained with rabbit polyclonal anti-FXYD1 (Abcam, for antibody information see also Supplementary Table 1) and either mouse monoclonal anti-α_1 antibody (Millipore) or goat polyclonal anti-PDE3A (Santa Cruz) antibodies in the blocking buffer (1:100 antibody dilution) overnight at 4°C. On the next day, cells were washed with PBS, followed by the incubation with the secondary fluorescent dye-coupled antibodies (Molecular Probes/Invitrogen/Life Technologies) at 1:500 dilution for 2 h at room temperature. For live staining of sarcolemmal membranes including T-tubules, PLM-Epac1 expressing ARVMs (48 h after transduction) were washed once with buffer A (see Methods section of the main manuscript) and incubated for 10 min with 1 µmol/L of CellMask® deep red (Life Technologies) membrane dye diluted in the same buffer. After washing the unbound dye two times with buffer A, cells were immediately subjected to confocal analysis. Confocal imaging was performed using Zeiss LSM 710 laser scanning confocal microscope (Carl Zeiss MicroImaging) equipped with a Plan-Apochromat x63/1.40 oil-immersion objective. Images were analysed using the ZEN software (Carl Zeiss MicroImaging).

Immunoblot analysis. ARVMs were lysed in a buffer containing 10 mmol/L HEPES, 300 mmol/L sucrose, 150 mmol/L NaCl, 1% triton X100, protease and phosphatase inhibitors (both from Roche). Protein samples and protein ladder (Precision Plus Protein TM Dual-Colour Standards, Bio-Rad) were run in SDS running buffer (0.025 mol/L Tris, 0.19 mol/L glycine, 1% SDS) in a Mini protean III Tetra Cell system (Bio-Rad). Next, proteins were transferred onto polyvinylidene fluoride membranes (GE Healthcare) pre-rinsed with methanol and transfer buffer (0.025 mol/L Tris, 0.19 mol/L glycine, 20 % methanol, 0.1 % SDS). The gels on

membranes were sandwiched between six layers of filter paper pre-soaked in transfer buffer. Next, the transfer in a Hoeffer TE77 semi-dry transfer unit was carried out at 10 V for 35 min with a current restricted to 250 mA per gel. Membranes were blocked PBST (phosphate buffer saline + 0.1 % Tween) + 5% milk powder (Marvel) over night at 4°C. Membranes was then treated with primary antibodies (diluted in blocking buffer) for 2 h at room temperature (RT). The specific dilutions for primary antibodies are in Supplementary Table 1. The membranes were washed for one hour (each washing step: 10-15 min). To visualize the resulting primary antibody binding, they were incubated with horseradish peroxidise (HRP)-conjugated secondary antibody (diluted in blocking buffer) for 2 h at RT. The specific dilutions for secondary antibodies incubations are in Supplementary Table 2. After another washing procedure, the membranes were processed for enhanced chemiluminescence (ECL) analysis. Here, the HRP conjugated antibodies formed a complex with the ECL reagent mixture, detected via Hyperfilm ECL high performance X-ray film (both from GE Healthcare) for different acquisition times and developed using a Fuji RGII automatic developer machine (Fuji). Upon scanning the protein bands, they were subjected to densiometric analysis.

To analyse PLM phosphorylation, ARVMs were freshly isolated from control or heart failure rats. After recalcification, cells were incubated (37 °C, 5% CO_2) for 90 min in calcium-free Tyrode solution (in mmol/L: NaCl 144, KCl 5.4, $MgCl_2$ 1, HEPES 10, pH 7.3) to reduce basal phosphorylation. Thereafter, cells were equilibrated for 15 min in the same buffer with added 1 mmol/L calcium chloride, pretreated for 2 min with 100 nmol/L CGP20712A and then stimulated for 5 min with 100 nmol/L isoproterenol, shock-frozen and lysed for immunoblot analysis using homemade PLM Phospho-Ser68 and GAPDH (HyTest) antibodies.

Cell surface biotinylation. ARVMs were transduced with PLM-Epac1 adenovirus (MOI300) for 48 h. Biotinylation solution containing 1 mg/mL sulfo-SS-NHS-biotin in PBS (Thermo Fischer Scientific) was added to the cells and incubated for 10 min at 37°C in incubator. After rinsing the cells to remove the excess biotinylation reagent, cells were lysed in 0.5 mL per well

of 1% triton X-100 in PBS supplemented with protease inhibitors (lysis buffer) for 30 min at 4°C. Samples were then transferred to tubes (scraped and harvested from plate surface) and spun down at 17.500 *g* for 5 min at 4°C. The supernatant was kept for further analysis, additionally one sample (50 µL) was retained from it and mixed 1:1 with 2x SDS PAGE sample buffer without β-mercaptoethanol (referred to as starting material). The retained supernatant was added to Streptavidin-Sepharose beads. Beforehand, ~30 µL beads per sample were washed multiple times, pre-equilibrated in lysis buffer at 4°C and recovered by centrifuging 1 min at 17.500 *g*. The beads were incubated with the supernatant of the lysed cell pellet for 1-4 hours at 4°C or overnight. The beads were spun down at 17.500 *g* for 2 min at 4°C. A small sample (100 µL) was taken and mixed 1:1 with 2x SDS-PAGE sample buffer with 5% β-mercaptoethanol (referred to as unbound fraction). The beads were washed with lysis buffer. Following final washing step, 100 µL 2x SDS-PAGE sample buffer with 5% β-mercaptoethanol was added. Before SDS-PAGE/immunoblot analysis, samples were heated at 60°C for 15 min to elute proteins in the bound fraction (referred to as the beads fraction).

Co-immunoprecipitation. ARVMs were transduced with PLM-Epac1 or Epac1-camps adenovirus for 48 h. On the day of experiment, all samples were handled at 4°C during the entire procedure. 20-30 µL of the GFP-Trap® reagent was washed 5 x 1mL in lysis buffer containing 2 mg/mL octaethylene glycol monododecyl ester in co-IP buffer (1 mol/L EDTA in PBS plus protease and phosphates inhibitors) and equilibrated overnight at 4°C. Upon lysing the cells in 500 µL co-IP lysis buffer, the cells were carefully scraped from the bottom of the well, mixed at 4°C and centrifuged (17000 *g*) for 5 min at 4°C. The lysates were incubated with the GFP-Trap® beads over night at 4°C. Thereafter, the beads were washed 5 times (1mL each) with 4°C co-IP buffer containing 1 mM EDTA in PBS plus protease and phosphates inhibitors and 0.5 mg/mL octaethylene glycol monododecyl ester. The beads were then resuspended in 100 µL 2x SDS PAGE sample buffer supplemented with 5% β-mercaptoethanol. The same protocol was applied for co-IP analysis by means of primary NKA antibody immobilized on Protein G Sepharose 4 Fast Flow® (GE Healthcare). 20 µL sepharose

beads were washed with co-IP lysis buffer (5 x 1mL) and equilibrated overnight at 4°C (or for 1-2 h at RT). 1 µg of primary antibody was pre-immobilized on the beads overnight at 4°C. The beads were incubated with solubilized and centrifuged supernatants of ARVMs overnight at 4°C. Samples were collected at each step throughout the entire procedure, and probed using immunoblots with specific antibodies.

