SUPPLEMENTARY MATERIAL

CAMKII-DEPENDENT PHOSPHORYLATION OF CARDIAC RYANODINE RECEPTORS REGULATES CELL DEATH IN CARDIAC ISCHEMIA/REPERFUSION INJURY

Di Carlo M.N. et al.

1. MATERIALS AND METHODS

1.1 *Ex-vivo* protocol of global I/R: Perfused mouse hearts

Hearts were perfused according to Langendorff technique, at constant temperature (37°C), coronary flow (3-4 ml/min) and heart rate (300 beats/min). The composition of the physiological bicarbonate buffer solution (BBS) was (in mmol/L): 128.3 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 20.2 $NaHCO_3$, 0.4 NaH_2PO_4, 1.1 MgSO4, 11.1 glucose and 0.04 Na_2EDTA. This solution was equilibrated with 95% O_2-5% CO_2 to give a pH of 7.4, as described [1]. The mechanical activity of the hearts was assessed by introducing into the left ventricle (LV) a latex balloon connected to a pressure transducer (ADInstruments MLT 0380, CO, USA) and filled with aqueous solution to achieve a left ventricular end-diastolic pressure (LVEDP) of approximately 5-10 mmHg. LV contractility was evaluated using the developed-pressure (LVDP) and the maximal rate of pressure development (dP/dt_{max}) [1]. After stabilization, hearts were perfused for 10 min and then, normothermic global ischemia was produced by interruption of the coronary flow for a period of 45 min. Coronary perfusion and electrical stimulation were then restored for 120 min. At the end of reperfusion, hearts in a group of experiments hearts were freeze-clamped at different times during reperfusion to assess the expression of PLN and of ryanodine receptor (RyR2), the phosphorylation of T17 and S16 sites of PLN, and that of S2814 and S2808 of RyR2.

1.2 *In vivo* protocolof regional I/R

Mice were anesthetized with sodium pentobarbital (45 mg/kg, i.p.) and then subjected to myocardial ischemia induced by left anterior descending (LAD) coronary artery occlusion for 1 hr followed by reperfusion for 24 hrs. Sham operated mice were subjected to the same surgical procedures without LAD ligation. Buprenorphine (0.1 mg/kg) was administrated subcutaneously to reduce postoperative pain. At the end of reperfusion, the LAD artery was re-occluded. Evans blue dye (2%), was injected into the carotid arterial root to stain the normally perfused region blue and outline the area at risk (AAR) [2]
After 24 hrs reperfusion, hearts were then freeze-clamped for phosphorylation determinations, or perfused with triphenyltetrazolium chloride to measure infarct size as described below.

1.3 Infarct size

At the end of reperfusion, hearts were perfused with 1% triphenyltetrazolium chloride (TTC) in phosphate buffer (pH 7.4) for 7 min and immersed in this solution for another 7 min. The hearts were frozen at −20°C for 1 hr and then cut into six transverse slices (2 mm thick) along the long axis of the LV, from apex to base. Infarct areas were enhanced by storage in 10% formaldehyde solution for 48 hrs before measurement. The infarct area was calculated by computer planimetry (Scion Image, Scion Corp, USA) and expressed as a percentage of the the area of risk, which in this preparation is the total area of the ventricle [3].
In the *in-vivo* experiments, the LAD artery was re-occluded at the end of reperfusion. 2% Evans blue dye was injected into the carotid arterial root to stain the normally perfused region blue and outline the area at risk (AAR). The left ventricle (LV) was rapidly excised and cut into 1-mm-thick transverse sections, which were then incubated in 1% 2,3,5- triphenyltetrazolium chloride (TTC) for 10 min at 37°C to differentiate the viable (red) and nonviable myocardium (pale) within the risk zone. The areas

of infarction, at risk, and non-ischemic LV were assessed by a blinded observer using Image J (NIH). The area at risk as a percent of the LV (AAR/LV) and the infarction size (IS) as a percent of the AAR (IS/AAR) were calculated.

