Online Supplemental Material

Materials

Chemicals and reagents:

[^{45}CaCl2] (10,000 dpm/nmol) (Perkin Elmer, Cat#NEZ013001); ^{3}H-cAMP (GE Healthcare, Cat#TRK304); Protease inhibitor cocktail (Sigma, Cat#P8340); Phosphatse inhibitor cocktail (Roche, Cat# 04906837001); Coomassie SimplyBlueTM SafeStain (Invitrogen, Cat#LC6060); trypsin (Invitrogen, Cat#MS10015); Cilostamide (Calbiochem, Cat#231085); Mil (Sigma, Cat#M4659); IBMX (Sigma, Cat#5879) Ionomycin (Calbiochem, Cat#407950); C18 ZipTip (Millipore, Cat# ZTC18S096); Ammonium molybdate (Santa Cruz, Cat#sc-227258); Malachite green (Sigma, Cat#38800); Liberase Blendzyme 2 (Roche Diagnostics, Cat#11988425001); Fluo-4 AM (Molecular Probes, Cat#F-142010); SuperSignal® Westpico and Westfemto chemiluminescent reagents (Pierce Cat#34080 and 34096, respectively). Other materials were obtained as indicated and were of the highest grade available.

Antibodies: anti-mouse SERCA2 (Sigma, Cat#S1314 and Abcam, Cat#ab2861and ab3625), anti-rabbit Calsequestrin (Sigma, Cat#C2491), anti-mouse PLN (Abcam, Cat#ab2865), anti-rabbit phospho-PLN (Abcam, Cat#ab15000), anti-rabbit RyR (Santa Cruz, Cat#SC-13942), anti-mouse Prohibitin (Abcam, Cat#ab1836), anti-mouse PKAc (BD, Cat#610980), anti-mouse PKA RIIα (BD, Cat#612242), anti-PP2A (BD Cat# 610556), anti PKARII (BD cat# 610626), anti-phospo-CREB (Millipore cat# 06-519), anti-CREB (Cell Signaling cat# 9104), anti-phospho-PKA substrate (Cell Signaling cat# 9621), anti-Desmin (DAKO cat# M0760), anti-SM-actin (Dako cat# M0851), anti-rabbit PDE3A (accession no. NP-061249) peptide against 1098TGENQSLDQVPLQHPSEQ1115 (raised in our lab), anti-mouse SERCA2 monoclonal antibody (gift from Dr. Jonathan Lytton, Univ. Calgary, Canada), and anti-mouse AKAP18 antibodies (gift from William Catterall, Univ Washington, Seattle).

Detailed Methods

Langendorff perfused hearts

10-12 week old, PDE3A wild type (WT) or KO littermate mice of either sex were heparinized (10 IU/g body weight) and 5 min later anaesthetized with intraperitoneal injection of ketamine/ xylazine mixture (1 mg/g ketamine plus 0.2 mg/g xylazine).

An appropriate level of anaesthesia was confirmed by the absence of a pedal reflex. Hearts were excised via midline thoracic incision, placed in warmed Krebs-Henseleit (KH) buffer for 10 seconds, transferred to ice-cold phosphate buffered saline solution (PBS) and immediately mounted on a 20-gauge blunted stainless steel canula to be retrogradely perfused via the aorta with warmed (37^{o}C) and oxygenated (95% O_2– 5% CO_2) modified KH buffer containing (in mmol/L): 118 NaCl, 23 $NaHCO_3$, 3.2 KCl, 1.2 KH_2PO_4, either 1.2 or 1.8 $CaCl_2$, 1.2 $MgSO_4$, 0.5 Na_2-EDTA, 11 Glucose, and 2 Na-pyruvate; pH 7.4. Once constant perfusion was established (75-80mmHg), the left atrial appendage was removed and a compliant fluid filled balloon – connected to a pressure transducer – was inserted into the left ventricle (LV) via the mitral valve to record LV pressure (BIOPAC Systems Inc., Goleta, CA). The balloon was inflated in stepwise increments of ~2mmHg followed by the proportionate increase in LV force development. At one point further increase in the left ventricular end diastolic pressure (LVEDP) would

not produce any further increase in left ventricular pressure suggesting optimal preloading conditions (in most cases 8-10mmHg was be sufficient). The preparation was allowed to equilibrate for 20-min, at which point a record of baseline function (1 min) was obtained. Milrinone a specific PDE3 inhibitor[1] (10mmol/L stock solution in dimethyl sulfoxide-DMSO) was dissolved in KH buffer, and administered by slow parallel injection into the perfusion line to obtain a final concentration of 10μmol/L. Milrinone (Mil) infusion rate (ml/min) was determined from the coronary outflow rate, measured by collecting venous effluent over 5-min. Heart rate and changes in the left intraventricular pressure were continuously monitored throughout the experiment. In experiments requiring control of the heart rate, hearts were electrically stimulated at frequency 1Hz higher than intrinsic heart rate, using 2ms pulses at twice the voltage threshold. The maximal rates of LV pressure development ($+dPdt_{max}$) and relaxation ($–dP/dt_{min}$) as well as the left ventricular developed pressure (end-systolic *minus* end-diastolic pressure) were calculated from our raw pressure tracings using commercially available software (Spike2 5.21, Cambridge Electronic Design, Cambridge, UK). In order to examine heart rate-independent effects of Mil on cardiac contractility hearts were electrically paced 1Hz above their intrinsic heart rate via platinum wire electrodes attached to the right atrium.