Ouabain sensitive ^{86}Rb uptake measurements. We measured the ouabain sensitive ^{86}Rb uptake as a surrogate for potassium to determine the NKA activity of transfected HEK293 cells stably expressing PLM-Epac1 sensor or untransfected control cells. Cells were cultured on 12-well plates until they reached 70% confluency. The measurements were performed in the presence of the sodium ionophore monensin (Sigma) to ensure consistent intracellular Na^+ and the Na/K/2Cl cotransporter inhibitor bumetanide (Sigma) to depress background uptake of ^{86}Rb. Cells were rinsed with the extracellular solution (in mmol/L: NaCl 20, NMDG-Cl 120, $MgCl_2$ 1, $CaCl_2$ 1, KCl 5, HEPES 10, glucose 10, bumetanide 0.1, pH 7.4, supplemented with 10 µg/mL monensin) and equilibrated with exactly 1 mL/well of same solution for 20 min at 37°C. During change of the medium, we added 100 µM ouabain to half of the wells and PBS (vehicle) to the other half and incubated at 37°C for 5 min before initiating ^{86}Rb uptake by the addition of 1 µCi/mL ^{86}Rb per well. Cells were incubated at 37°C for exactly 15 min prior to stopping the uptake by rapidly washing steps in three baths of ice cold PBS. The complete wash protocol should take ~30 s. Hereupon, cells were lysed in 0.2 mL lysis buffer per well (1% Triton-100 in PBS). The specific activity of the ^{86}Rb solution is determined through 3x measurements of 1 µL ^{86}Rb solution in 3 scintillation vials ('total counts vials'). Next to this, 3x 50 µL of each cell lysate per well was transferred to 3 separate scintillation vials for counting. The number of ^{86}Rb molecules of the lysates were measured in a liquid scintillation counter, and the protein content of lysates was measured in a spectrophotometer using a Bradford assay with bovine serum albumin as a standard.

Quantitative real-time PCR. Total RNA was isolated from ARCMs using RNeasy Plus Micro Kit (QIAGEN, Düsseldorf, Germany). The concentration of the extracted total RNA was determined using the DS-11 spectrophotometer (DeNovix; Wilmington, DE; USA). 15 ng of total RNA was used for cDNA synthesis by iScript cDNA Synthesis Kit (Biozym, Hessisch Oldendorf; Germany) and the GeneTouch thermal cycler (Bioer, Hangzhou, China). qPCR reactions were performed in triplicates using PerfeCTa SYBR Green SuperMix (Quanta, Beverly, MA, USA) and Rotor-Gene Q (QIAGEN, Düsseldorf, Germany). A three-step protocol was repeated for 50 cycles (95°C for 10 sec, 60°C for 15 sec, 72°C for 20 sec). The data are expressed as the diffecence between the cycle threshold (Ct) of the gene of interest (goi) and calsequestrin (CSQ) $\left(2^{-(Ct_{goi}-Ct_{CSQ})}\right)$.

Primers used for qPCR were as follows:

Gene	**Number of bp**	**Primer sequence**
AC5 (ADCY5)	22	For.: CACCGCCAATGCCATAGACTTC
	21	Rev.: CAGAATGTTGTGCAGCAAGCG
AC6 (ADCY6)	20	For.: TGACCGAATACAGGTGACCA
	21	Rev.: GGTTGAATTTCCACTGCTGGC
CSQ (CASQ)	20	For.: TACGATGGGAAGGACCGAGT
	20	Rev.: GCCACAAGCTCCAGTACGAT

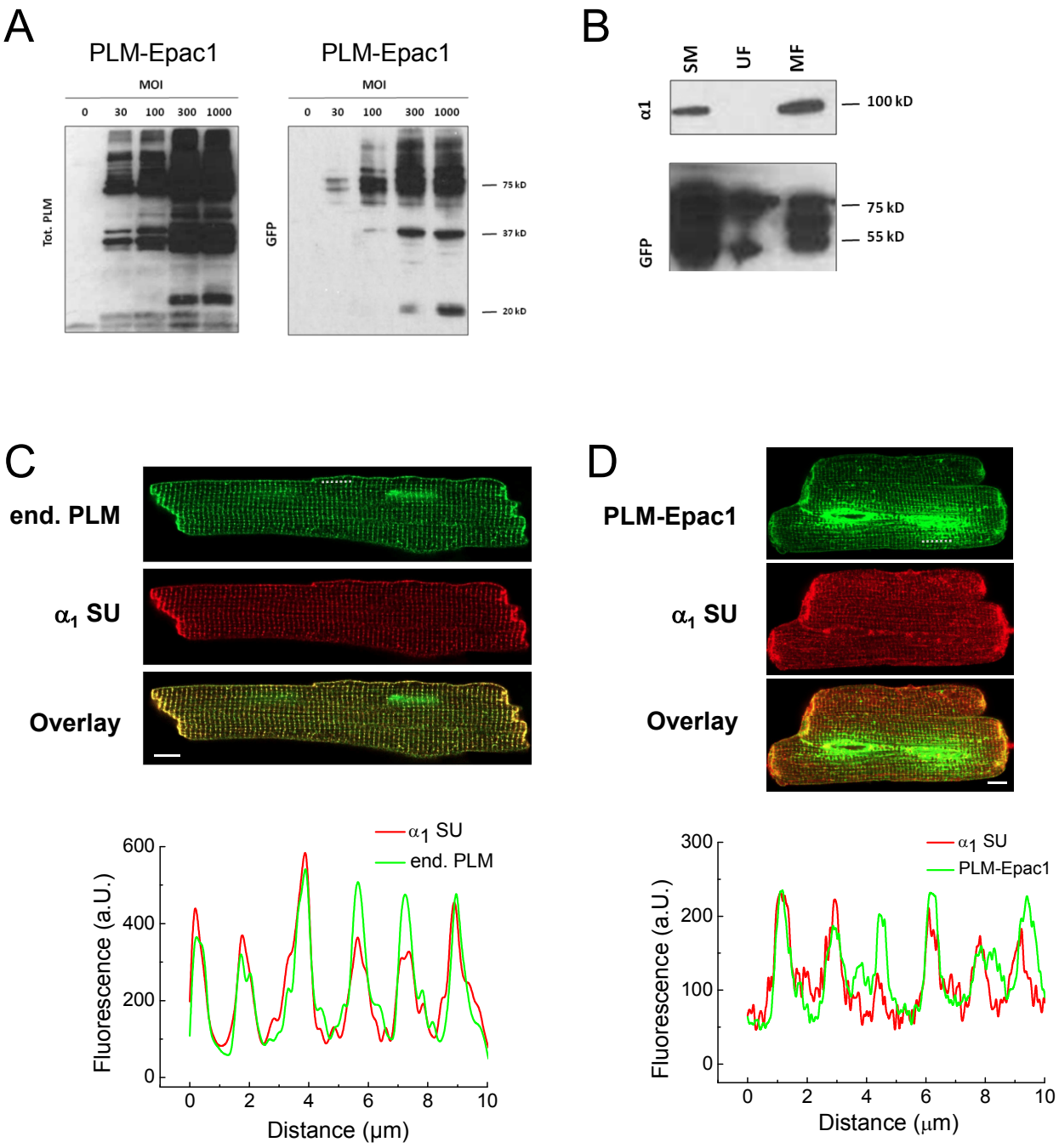
Supplementary Tables

Supplementary Table 1. **Primary antibodies** are used for immunoblotting and immunofluorescence analysis. Antibodies for immunoblot analysis were diluted in a blocking buffer containing TBS + 1% Tween + 5% milk powder. For immunofluorescence experiments the blocking buffer contained PBS +10% fetal calf serum + 0.2% Triton-X-100.