1.4 TUNEL determinations

Terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL) assay was performed in myocardial slices obtained from the middle of the long axis of the LV, fixed in formalin. Deparaffinized sections (5 μm thick) were processed for histological examination, and apoptosis was detected with a Cell Death Detection kit (Roche) according with the supplier's instructions. Total nuclei were stained with DAPI. The sections were captured with a digital RGB video camera (Evolution VF, Qimaging, Canada) attached to a microscope (Olympus BX- 50, Tokyo). Twenty areas at high magnification (objective 40X) were randomly chosen in each stained section and approximately 500 myocytes were analyzed per section. Images were processed by a digital image analyzer program (Image-Pro Plus V6.O, Media Cybernetics Silver Spring, MA) and percentage of TUNEL-positive myocytes was calculated by two blinded independent investigators. Cardiac myocytes were identified following morphologic (nuclei with elliptical shape and striated cytoplasm) and morphometric parameters [4].

1.5 Electrophoresis and Western blot analysis

Proteins from cardiac homogenates (30 μg, Bcl2 and Bax; 25 μg, P-T17 and P-S16 of PLN and total PLN, 80 μg, total RyR2, S2814 and 2808 of RyR2) were separated on appropriate Laemmli SDS-polyacrylamide gels (buffer: 25 mmol/L Tris–HCl, 190 mmol/L Glycine and 0.1% SDS) and transferred to PVDF membranes as previously described [5]. Blots were probed with antibodies raised against S16 and T17-phosphorylated PLN (Badrilla, Leeds, UK), total PLN (ABR Rockford, IL, USA), S2814 and 2808 (both from Badrilla, Leeds, UK), total RyR2 (ABR Rockford, IL, USA), total RyR (Affinity Bioreagents), Bcl-2 and Bax (Santa Cruz Biotechnology, CA, USA). Immunoreactivity was visualized by peroxidase-based chemiluminescence detection kit, ECL Plus (Amersham Biosciences, Pittsburgh, PA, USA). The signal intensity of the bands in the immunoblots was quantified by densitometry using Image J software (NIH, USA).

1.6 Ca^{2+}_i and Cell shortening in isolated myocytes

Isolated myocytes were loaded with Fura-2/AM (10 μmol/L for 15 min). Ca^{2+}_i was measured with an epi-fluorescence system (Ion Optix, Milton, MA). Briefly, dye-loaded cells were placed in a chamber on the stage of an inverted microscope (Nikon.TE 2000-U) and continuously superfused with a HEPES buffered solution at a constant flow of 1 ml/min at room temperature (20-22°C) Myocytes were stimulated via two-platinum electrodes on either side of the bath at 0.5 Hz. Fura-2 fluorescence was taken as an index of the Ca^{2+}_i. Resting cell length and cell shortening were measured by a video-based motion detector (Ion Optix). Fluorescence and cell shortening data were stored for off-line analysis (ION WIZARD fluorescence analysis software) [6].