Cardiomyocyte isolation for imaging

Ventricular cardiomyocytes were isolated from WT and $PDE3A^{-/-}$ adult mice (~15 weeks) by modified perfusion method[2, 3]. Briefly, hearts were excised from mice anesthetized with sodium pentobarbital. Using a Langendorff apparatus, hearts were perfused with Ca^{2+}-free Tyrode's buffer (mmol/L): 140 NaCl, 4 KCl, 1 $MgCl_2$, 5 HEPES, pH 7.4, 10 D-glucose, bubbled with 100% O_2 for 5 min at 37°C. Perfusion was then switched to the Tyrode's buffer containing 0.1 mmol/L $CaCl_2$ and Liberase Blendzyme 2 (Roche Diagnostics, Cat#11988425001). Hearts were digested until the flow rate increased to 1.5 fold. After perfusion/digestion, the ventricular tissue was minced, gently triturated and incubated in 5 ml of same digestion buffer for 3 min at 37°C; the dissociated myocytes were transferred into an equal volume of the Tyrode's buffer containing 0.2 mmol/L $CaCl_2$ and 10 mg/ml bovine serum albumin (Sigma, Cat#A6003) to stop the digestion. This process was repeated three times. After gradually increasing $CaCl_2$ to 1.2 mmol/L, the isolated cardiomyocytes were suspended into modified MEM buffer containing 100 U/ml penicillin-streptomycin. The cardiomyocytes were plated onto laminin-coated glass coverslip and cultured at 37°C.

Cardiomyocytes isolation for electrophysiological studies

Mice were injected with 0.2 ml heparin (1000 U/ml, I.P.), anaesthetised with isoflurane followed by a cervical dislocation. Hearts were surgically excised and placed in modified Ca^{2+}-free Tyrode's solution (4°C) containing (mmol/L): 130 NaCl, 0.4 NaH_2PO_4, 5.4 KCl, 0.5 $MgCl_2$, 25 HEPES (-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid) and 22 glucose; pH 7.3 with NaOH. The aorta was canulated and perfused in retrograde fashion with Ca^{2+}-free Tyrode's at a constant flow (37°C). Following 10-min perfusion with Ca^{2+}-free Tyrode's, collagenase (type II, 25.2 U/ml) and protease (type XIV, 0.05 U/ml) were introduced until the myocardium was digested (typically 8-10-min). The septum was then placed in modified Kraft Bruhe solution for storage up to 8-hrs at 4°C. The Kraft Bruhe solution contained (in mmol/L): 120 K-glutamate, 10 KCl, 10 KH_2PO_4, 2 $MgSO_4.7H_2O$, 10 taurine, 5 creatine, 10 HEPES, 10 glucose, 0.5 EGTA and

0.1% bovine serum albumin; adjusted to pH 7.3 with KOH. The tissue was gently agitated to facilitate release of individual myocytes.

I_{CaL} and Ca^{2+} transient measurements

Electrophysiological responses were examined in rod shaped, Ca^{2+}-tolerant myocytes. Cells were placed in a glass bottomed recording chamber (~2ml volume), allowed to settle and adhere, then superfused with a bathing solution containing (in mmol/L): 140 NaCl, 0.5 $MgCl_2$, 1.8 $CaCl_2$, 5 CsCl (to block K^+ currents), 5.5 glucose and 5 HEPES; adjusted to pH 7.4 with NaOH. All electrophysiological recordings were conducted at room temperature (21-23^{o}C) at a perfusion rate of 1.5-2ml/min. All drugs (except R_p-cAMPS) were delivered via the bathing solution

Patch pipettes with tip resistances of 1.5-2 MΩ, where utilized for whole cell recordings of L-type Ca^{2+} currents (I_{CaL}). The intracellular pipette solution contained (in mmol/L): 130 CsCl, 2 KCl, 1 $MgCl_2$, 1 NaH_2PO_4, 10 HEPES, 5.5 glucose, 3.6 Na_2-phosphocreatine, 5 Mg-ATP and 0.05 Fluo-3 (pentapotassium salt); adjusted to pH 7.2 with CsOH. Corrections for series resistance and cell capacitative currents in conjunction with 80% compensation were routinely employed. L-type Ca^{2+} currents were evoked by depolarizing voltage pulses ranging from –40mV to +30mV (200ms duration, 0.2 Hz) at 10mV increments, delivered from a holding potential of –85mV. To inactivate cardiac Na^+ channel currents, depolarizing pulses were preceded by a 500ms voltage ramp from –75mV to –45mV followed immediately by a 100ms hold at –45mV. Command potentials were generated and membrane currents recorded using an Axopatch 200A, patch-clamp amplifier driven by pClamp-8.2 software (Axon Instruments, Foster City, USA). Whole-cell currents were low-pass filtered (1KHz), sampled and digitized at 5KHz (DigiData 1320A A/D converter, Axon Instruments) and stored on a local hard-drive for subsequent analysis.

Electrophysiological recordings did not commence until 8-min following membrane rupture, to allow for adequate diffusion of Fluo-3 throughout the cytosol. In all drug exposure protocols – excluding R_p-cAMPS – each cell acted as it own control and was only subjected to one treatment. Control traces were obtained 8-min following membrane rupture, then appropriate drugs were added and responses measured 8-min latter (i.e. 16-min following membrane rupture). Cells were stimulated every 2-min by applying a series of depolarizing voltage steps from –40mV to +40mV (as described above) and subjected to a single depolarizing pulse to 0mV (100ms duration) every 10-sec during the interim. In control cells not subjected to any drug treatments, there was no difference in I_{CaL} density or Ca^{2+} transient amplitudes at the 8 and 16-min time points.

Ca^{2+} transients were recorded in voltage-clamped myocytes using a Fluoview FV200 confocal system equipped with an Argon laser (488nM), connected to an Olympus IX70 inverted microscope employing a 60x LCPlanFl objective (0.7 NA). Myocytes were dialyzed with 0.075μmol/L Fluo-3 (pentapotassium salt) and allowed to equilibrate 8-minutes before intracellular Ca^{2+} responses were assessed. Changes in fluorescence were measured by line-scans through the short axis of the cell at a temporal resolution of 2ms and a spatial resolution of 0.3μm. Acquisition was triggered to coincide with the stimulation of I_{CaL} by pClamp software outputs and each TIFF file generated (Fluoview TIEMPO ver 2.1.39) consisted of 8000 continuous line scans (512x8000 pixels) representing 16.4s of raw data. Under these conditions, the intracellular Ca^{2+} concentration is estimated to be ~75nmol/L as the Ca^{2+} buffering capacity of the cytosol is largely determined by the K_d of Fluo-3 for Ca^{2+3}.