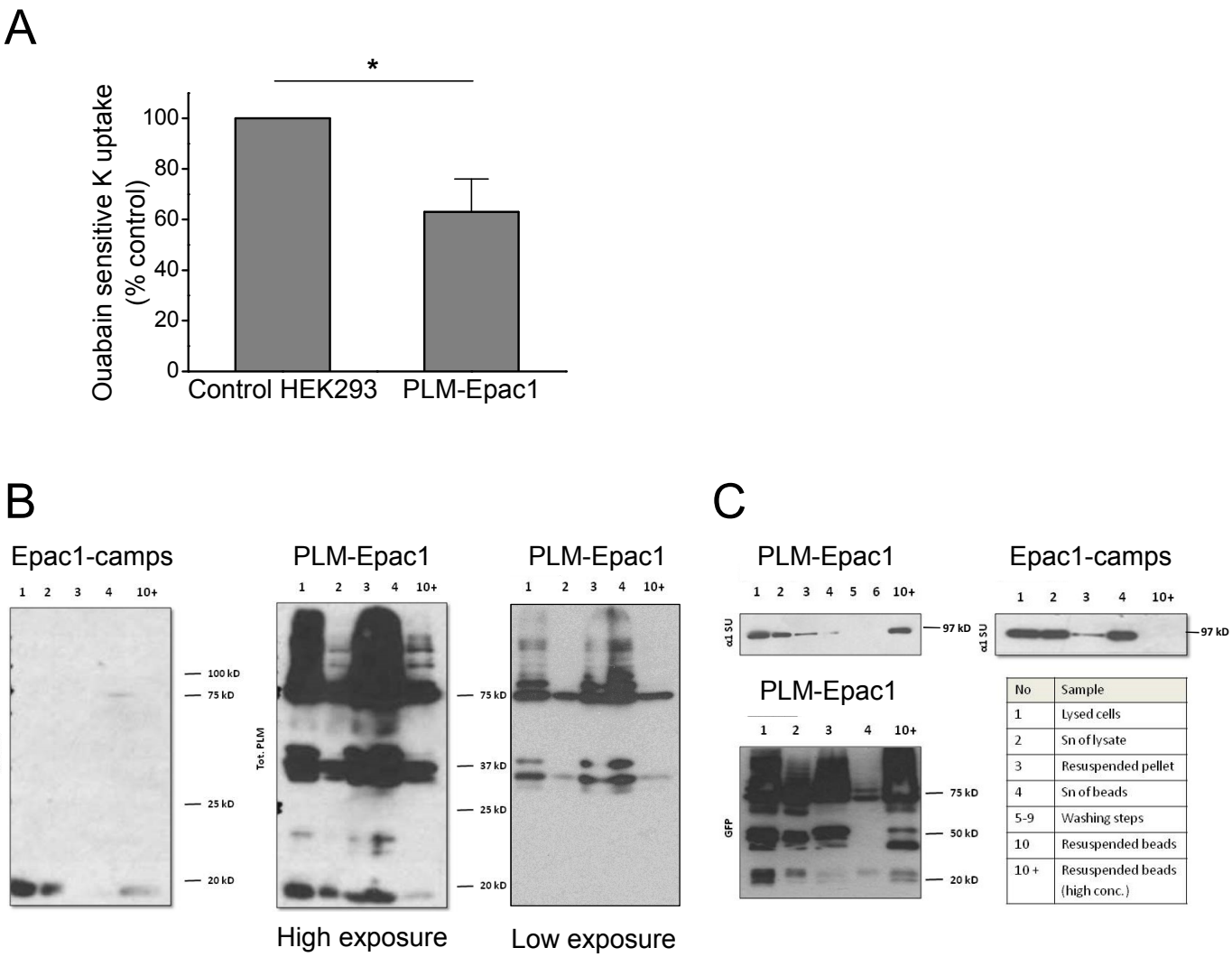
Antibody	Dilution	Manufacturer
anti-α_1 mouse monoclonal C464.6	Immunoblot: 1:10000 Immunofluorescence: 1:100	Millipore, # 05-369
anti-FXYD1 (PLM) rabbit polyclonal	Immunoblot: 1:10000 Immunofluorescence: 1:100	Abcam, # ab76597
anti-GFP rabbit monoclonal	Immunoblot: 1:10000	Abcam, # ab32146
anti-PDE3A goat polyclonal	Immunofluorescence: 1:100	Santa Cruz, # sc11834
anti-caveolin 3 mouse monoclonal	Immunofluorescence: 1:300	BD Biosciences, #610421
anti-β_1-adrenergic receptor (A20)	Immunoblot: 1:500	Santa Cruz, # sc-567
anti-β_2-adrenergic receptor (H20)	Immunoblot: 1:500	Santa Cruz, # sc-9042
anti-PKA RIα	Immunoblot: 1:1000	BD Biosciences, #610165
anti-PKA RIIα	Immunoblot: 1:1000	BD Biosciences, #612242
anti-GAPDH	Immunoblot: 1:160000	HyTest, # 6C5
anti-Calsequestrin (CSQ)	Immunoblot: 1:10000	Thermo, # PA1-913,

Supplementary Table 2. Secondary antibodies used for immunoblot and immunofluorescence analysis.

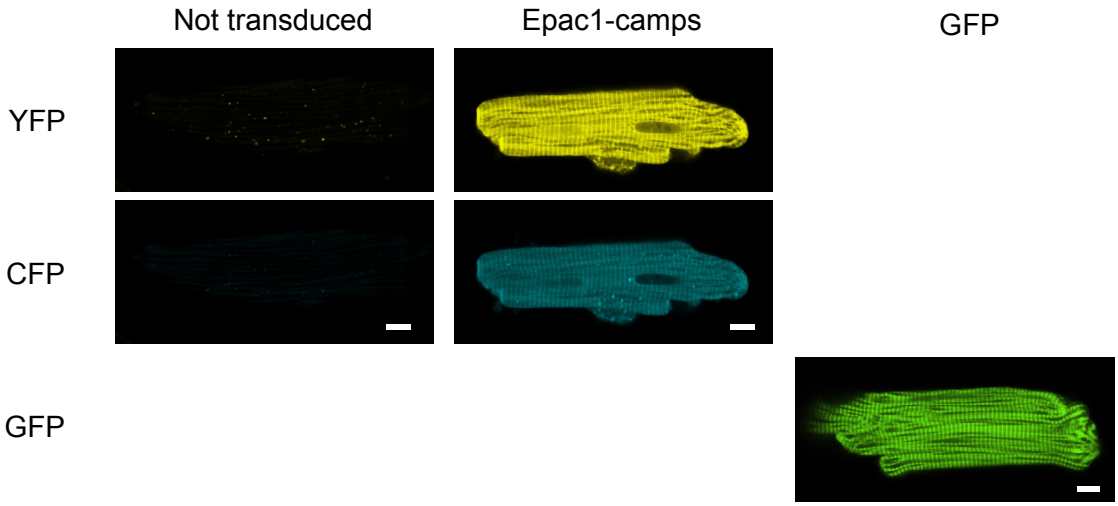
Antibody	Dilution	Manufacturer
Alexa Fluor® 488 donkey anti-goat	Immunofluorescence: 1:500	Invitrogen, # A11055
Alexa Fluor® 633 goat anti-rabbit	Immunofluorescence: 1:500	Invitrogen, # A21070
Alexa Fluor® 488 goat anti-rabbit	Immunofluorescence: 1:500	Invitrogen, # A11034
Alexa Fluor® 633 rabbit anti-mouse	Immunofluorescence: 1:500	Invitrogen, # A21063
Immun-Star™ goat anti-mouse-HRP	Immunoblot: 1:5000	Biorad, # 170-5047
Immun-Star™ goat anti-rabbit-HRP	Immunoblot: 1:5000	Biorad, # 170-5046