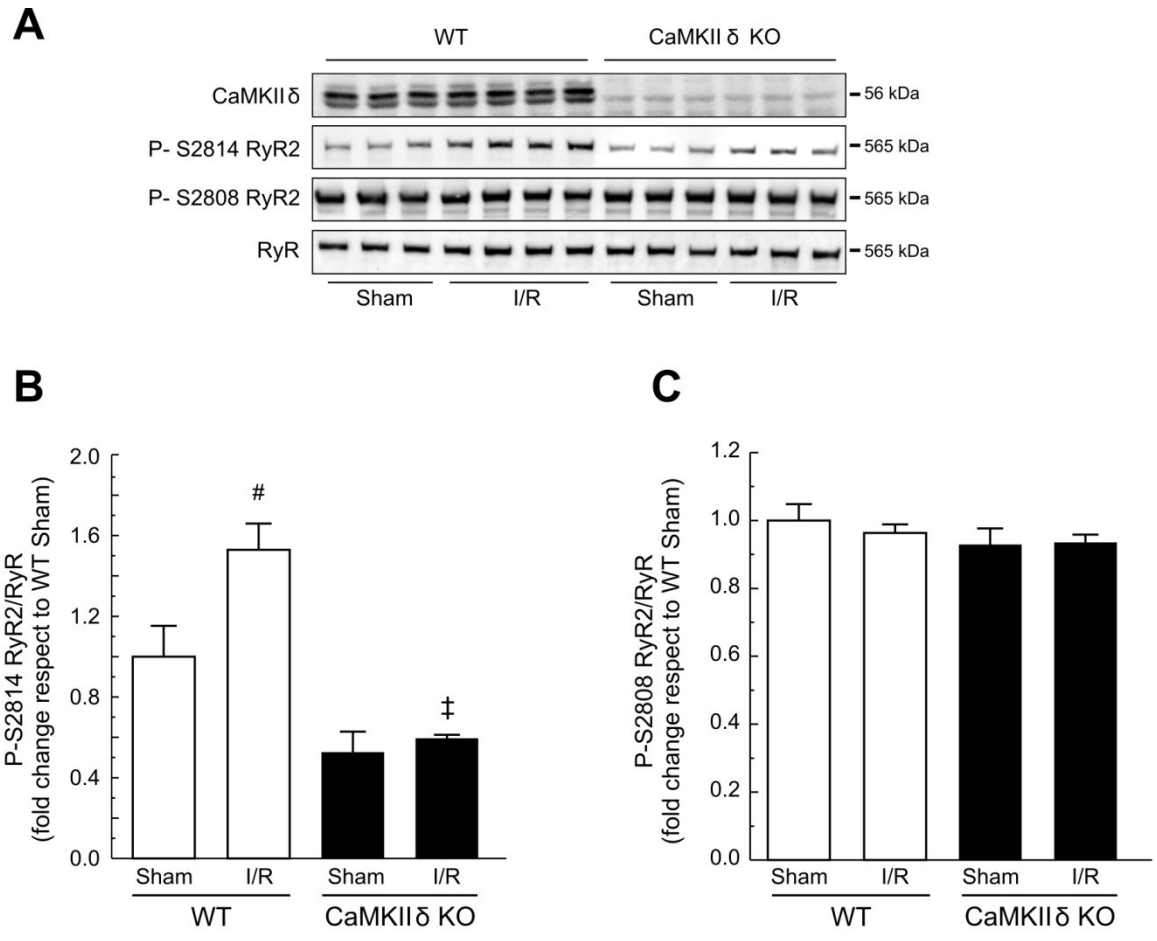
1.7 I_{Ca} in isolated myocytes

Mice ventricular myocytes were placed in a perfusion chamber and superfused with bath solution at a flow rate of 1.5 ml/min. The standard whole-cell configuration of the patch-clamp technique was used for voltage-clamp recordings with a patch-clamp amplifier (Axopatch 200A; Axon Instruments, Foster City, CA). Patch pipettes were pulled with a PP-83 puller (Narishige; Tokyo, Japan) and fire polished with an MF-83 Microforge (Narishige) to a final resistance of 1–3 MΩ when filled with pipette solution. The tip of the pipette was positioned above the cell, and its potential and capacitance were nullified. Currents were digitally recorded directly to hard disk via an analog-to-digital converter (Digidata 1200, Axon Instruments) interfaced with an IBM clone computer running pCLAMP and Axotape software (Axon Instruments). Data analysis was performed with pClamp (Clampfit).

Voltage-clamp depolarizing pulses (250 ms) were delivered at 0.2 Hz. A holding potential of −80 mV was used in all protocols to prevent slow inactivation and to minimize current rundown. A 500 msprepulse to −40 mV, used to inactivate Na+ channels and potential T-type Ca^{2+} channels, preceded the depolarizing test pulses to different potentials. The currents evoked by the test pulses exhibited activation and inactivation kinetics consistent with those of L-type I_{Ca}. The I_{Ca} amplitude was measured as peak inward current with reference to the current measured at the end of the test pulse. For each cell, capacitative current was recorded to determine the membrane capacitance, and the currents were normalized for cell capacitance.