In addition to providing the capacity to measure relative changes in cytosolic Ca^{2+} levels induced by SR Ca^{2+} release events, these experimental conditions also allow for global elevations in Ca^{2+} to occur in a somewhat unrestricted fashion that promotes actin-myosin interactions (i.e. cell shortening) and maintains Ca^{2+}/calmodulin-dependent feedback mechanisms on L-type Ca^{2+} channels, thereby providing a more physiological context in which to explore the role of cAMP/PKA-signalling in key cardiac EC-coupling events. Furthermore, by employing confocal microscopy to measure $[Ca^{2+}]_i$ events, we obviate any effects that cell shortening may have on Ca^{2+} transient amplitudes since the thickness of confocal plane does not change throughout experiment.

In the experiments where $I_{Ca,L}$ currents were measured in wild type and PDE3A$^{-/-}$ cardiomyocytes under the conditions of maximal phosphorylation using the β-adrenergic agonist isoproterenol and broad spectrum PDE inhibitor IBMX (100nmol/L ISO + 100µmol/L IBMX) electrophysiological responses were examined using a bathing solution containing (in mmol/L): 140 TEACl, 5 CsCl (both used to block K^+ currents), 0.5 $MgCl_2$, 1.8 $CaCl_2$, 5.5 glucose and 5 HEPES; adjusted to pH 7.4 with CsOH. The intracellular pipette solution contained (in mmol/L): 140 CsCl, 5 TEA Cl, 1 $MgCl_2$, 4 MgATP, 10 HEPES, 10 EGTA, 0.3 Na GTP adjusted to pH 7.2 with CsOH. Control traces were obtained 8-min following membrane rupture. Second measurement was taken 8 minutes after perfusion of ISO+IBMX (100nmol/L, 100µmol/L respectively). Cells were stimulated every 2-min by applying a series of depolarizing voltage steps from –40mV to +60mV, during the interim cells were subjected to a single depolarizing pulse to 0mV (100ms duration) every 20-sec. inclusion of 10mmol/L EGTA in our pipette solutions reduced $[Ca^{2+}]_i$ to subnanomolar levels. With EGTA in the pipette, $I_{Ca,L}$ couls be recorded in the absence of Ca^{2+} transients, which can strongly affect amplitudes and and decay rates. This allowed us to assess differences in $I_{Ca,L}$ between the WT and PDE3A$^{-/-}$ cardiomyocytes under conditions of maximal phosphorylation. Recordings were conducted under exactly same conditions as described above.

Estimation of SR Ca^{2+} load

SR Ca^{2+} content was estimated by integrating the forward-mode Na^+/Ca^{2+} exchanger current ($\int I_{NCX}$) evoked by SR Ca^{2+} release following a 10s application of 20 mmol/L caffeine (delivered via wide-bore pipette positioned up-stream of the cell)[4-6]. I_{NCX} was measured at a holding of -80 mV, employing 1-2 MOhm pipettes containing the following (in mmol/L): 125 K-aspartate, 20 KCl, 0.5 $MgCl_2$, 10 HEPES, 5.5 glucose, 0.4 Na-GTP, 5 Na_2.-phosphocreatine, 7 Mg-ATP and 0.05 Fluo-$_3$ (pentapotassium salt); pH 7.2 with KOH. The accompanying $[Ca^{2+}]_i$ responses were recorded as described above, with a temporal resolution of 4ms per/scan for a total record length of 32.8s.

Laser scanning confocal immunofluorescence

After WT C57 mice (~16 weeks) were sacrificed, hearts were immediately embedded and frozen sections were cut at a thickness of approximately 6 µm. Sections were fixed in 4% paraformaldehyde for 10 mins, washed in PBS 3x5 min, blocked and permeabilized in 10 % donkey serum containing 0.05% Triton X-100 for 6 h at 4^{o}C. Slides were incubated in blocking buffer with primary antibody overnight and washed with PBS (3 x 5 min) before incubating in blocking buffer for 2 h with secondary antibodies (Alexa Fluor 488 or alexa fluor 594) (Molecular Probe). As controls, samples were also incubated with nonimmune IgG or with primary antibody incubated with

blocking peptides prior to staining with secondary antibody. Slides were viewed with a Zeiss LSM510 laser scanning confocal microscope.

PDE activity assay

PDE activity was measured as described previously[7] Samples (usually 5~15 μg protein in 100 μl) were incubated for 10~15 minutes in a total volume of 0.3 ml assay buffer containing (in mmol/L): 50 HEPES, pH 7.4, 0.1 EGTA, 8.3 $MgCl_2$, and 0.1 μmol/L [^{3}H]-cAMP. PDE3 and PDE4 activities are those portions of total PDE activity inhibited by 1 μmol/L cilostamide (a specific PDE3 inhibitor) (Calbiochem, Cat#231085) and 10 μmol/L rolipram (a specific PDE4 inhibitor) (Calbiochem, Cat#557330), respectively. Inhibitor vehicle, DMSO, added in equal quantities to samples without inhibitors, did not alter PDE activity. Protein concentration was determined by BCA protein assay.