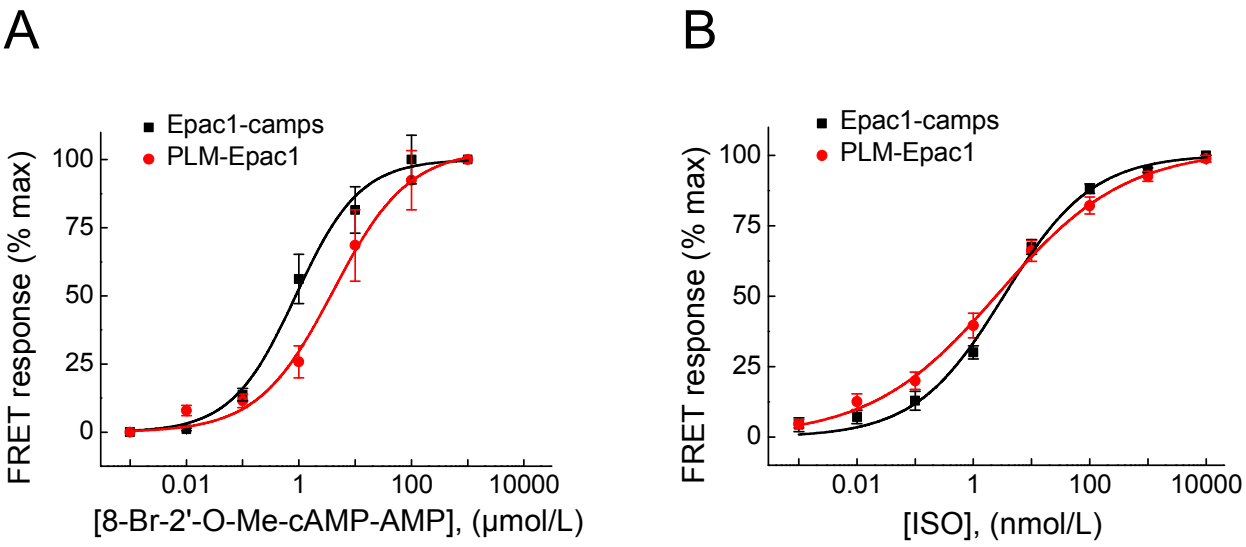
Supplementary Figure 1. Expression and co-localization of PLM-Epac1 with the NKA α_1 subunit. (**A**) Immunoblot analysis for the adenoviral expression of PLM-Epac1 in ARVMs at the multiplicity of infection (MOI) of 10, 30, 100, 300 or 1000. Cell lysates were subjected to 10 % SDS-PAGE and immunoblotting using anti-PLM and anti-GFP antibodies (which detects CFP and YFP moieties of the biosensor). Blots showing PLM-Epac1 at ~ 75 kD are representative of at least three individual experiments. (**B**) Representative immunoblot (n=3) showing cell surface biotinylation of PLM-Epac1 transduced ARVMs. Biotinylated cell surface proteins that were not degraded during lysis (Starting Material, SM) were purified using streptavidin-sepharose beads. Collected unbound fraction (UF) and cell surface proteins in the membrane fraction (MF) were immunoblotted for PLM-Epac1 using anti-GFP and anti-NKA α_1-subunit antibodies. (**C,D**) Representative confocal images (n=7 each) of untransduced and PLM-Epac1 transduced (MOI 300) ARVMs immunostained for co-localization of PLM with NKA α_1-subunit (α_1 SU) confirmed by the intensity overlay of both fluorescent signals. end. PLM, endogenous PLM. Scale bars, 10 μm.



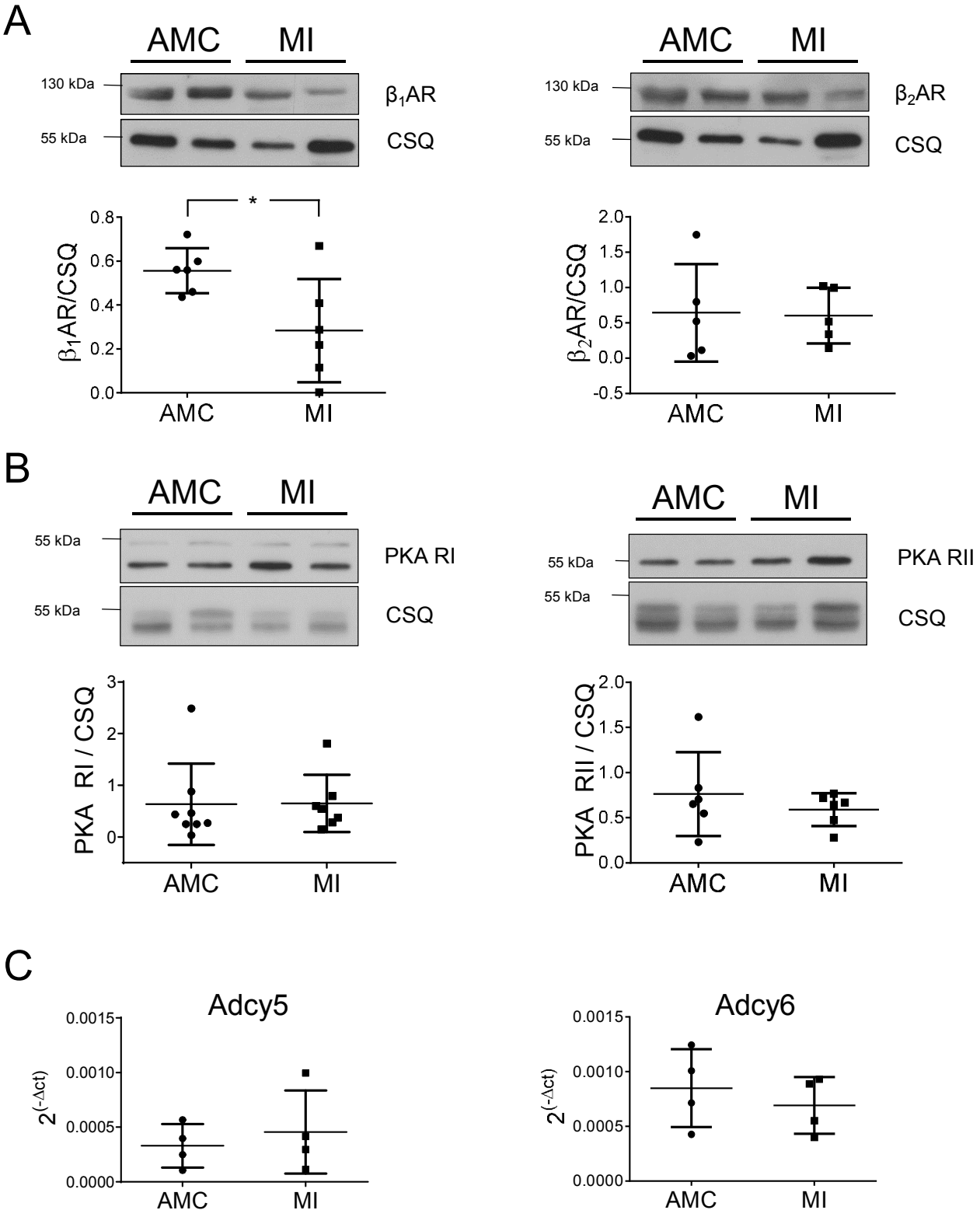
Supplementary Figure 2. Functionality and physical association of PLM-Epac1 with the sodium-potassium pump. (**A**) Quantification of PLM-Epac1 mediated inhibition of NKA activity. Sodium pump activity was measured as ouabain-sensitive ^{86}Rb uptake in HEK293 cells stably expressing PLM-Epac1. In this assay, pump activity is expressed as a percentage of the activity measured in untransfected HEK293 cells which do not express PLM. Means ± SE, n=8 from 3 independent assays, *—significant differences, P<0.05 by one-way ANOVA. (**B**) ARVMs were transduced with PLM-Epac1 or Epac1-camps (negative control), immunoprecipitatied using anti-NKA α_1-subunit monoclonal antibody preimmobilized on protein G-sepharose beads and immunobloted for total PLM. Shown are representative immunoblots (n=3) for various samples numbered as described in the table (see C, right). Both the sensor PLM-Epac1 (~75 kD) and the endogenous PLM (~17 kD) are pulled down using this antibody. (**C**) Representative immunoblots for co-immunoprecipitation experiments (n=3) with PLM-Epac1 and Epac1-camps precipitated using GFP-Trap® under identical conditions as in B and immunoblotted for NKA α_1-subunit or GFP as shown. All blots are representatives for at least three individual experiments.