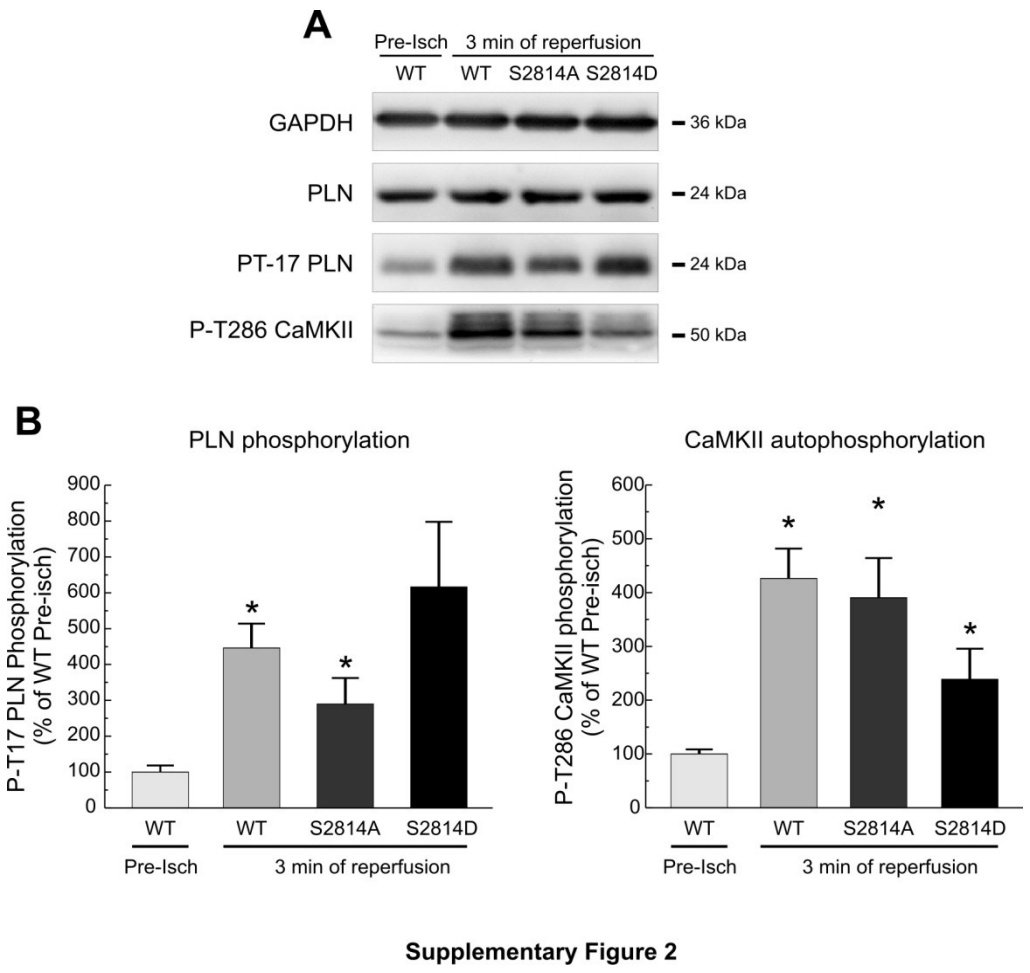
The superfusion medium used to measure I_{Ca} had the following composition (in mmol/L): 5 CsCl, 133 NaCl, 1 MgCl2, 1.2 MgSO4, 10 HEPES, 10 tethraethylammonium chloride (TEA), 1.35 CaCl2, and 10 glucose; pH was adjusted to 7.4 with NaOH. The internal (pipette) solution used contained (in mmol/L): 140 CsCl, 1 MgCl2, 5 Na2ATP, 5 EGTA and 10 HEPES; pH was adjusted to 7.2 with NaOH[7].