Preparation of SR vesicles

SR vesicles were obtained as described[8-10] C57 male mice (8-16 weeks old) were sacrificed, the hearts were excised and placed in ice-cold PBS. Ventricles from 3-6 mice were quickly removed, washed 3 times in ice-cold PBS, chopped to small pieces, washed and homogenized in buffer containing 0.6 mol/L sucrose and 10 mmol/L imidazole, pH 7.0 (buffer A), protease inhibitor and phosphatase inhibitor. Large particles from homogenates were removed by centrifugation (12,000 *g*, 30 min, 4^{o}C). The resultant supernatants were diluted with 300 mmol/L KCL buffer to solubilize myofibrillar proteins. After setting in ice for 30 minutes, supernatants were centrifuged (43,666 *g*, 30 min, 4^{o}C). Pellets were resuspended in buffer containing (mmol/L): 250 sucrose, 10 histidine, (pH 7.0) protease inhibitor and phosphatase inhibitor. Samples were centrifuged (43,666 *g*, 30 min). Final pellets containing SR fractions were suspended in buffer containing 250 mmol/L sucrose and 10 mmol/L histidine (pH7.0), aliquoted, frozen in liquid nitrogen, and stored at -80^{o}C.

Density gradient separation of microsomes

Crude microsomes were isolated and loaded onto a discontinuous sucrose gradient, as previously described by Frias[11]. The gradient consisted of four steps of 2.4 ml (27–32–34–38% sucrose, w/w) and one step of 1.4 ml (40% sucrose, w/w) sucrose in 5 mmol/L imidazole–HCl pH 7.4. Gradients were centrifuged (70,000 *g*, 12~16h, 4^{o}C). Fractions were collected at the each interface of the density gradient from most to least dense, diluted 3 fold with 5 mmol/L imidazole-HCl pH7.4, and then centrifuged (43,666 *g*, 30 min, 4^{o}C). Pellets were collected in RIPA buffer containing 1X protease inhibitor cocktail and 1X phosphatase inhibitor cocktail and stored at -80^{o}C.

Immunoprecipitation and immunoblotting

WT C57BL/6J and PDE3A-/- male mice (5 months old, n=3 per group) were killed by CO_2 asphyxiation, followed by cervical dislocation. Dissected heart tissues were quickly washed in ice-cold PBS, chopped with scissors, and homogenized (5 ml/g tissue) in buffer containing (in mmol/L): 50 HEPES, 50 sucrose, 1 EDTA, 10 pyrophosphate, 5 $MgCl_2$, 5 NaF, 100 NaCl, 0.1μmol/L okadaic acid, phosphatase inhibitor cocktail set II (Calbiochem) and Roche protease inhibitor cocktail (pH 7.5) with a rotor-stator homogenizer (Omni International, Marietta, Georgia, USA) at 30,000 rpm (60-70 seconds, on ice), and sonified (on ice, 20 pulses, 40% duty cycle, output scale 4). After

homogenization (on ice, 20 strokes in a glass Dounce homogenizer), in 1% NP-40 (Calbiochem), samples were incubated (on ice, 1 hour), before centrifugation (15,000 *g*, 20 minutes, 4°C). Supernatants (total heart lysates) were used for Western immunoblotting. Protein was determined by BCA protein assay (Pierce, Rockford, Illinois, USA).

For most experiments, samples were cleared by incubation (1 h, 4 ^{0}C) with 5 μg of normal rabbit IgG, and then (30 min) with 50 μl of protein G–sepharose (GE Healthcare), before centrifugation (2800 g, 4 ^{0}C, 5 min). Cleared fractions were incubated overnight (4 ^{0}C) with control IgG or specified antibodies, followed by incubation with fresh protein G–sepharose (1 h) before centrifugation (2800 g, 4 ^{0}C, 5 min). Immunoprecipitates were washed three times with buffer containing (in mmol/L): 50 Hepes, 50 sucrose, 1 EDTA, 10 pyrophosphate, 5 MgCl2, 5 NaF, 100 NaCl, 1 Na3VO4, 0.1 uM okadaic acid, and Roche protease inhibitor cocktail, pH 7.5. Immunoprecipitated proteins were eluted from protein-G-sepharose by boiling in SDS sample buffer. Lysate samples (20 ug/lane) and immunoprecipitated samples were subjected to SDS/PAGE and electrotransferred to membranes in Tris-glycine buffer (25 mmol/L Tris/base and 192 mmol/L glycine, pH 8.3), containing 20% (v/v) methanol. Membranes were incubated (4 ^{0}C overnight) with 5% (w/v) NFDM (non-fat dry milk) in DPBS (Dulbecco's PBS) and then with the appropriate primary antibody (usually for 2 h, but sometimes longer, depending on antibody quality and sensitivity) in 0.5% NFDM in DPBS, washed (DPBS), and incubated with HRP (horseradish peroxidase)-labelled secondary antibody (Pierce). Immunoreactive proteins were reacted with SuperSignal® Westpico or Westfemto chemiluminescent reagents; signals were detected with Imagereader LAS3000 (Fuji, Stanford, CT, U.S.A.).

SERCA2a activity assay

Ca^{2+}-ATPase activity in SR fractions was determined by measuring the amount of Pi released after addition of ATP, using the malchite green ATPase method. Samples were assayed in the presence or absence of thapsigargin (10 μmol/L), since SERCA2a activity was determined as that portion of total activity inhibited by thapsigargin. The assay mixture (total volume, 125 μL) contained (in mmol/L): 125 KCl, 20 imidazole, pH7.0, 0.1 EGTA, 0.103 Mmol/L $CaCl_2$, 1 μM Ionomycin (Calbiochem, Cat#407950) and 10 μg SR. To initiate the reaction, 25 μL of substrate Mg^{2+}-ATP was added to a final concentration of 0.25 mmol/L. The mixture was incubated at room temperature for 0.5~3 minutes. The reaction was terminated by adding 25 μl of 250 g/L TCA, vortexed quickly, and centrifuged (8,000 *g*, 3 min). Supernatants (20 μL) were added to 96 well plates, followed by adding color reagent (100 μl per sample) which consisted of 54 mM ammonium molybdate and 0.73 mM Malachite green. After 1 min, NaCitrate (340 g/L, 10 μl per sample) was added, with gentle shaking (room temperature for more than 20 min), after which plates were scanned at 650 nm. Pi was calculated by converting OD650 nm to nmoles by means of a standard curve.