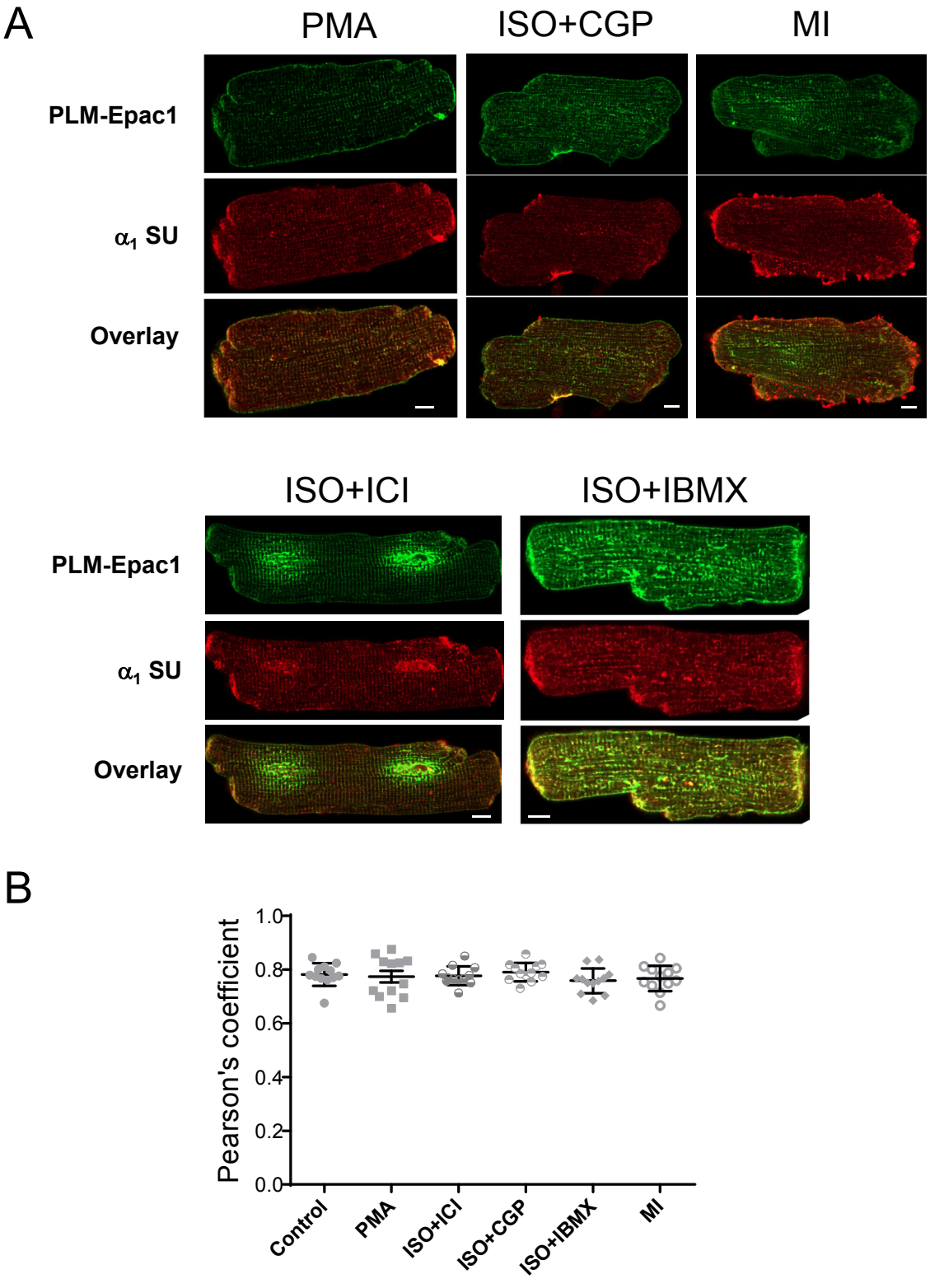
Supplementary Figure 3. Autofluorescence and localization control for confocal images shown in Figure 1B. ARVM without biosensor expression and ARVM expressing Epac1-camps or GFP 48 h after transduction. Representaive confocal images (n=5) acquired with the same microscope settings. Scale bars, 10 µm.