2. RESULTS



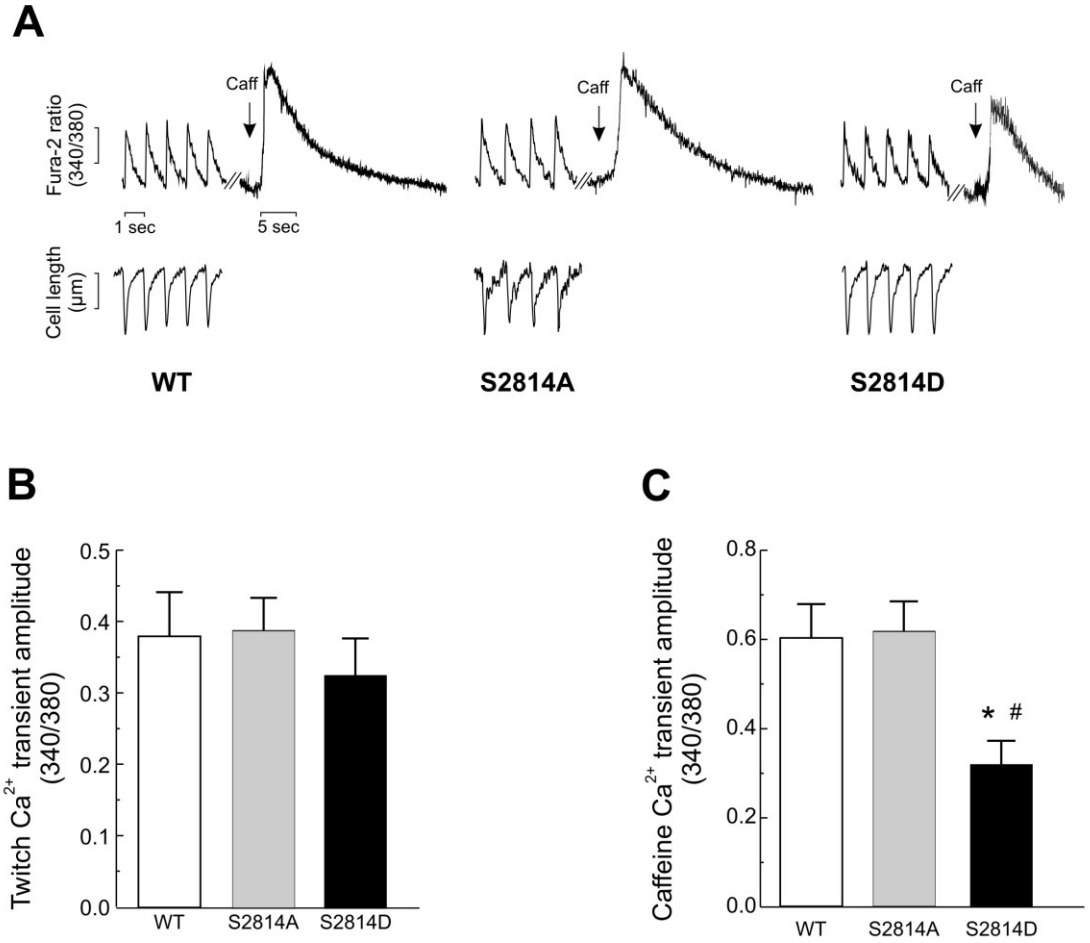
Supplementary figure 1

S Figure 1. Reperfusion significantly increased CaMKII-dependent phosphorylation levels in an *in-vivo* model of regional infarction.
WT and CaMKIIδ-KO mice were subjected to 1 hr occlusion of left anterior descendent coronary artery followed by 24 hrs reperfusion. No significant changes were observed in either RyR2 expression or in the phosphorylation of S2808 site at the end of reperfusion period. In contrast, there was an increase in CaMKII-dependent phosphorylation of RyR2 in WT mice, which did not occur in CaMKIIδ-KO mice. Data represent the average ± SEM of values from 3-9 hearts per group.* P<0.05 vs. the corresponding pre-ischemic values # P<0.05 vs. WT sham. ‡P<0.05 vs. WT I/R.



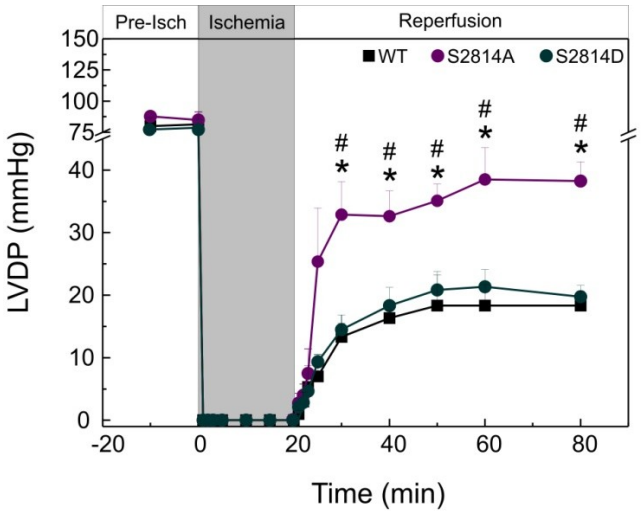
Supplementary Figure 2

S Figure 2. CaMKII phosphorylation and CaMKII-dependent phosphorylation of PLN increased at the onset of reperfusion in S2814A/D vs. Pre-Isch. A. Western blots of CaMKIIautophosphorylation and CaMKII-dependent phosphorylation of PLN, in Langendorff perfused WT and S2814A and D mouse hearts subjected to 45 min ischemia followed by 3 min reperfusion. **B.**Sumary of results. Comparing with pre-ischemic values (Pre-Isch) there was a similar and significant increase in CaMKII and T17 phosphorylation of PLN at 3 min of reperfusion in WT and S2814A/D mice with respect to pre-ischemia. Data represent the average ± SEM of values from 3-8 hearts per group.* P<0.05 vs. the corresponding pre-ischemic values.