Effect of cAMP on SERCA2a activity

SR fractions (50~100 μg) were incubated (30 min, 30^{o}C) with indicated concentrations of cAMP in buffer containing (in mmol/L):10 imidazole-HCl (pH7.0), 10 $MgCl_2$, 5 DTT, 0.5 ATP, 1 μM okadaic acid, and 1X phosphatase inhibitor cocktail. The reaction mixture was centrifuged (43,666 *g*, 30 min) at 4^{o}C. Pellets were suspended in buffer containing 0.6 M KCl, 20 mM Tris-HCl (pH6.8), 1X protease inhibitor and 1X

phosphatase inhibitor. After setting in ice for 30 minutes, samples were centrifuged (43,666 *g*, 30 min). Final pellets were re-suspended in buffer containing 250 mM sucrose and 10 mM histidine (pH7.0), aliquoted, frozen in liquid nitrogen, and stored at -80^{o}C for calcium uptake and SERCA2a activity assays.

Sarcoplasmic reticulum $^{45}Ca^{2+}$ uptake

Oxalate-dependent Ca^{2+} uptake was performed as described previously. [12] Briefly, 50 µl SR(10-20 µg) were incubated at 37^{o}C for 0.5~3 minutes in 0.4 ml Ca^{2+} uptake buffer, consisting (in mmol/L): 50 imidazole-HCl, pH7.0, 100 KCl, 6 $MgCl_2$, 10 NaN_3, 10 potassium oxalate, 0.1 EGTA, 20 µmol/L ruthenium red, $^{45}CaCl_2$ and unlabeled $CaCl_2$ (0.5 µmol/L free Ca^{2+}) . The reaction was initiated by adding 50 µl of 50 mmol/L ATP, and terminated at indicated times by filtration through 0.45 µm Millipore filters. After washing 4 times with 4 ml buffer containing (in mmol/L): 140 KCl, 10 NaCl, 2 $MgCl_2$, 1 $CaCl_2$ and 50 imidazole-HCl, pH7.0, radioactivity retained on the filters was quantified by liquid scintillation counting. Free calcium was calculated using a program obtained at http://www.stanford.edu/~cpatton/webmaxc/webmaxcS.htm

To assess effects of cilostamide on cAMP-stimulated Ca^{2+} uptake, WT SR fractions (20µg) were incubated (30^{o}C, 30 min) with or without 0.3µmol/L cAMP (final concentration) or cAMP plus 1µmol/L cilostamide, a specific PDE3 inhibitor, followed for assay of $^{45}CaCl_2$ uptake for 1 min, as described above.

LC-MS/MS of PDE3A immunoprecipitates

Cardiac muscle from 8~16 weeks old C57 wild type mice was homogenized in ice-cold buffer containing 10% glycerol with (in mmol/L): 50 HEPES, 100 sodium pyrophosphate, 100 sodium fluoride, 1 EDTA, 1X Protease inhibitor cocktail (Sigma, Cat# P8340) and 1% Triton X-100 or 1% NP-40. Insoluble material was removed by centrifugation (100,000 *g*, 30 min, 4^{o}C). Pre-cleared lysates (500~1000 µg) were incubated (overnight, 4^{o}C) with 5 µg non-immune IgG or anti-rabbit PDE3A antibody raised in our lab, (CT(1098TGENQSLDQVPLQHPSEQ1115)). After incubation with Protein G beads (2 hours, 4^{o}C) and centrifugation (2,000 g, 30 s), the collected beads were washed 5 times with ice-cold lysis buffer, and heated in 1X SDS sample buffer containing 5% β-mercaptoethanol at 95^{o}C for 5 minutes to release bound protein. PDE3A immunoprecipitates were separated by 4~20% SDS-PAGE. Gels were stained by Coomassie SimplyBlueTM SafeStain (Invitrogen, Cat#LC6060) for 1 hour at RT, followed by destaining (overnight, RT) in pure water. Gels were sliced, as indicated. Each gel slice was diced into small cubes (1~2 mm^3) and transferred to 1.5 ml tubes. Gel pieces were further destained in the presence of 25mM NH4HCO3/50% ACN (3X10 min), and then taken to complete dryness in a speed Vac. After reduction (10 mmol/L DTT, 25 mmol/L NH4HCO3) (56^{o}C, 1h) and alkylation (55 mmol/L iodoacetamide, 25 mmol/L NH4HCO3)(45 min, RT), gel pieces were taken to dryness in a speed Vac and digested in the presence of 12.5 ng/µl trypsin (Invitrogen, Cat#MS10015) (overnight, 37^{o}C). Peptides were extracted and cleaned by C18 ZipTip (Millipore), final samples were stocked in 0.1% FA for LC-MS/MS scanning.

Data analysis

All values are reported as a mean ± standard error of the mean (SEM), with n-values varying between experiments. For the *in vivo* mouse studies and the Langendorff heart studies, the n-values refer to number of animals or hearts. For the patch-clamp studies, n-values refer to the number of myocytes which were always derived from at least 3 mice. For the biochemical studies n-values refer to the number of experiments performed (each using a different set of samples for both WT and PDE3A$^{-/-}$).