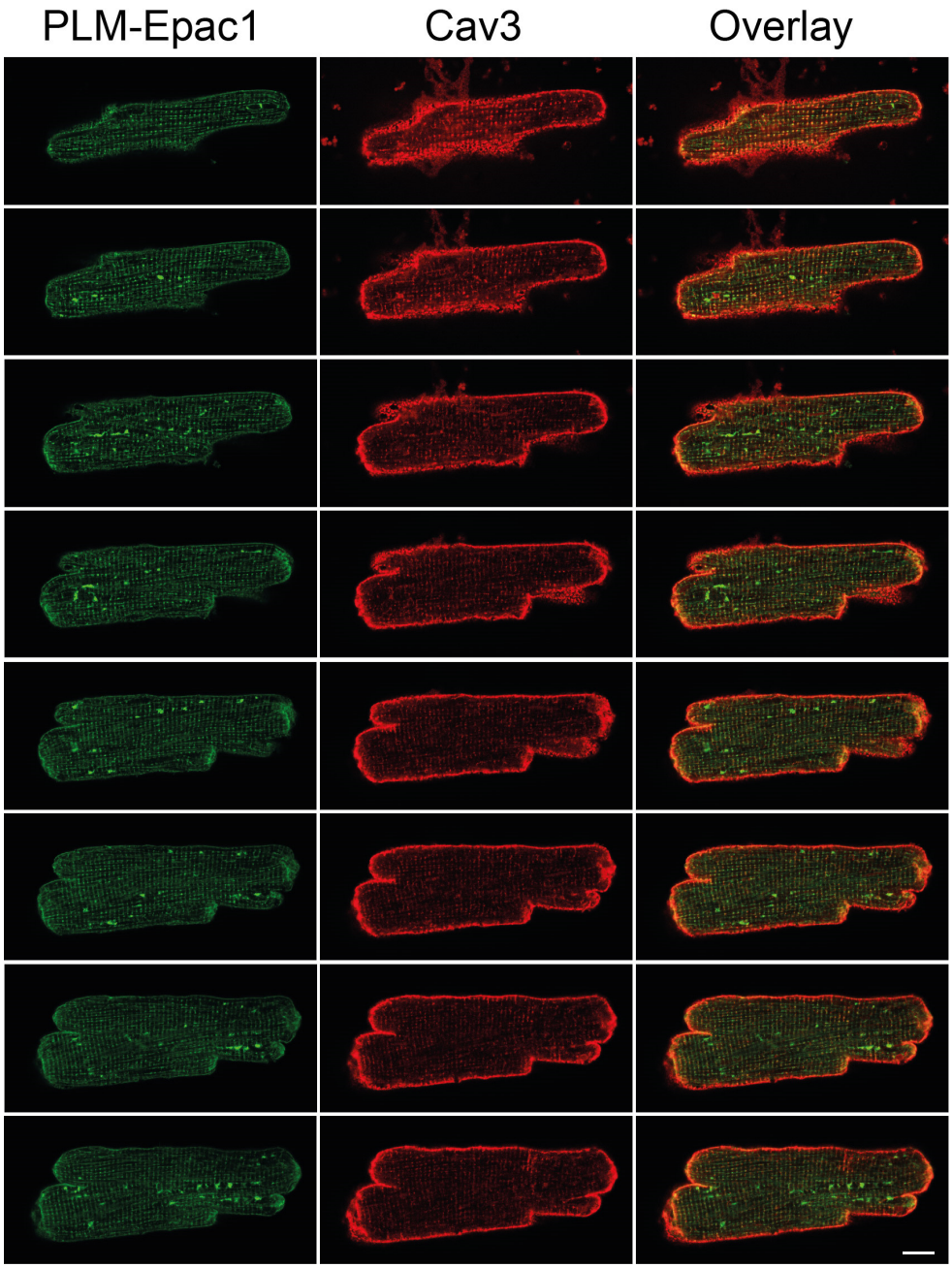
Supplementary Figure 4. Sensitivity of Epac1-camps and PLM-Epac1. (**A**) Concentration response dependencies measured in PLM-Epac1 and Epac1-camps transduced ARVMs pretreated with the adenylyl cyclase inhibitor MDL12,330A (100 µmol/L) for 10 minutes and stimulated with increasing concentrations of the cell-permeable cAMP analogue cAMP 8-Br-2'-O-Me-cAMP-AM. This analogue can be used for direct in cell sensor calibration since it has the same affinity for the sensor binding domains as native cAMP (see Ref. 23). PLM-Epac1 shows a slightly lower affinity than the cytosolic Epac1-camps sensor. EC_{50} values were 4.1±1.1 and 0.9±0.2 µmol/L, respectively. Means±SE, n=7 and 12 cells from 4 and 5 rat hearts, respectively. (**B)** FRET responses to increasing ISO concentrations in Epac1-camps and PLM-Epac1 expressing ARVMs show no significant differences in terms of ISO potency. EC_{50} values were 4.4±1.2 and 4.3±0.7 nmol/L, respectively. Means±SE, n=15 and 10 cells from 4 rat hearts each.



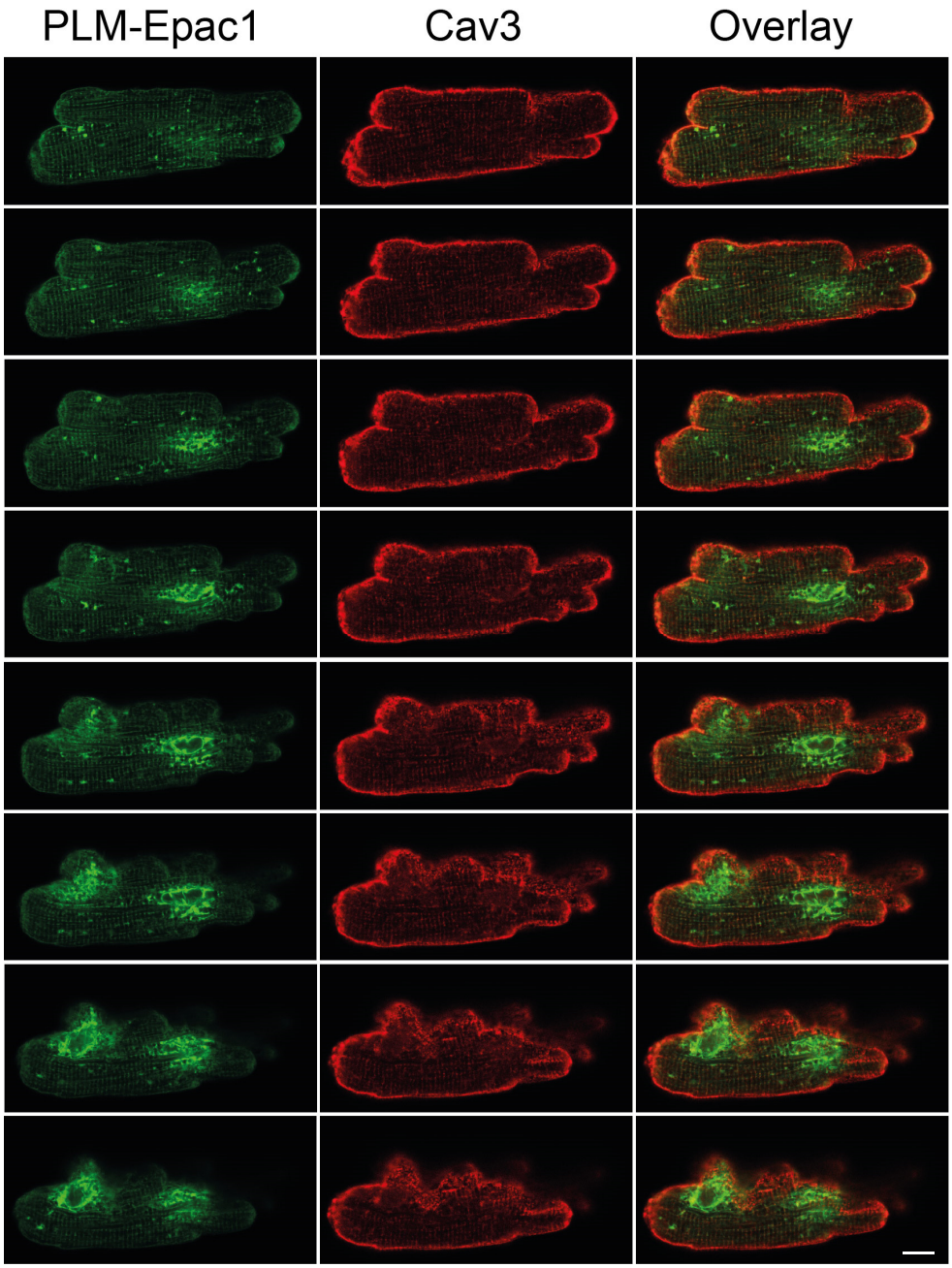
Supplementary Figure 5. qPCR and immunoblot analysis for crucial components of the β-adrenergic/cAMP pathway in healthy (AMC) and failing (MI) myocytes. (**A**) ARVMs lysates were immunoblotted for β_1- (n=6) and β_2-AR (n=5). (**B**) Levels of PKA regulatory subunits RI (n=8) and RII (n=6 each) were quantified by immunoblot. (**C**) qPCR results for the major cardiomyocyte adenylyl cyclase families AC5 (Adcy5) and AC6 (Adcy6), n=4 each. * - significant difference ($p<0.05$) by one-way ANOVA. All other differences are not significant at $p=0.05$ by the same test.