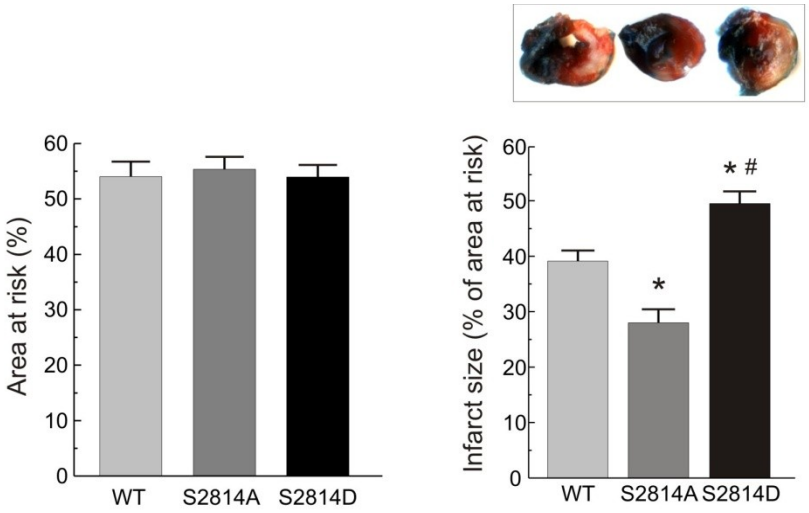
Supplementary Figure 3

S Figure 3. Basal intracellular Ca^{2+} characteristics of S2814A and S2814D mice. A to **C**, typical records and overall results of myocyte twitch and caffeine Ca^{2+} transient amplitudes in WT, S2814A and S2814D mice. No significant differences were observed in myocyte twitch Ca^{2+} transient amplitude and cell length when compared S2814A, S2814D and WT mice (**A** and **B**). In contrast, whereas caffeine-induced Ca^{2+} transient amplitude was similar in S2814A vs. WT mice, it was significantly lower in S2814D, revealing a decrease in SR Ca^{2+} load (**C**). These results indicate that S2814D mice present an increase in fractional Ca^{2+} release, as previously described [8]. Data represent the average ± SEM of n = 8 -14 per group. **P*<0.05 vs. WT. #*P*<0.05 vs. S2814A.