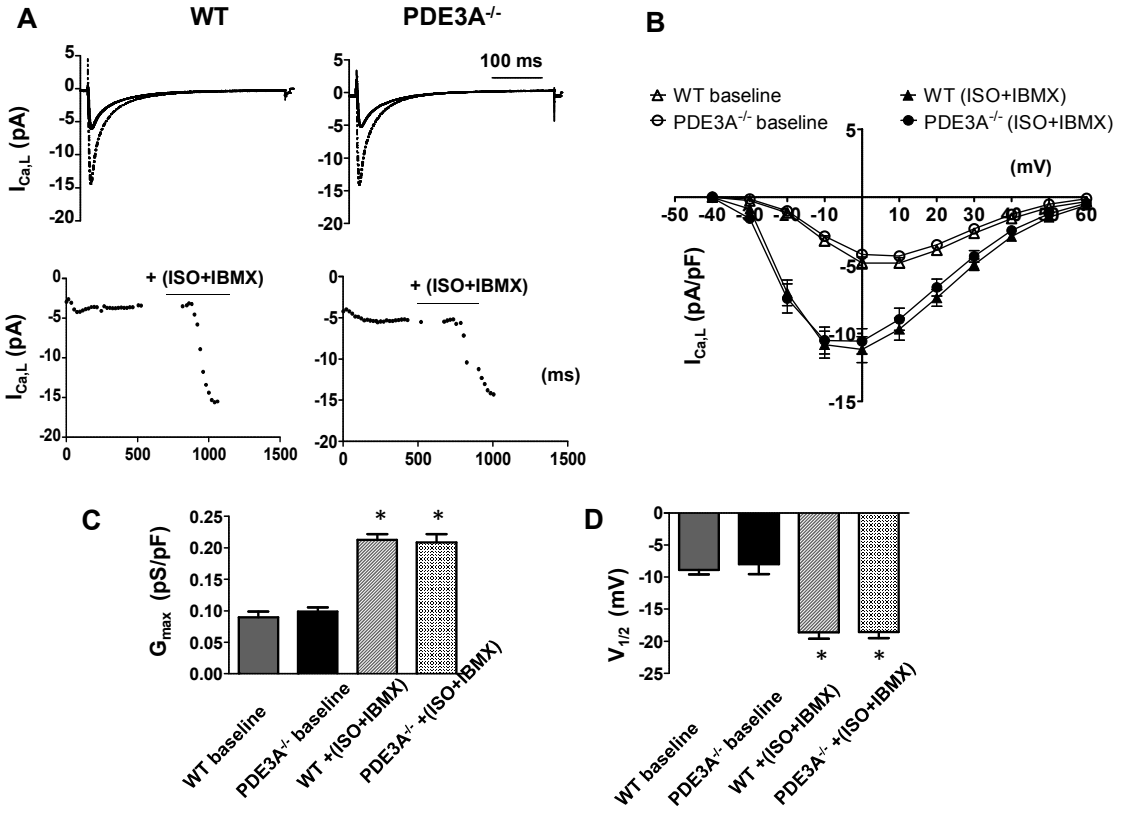
Statistical significance was assessed as appropriate by utilizing a paired or unpaired Students t-test, or a two-way ANOVA. *P-values* <0.05 were considered significant.

In Langedorff studies the left ventricular developed pressure (LVDP) was calculated as the difference between the end-systolic and end-diastolic pressure. Indices of cardiac contractility ($+dPdt_{max}$) and relaxation ($–dP/dt_{min}$) were calculated as the maximum and minimum first time derivatives of the LV pressure (mmHg) using commercially available software (Spike2 5.21, Cambridge Electronic Design, Cambridge, UK).

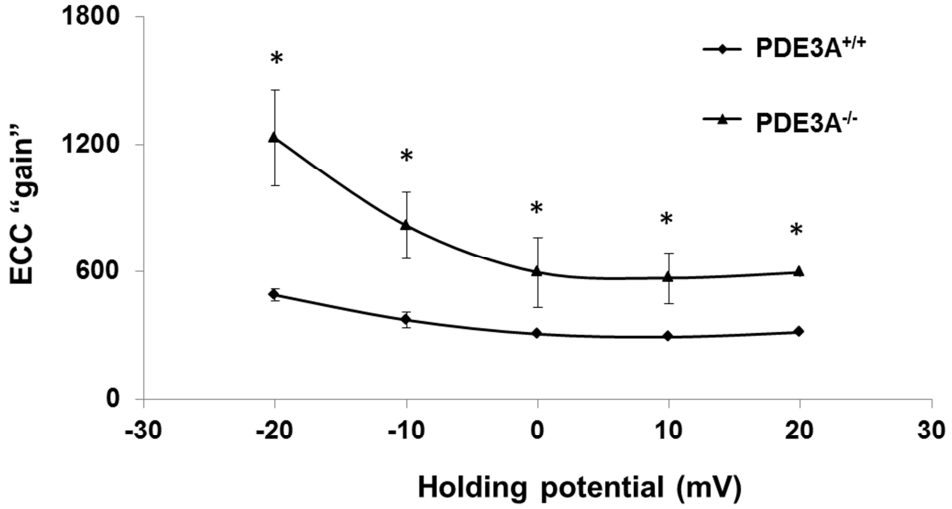
I_{CaL} amplitude was measured as the difference between the peak current evoked by a 200ms depolarizing pulse and that remaining at the end of the pulse. I_{CaL} density (pA/pF) was calculated by normalizing the raw current amplitudes to the cell capacitance. Cord conductance for each cell was calculated using the following equation: $G = \text{peak } I_{CaL} / (V_m - E_{rev})$, where V_m represents the membrane potential and E_{rev} represents the estimated reversal potential extrapolated from I_{CaL}-voltage relationships using linear regression (least squares method). Conductance data was then fit by a Boltzmann function: $G(V_m) = G_{max}/(1+\exp[(V_m-V_{1/2})/k])$, where G_{max} is the maximal conductance, $V_{1/2}$ the voltage for 50% activation of I_{CaL} and k is the slope factor. Both the fast (τ_{fast}) and slow (τ_{slow}) time constants of inactivation were acquired by fitting the I_{CaL} decay at 0 mV with a biexponential function.

Fluo-3 fluorescence was quantified by spatially averaging the Fluo-3 fluorescence (F). At each time point we then calculated the difference (ΔF) between F and the fluorescence recorded over a 40 ms period during diastole (F_O, i.e. fluorescence observed at a membrane potential of -85 mV). The Ca^{2+} changes in response to depolarizations were quantified as $\Delta F/F_o$. All analysis of line-scans was performed using Image-Pro Plus 4.5.0.25 (Media Cybernetics, Inc., Bethesda, MD). Due to the inherent level of noise present in the line-scans obtained, each trace was filtered using 30 points average substitution. The time constant of decay (τ_{decay}) was obtained by fitting the recovery phase of the Ca^{2+} transient with a standard monoexponential function.