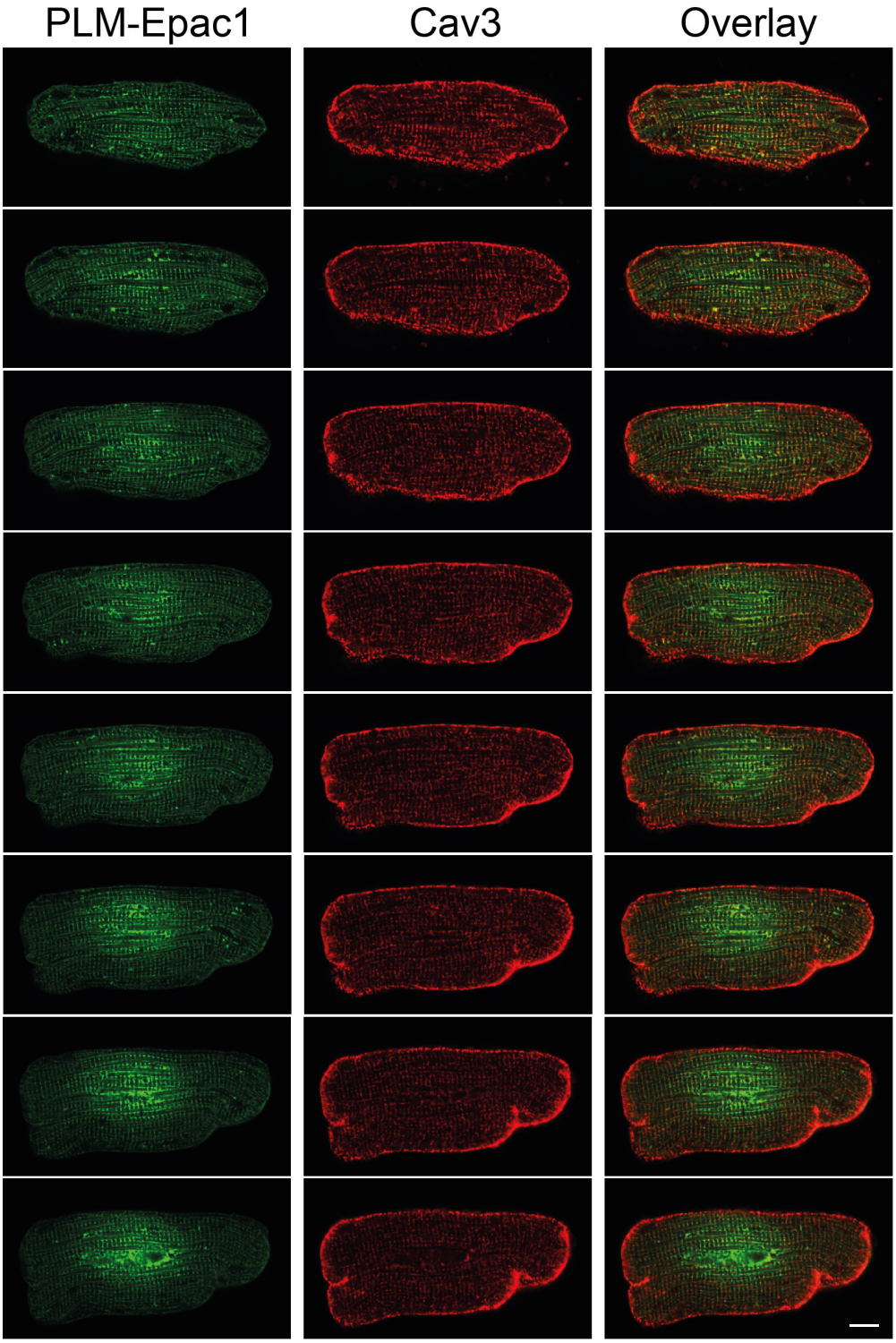
Supplementary Figure 6. Co-localization of PLM-Epac1 with NKA under various conditions. (A) Representative confocal images of PLM-Epac1 transduced (MOI 300) healthy or failing (MI) ARVMs. Healthy cells were stimulated for 5 min with 10 µmol/L phorbol 12-myristate 13-acetate (PMA), β_2-AR ligands (100 nmol/L ISO plus 100 nmol/L CGP20712A=CGP), β_1-AR ligands (100 nmol/L ISO plus 50 nmol/L ICI118551=ICI) or with ISO in combination with a broadband PDE inhibitor (IBMX, 100 µmol/L), fixed with 4% PFA and immunostained for NKA α1-subunit (α1 SU). Scale bars, 10 µm. Example of an unstimulated healthy cell is in Supplementary Figure 1D. (B) Quantification of the data from A using Pearson's co-localization coefficient (N/n=3/12 each) shows no significant differences between unstimulated and any stimulated groups, by mixed ANOVA followed by Wald χ^2-test.



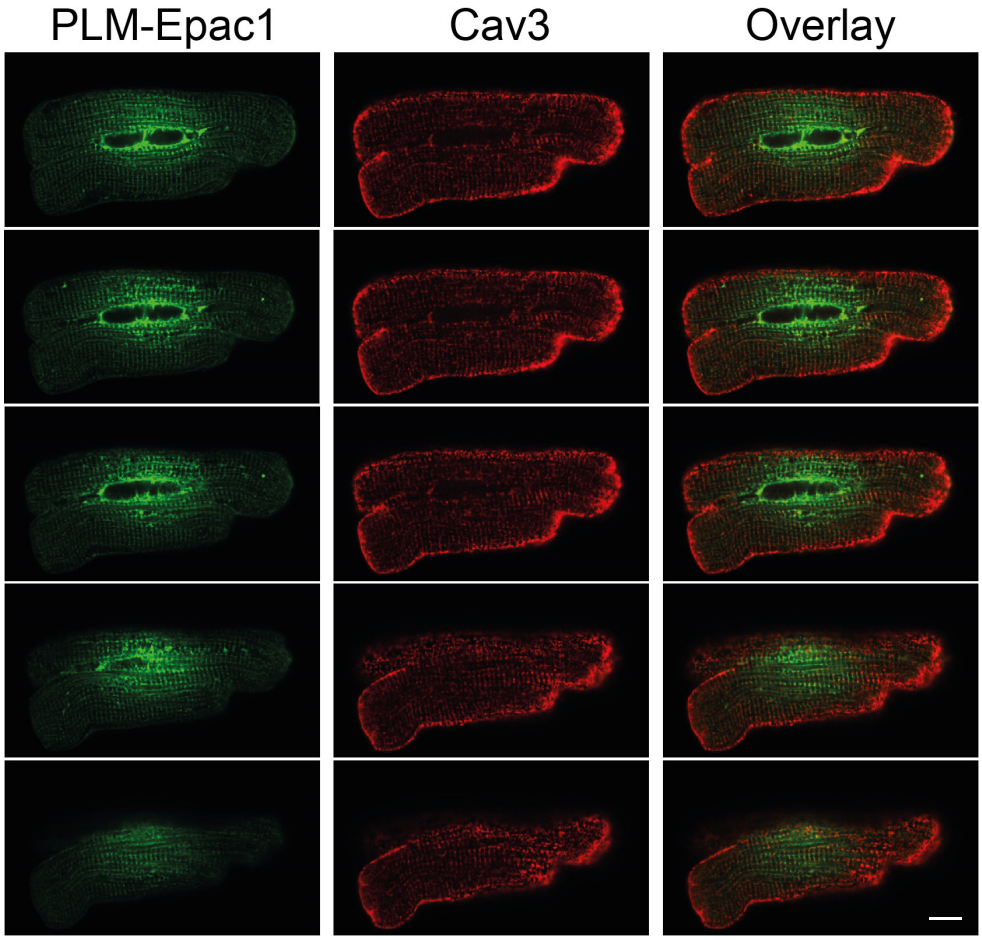
Supplementary Figure 7. Confocal microscopy analysis of PLM-Epac1 transfected healthy cardiomyocytes stained with caveolin 3 antibody. Representative z-stack images (1.5 µm steps from bottom/glass surface to the top) acquired for cells isoated from AMC animals (N/n=3/10). Scale bar, 15 µm.