Supplementary Figure 4

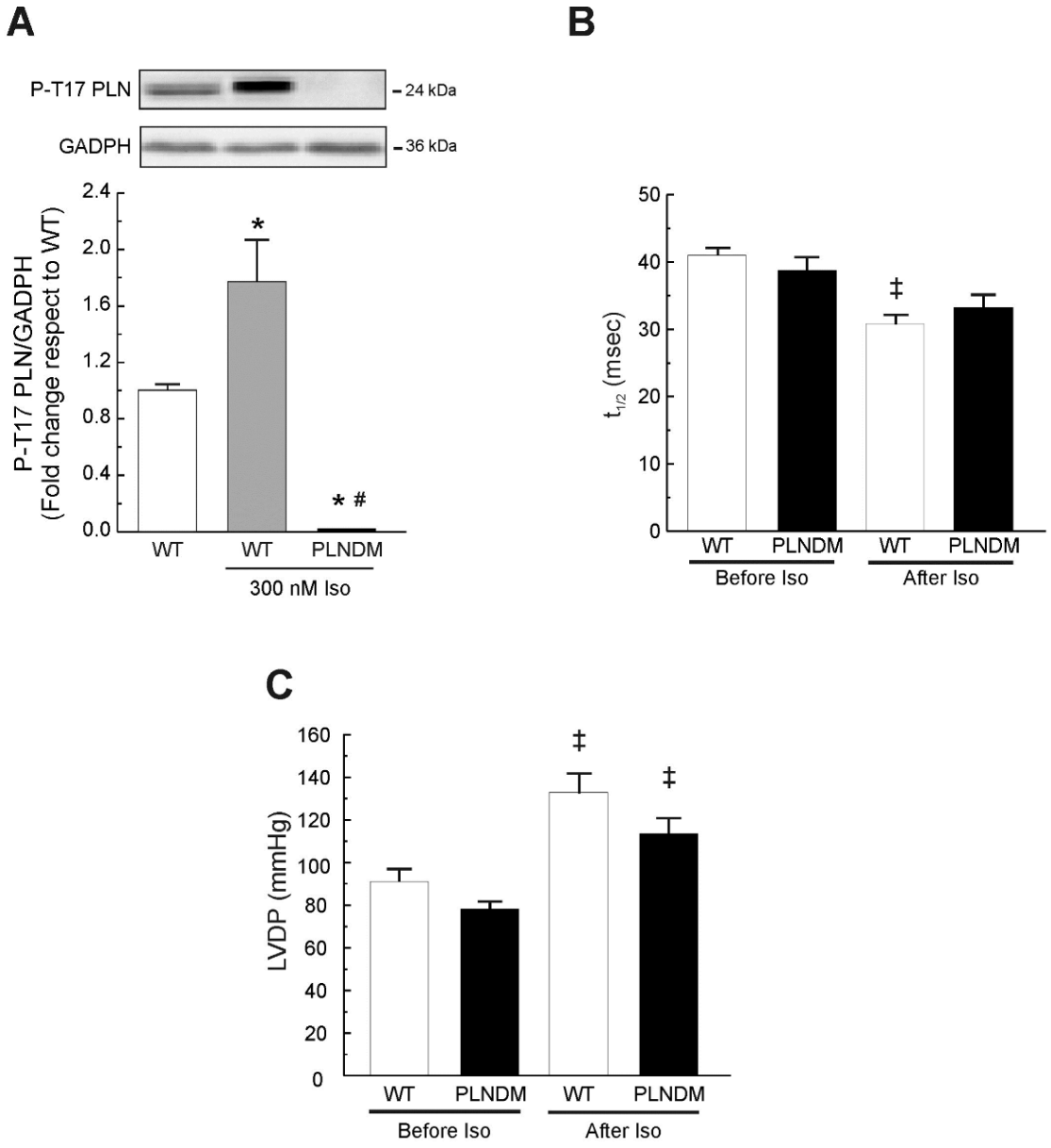
S Figure 4. Mechanical recovery in ischemic/reperfused hearts of S2814A and D mice and their control littermates, after a 20 min of ischemia. Left ventricular developed pressure (LVDP), was significantly increased in S2814A and did not change in S2814D mice with respect to WT mice in perfused hearts subjected to 20 min ischemia followed by 60 min reperfusion. Data represent the average ± SEM of n = 3 -4 per group. **P*<0.05 vs. WT. #*P*<0.05 vs. S2814D.



Supplementary figure 5

S Figure 5. CaMKII-dependent phosphorylation of RyR2 is responsible for increased I/R injury in an *in vivo* model of regional infarct. S2814A and S2814D and the corresponding WT mice, were subjected to 1 hr occlusion of left anterior descendent coronary artery followed by 24 hrs reperfusion. At the end of reperfusion, the infarct size was significantly decreased in S2814A and increased in

S2814D relative to WT mice. Data represent the average ± SEM of n = 3 - 6 mice per group.*P<0.05 vs. WT. # P<0.05 vs. S2814A.



Supplementary figure 6

S Figure 6. Lack of phosphorylation of T17 and S16 sites of PLN, blunts the relaxant effect but not the inotropic action of β-adrenergic stimulation. Stimulation of perfused mice hearts with isoproterenol induces CaMKII dependent T17 phosphorylation in WT but not in PLNDM mice (**A**). PLNDM mice lack the relaxant effect produced by β-adrenergic stimulation (**B**), although preserve the positive inotropic effect (LVDP) (**C**). Data represent the average ± SEM of n = 3-16 per group. *P<0.05 vs. WT. # P< 0.05 vs. WT + Iso. ‡P<0.05 vs. before Iso in the same strain.

References

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