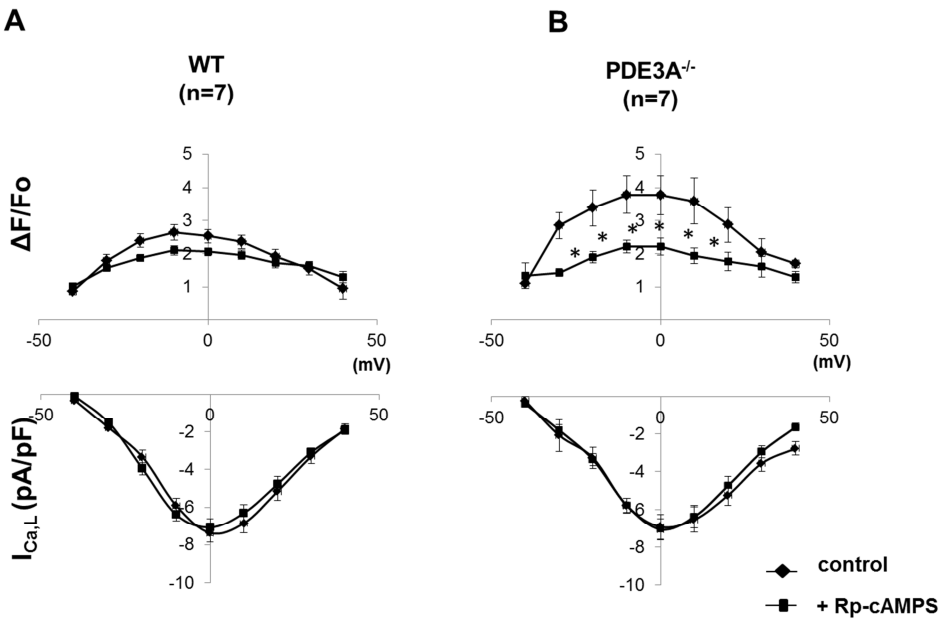
The time integral of I_{NCX} (pA·ms) was calculated using Clampfit 9.2 (Axon Instruments, Foster City, USA), normalized to cell size (pA·ms/pF) and divided by a correction factor of 0.87, since it is estimated that NCX removes ~87% of Ca^{2+} released by the SR [7]. In the majority of cases, I_{NCX} recovered (i.e. returned to baseline) prior to cessation of caffeine application (i.e. 4 ± 0.5 s, n= 13). Where I_{NCX} did not completely recover towards baseline (5/18 cells), integration was cropped at 10 s following current activation (to coincide with caffeine application).



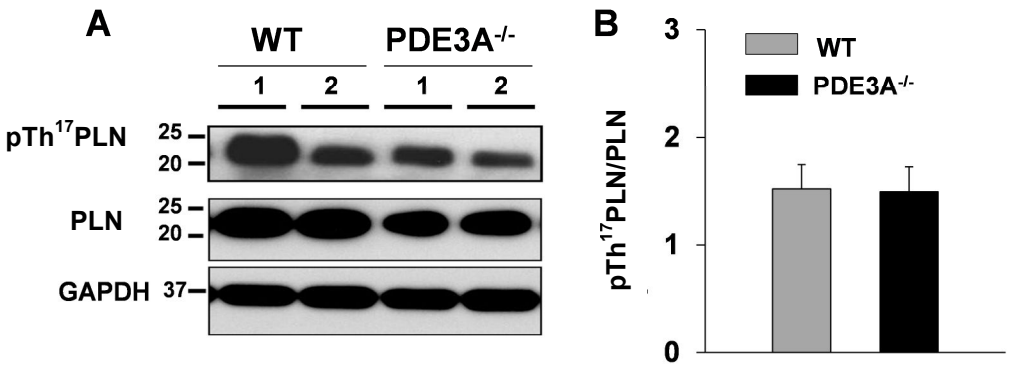
Online Figure I: $I_{Ca,L}$ currents recorded from the WT and PDE3A$^{-/-}$ cardiomyocytes under the conditions of maximal cAMP activation with IBMX [100umol/L] and isoproterenol [100nmol/L; ISO]. A) Raw traces (top panel) and time plot (bottom panel) of $I_{Ca,L}$ measured in response to depolarizing step to 0mV illustrating the effects of IBMX and ISO infusion in WT and PDE3A$^{-/-}$ cardiomyocytes. The holding potential was -85mV and 200ms depolarization ramp to -45mVpreceeded voltage steps. After the cell rupture, cardiomyocytes were allowed 8 min for full equilibration; then ISO+IBMX were perfused and cardiomyocytes were allowed to equilibrate for another 8 minutes before measuremnts of $I_{Ca,L}$ were made. B) Mean data illustrating the effects of IBMX and ISO on $I_{Ca,L}$ in WT (n=11) and PDE3A$^{-/-}$ (n=9) cardiomyocytes over the voltage range from -40 to +40mV. The protocol was the same as described in panel A, except in this case depolarizations were varied in 10mV increments. C) Mean data illustrating the effects of IBMX and ISO on maximal $I_{Ca,L}$ current conductance (G_{max}). D) Mean results for half-maximal $I_{Ca,L}$ activation voltage ($V_{1/2}$).