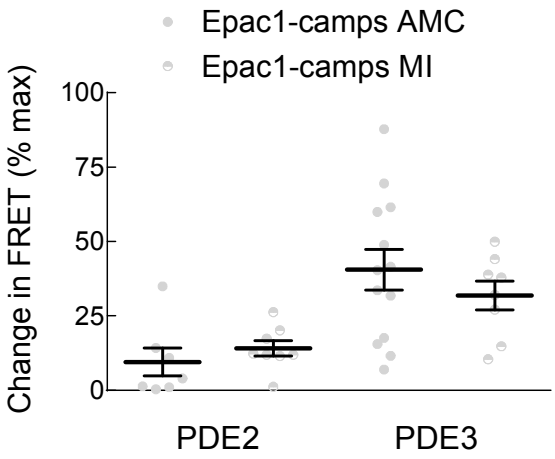
Supplementary Figure 7 (continued). Confocal microscopy analysis of PLM-Epac1 transfected healthy cardiomyocytes stained with caveolin 3 antibody. Representative z-stack images (1.5 µm steps from bottom/glass surface to the top) acquired for cells isoated from AMC animals (N/n=3/10). Scale bar, 15 µm.



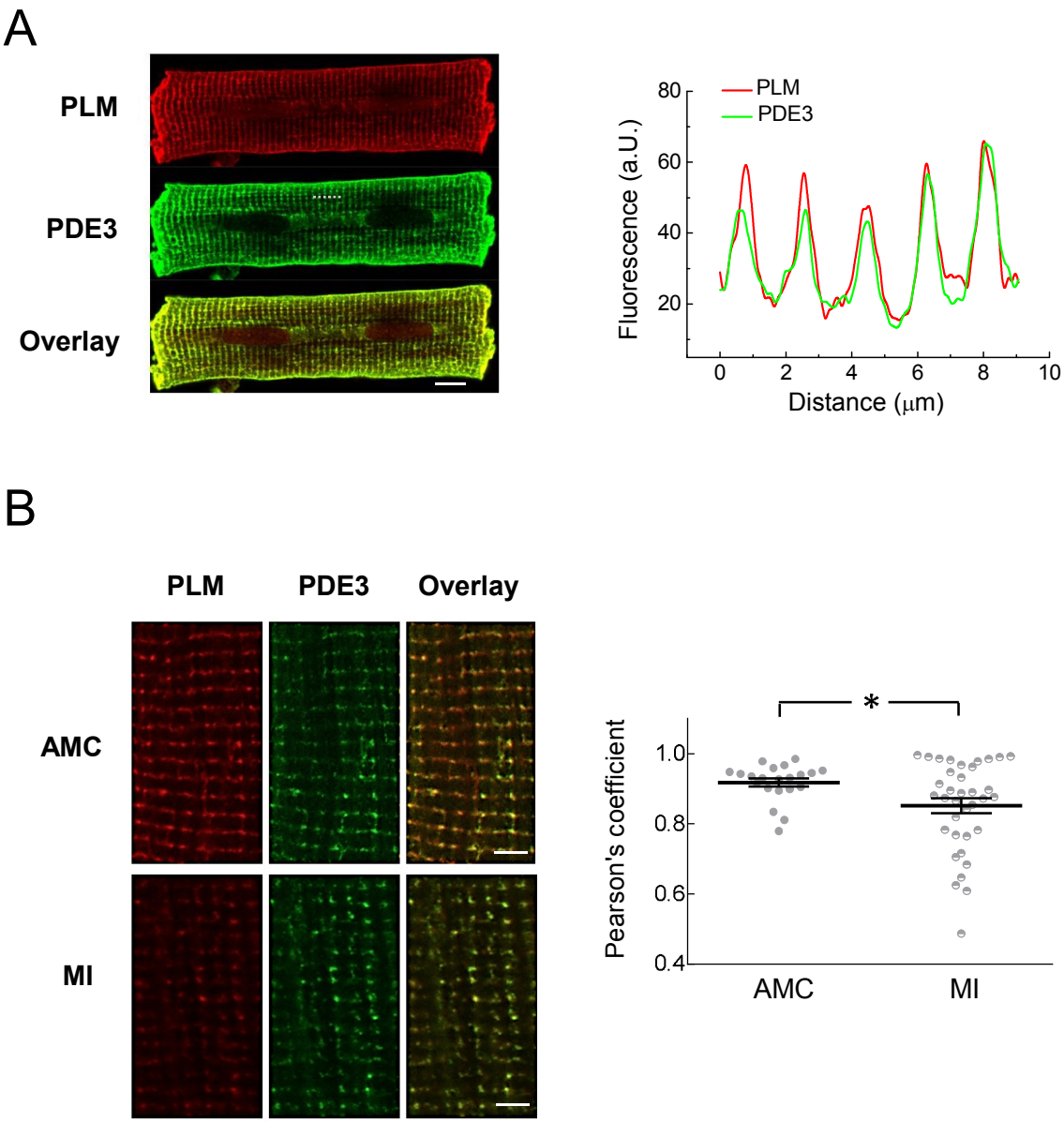
Supplementary Figure 8. Confocal microscopy analysis of PLM-Epac1 transfected failing cardiomyocytes stained with caveolin 3 antibody. Representative z-stack images (1.5 µm steps from bottom/glass surface to the top) acquired for MI cells (N/n=3/10). Scale bar, 15 µm.



Supplementary Figure 8 (continued). Confocal microscopy analysis of PLM-Epac1 transfected failing cardiomyocytes stained with caveolin 3 antibody. Representative z-stack images (1.5 µm steps from bottom/glass surface to the top) acquired for MI cells (N/n=3/10). Scale bar, 15 µm.



Supplementary Figure 9. Effects of PDE2 and PDE3 inhibitors on cytosolic cAMP after β_2-AR stimulation in healthy and failing myocytes. Cells were prestimulated with 100 nmol/L ISO plus 100 nmol/L CGP20712A, after which 100 nmol/L BAY 60-7550 for PDE2 and 10 µmol/L cilostamide for PDE3 were applied as described in Figure 5A-D. Data analysis shows no significant differences for PDE2 and PDE3 inhibitor effects measured by Epac1-camps between healthy and failing cells. Means ± SE, number of cells (n) and hearts (N) were as follows - N/n=3/7, N/n=3/8 for PDE2 and N/n=3/13, N/n=3/8 for PDE3. There are no significant differences between AMC and MI groups by mixed ANOVA followed by Wald χ^2-test.



Supplementary Figure 10. Confocal microscopy analysis of PDE3A/PLM co-localization. (**A**) Representative confocal image (n=10) of a healthy ARVM immunostained with specific anti-PLM and anti-PDE3A antibodies showing a high degree of co-localization by the intensity overlay of both fluorescent signals. Scale bar, 10 μm. (**B**) Representative confocal images of age-matched control (AMC) and MI cells immunostained under the same conditions. Scale bar, 5 μm. Significant decrease of co-localization between PLM and PDE3 in diseased cells as calculated using Pearson's co-localization coefficient. Means ± SE, number of cells (n) and hearts (N) was N/n=3/21, N/n=3/36 for AMC and MI, respectively. * — significant differences, P<0.05 by Mann-Whitney test.