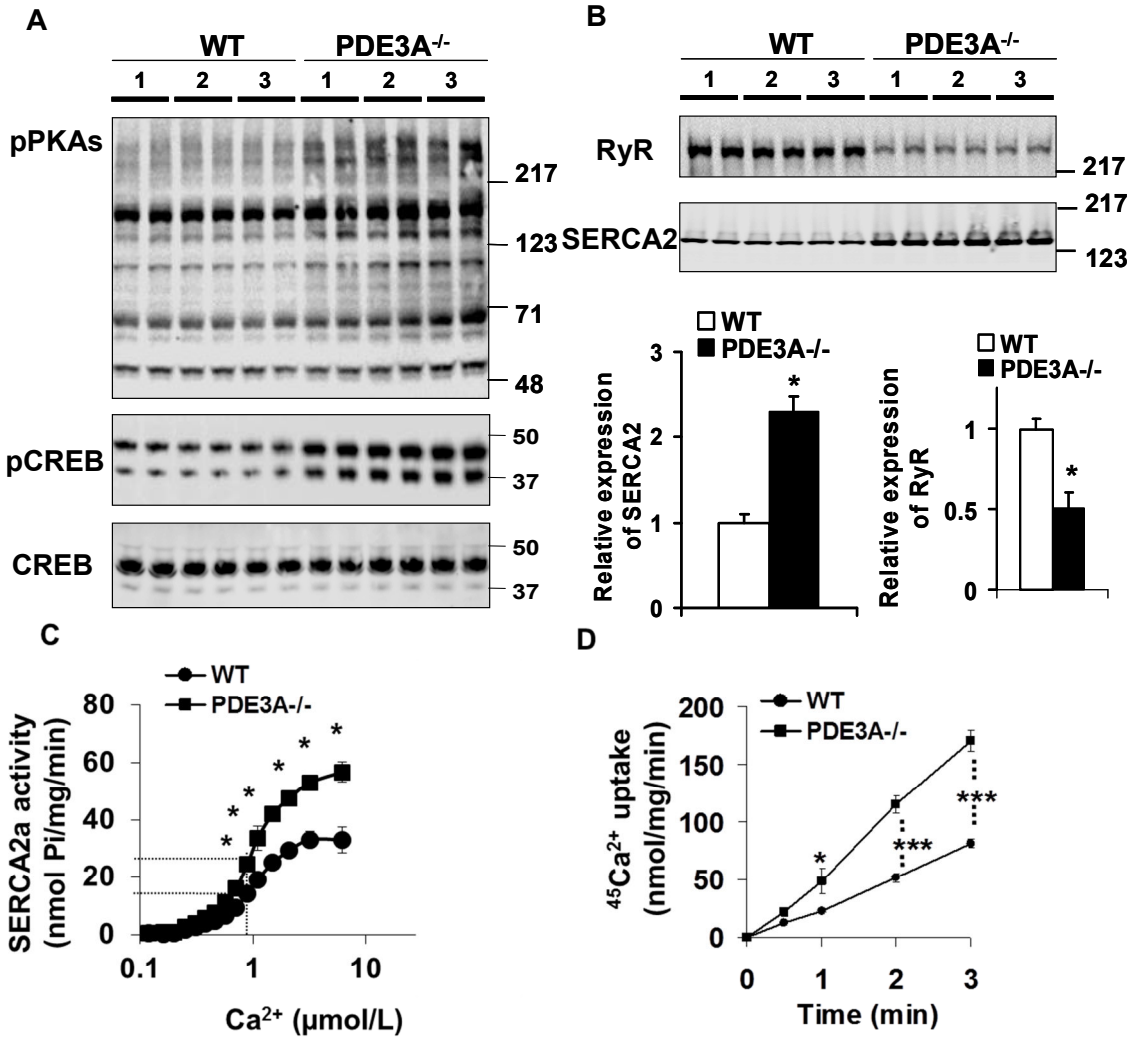
Online Figure II: PDE3A ablation enhances excitation-contraction (ECC) coupling gain. Mean data illustrating that PDE3A regulates the efficiency (i.e. gain) of excitation-contraction coupling. ECC gain was calculated by dividing the Ca^{2+} transient time derivative by the peak $I_{Ca,L}$ current amplitude. Data was plotted over a range of holding potentials ($P<0.05$; n=16 for the WT, n=9 for the $PDE3A^{-/-}$).



Online Figure III: Effects of PDE3A ablation on Ca^{2+} transients and I_{CaL} are cAMP dependent. A) Mean data illustrating the effects of Rp-cAMPS infusion on Ca^{2+} transient amplitudes and I_{CaL} densities from WT (n=7) and B) PDE3A$^{-/-}$ mice (n=7). I_{CaL} was evoked by depolarizing pulses from –40mV to +40mV delivered from a holding potential of –85mV (values indicate membrane potential). *p<0.05

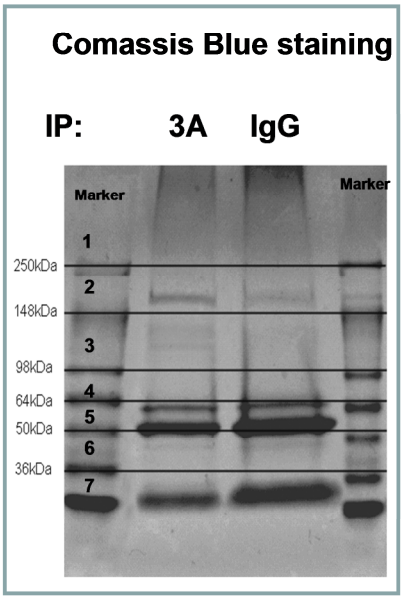


Online Figure IV: Unchanged levels of CaMKII dependent phosphorylation of phospholamban in PDE3A$^{-/-}$ hearts. Western blots of WT and PDE3$^{-/-}$ heart lysates (40 µg), illustrating the effect of PDE3A ablation on phosphorylation of PLN CaMKII dependent site Threonine 17 (Th^{17}). A) PDE3A ablation had no effect on the phosphorylation of PLN (pPLN) at residue Th-17, with respect to total PLN. GAPDH was used as indicator of equal loading conditions. B) Bar graph summarizing pTh^{17}PLN/PLN$_{total}$ ratios in WT and PDE3A $^{-/-}$ hearts; (n=3 experiments, 1 heart per lane). As a result of the protein samples heating, PLN run on a SDS-PAGE gel in its monomeric (5 kDa) and pentameric forms (25 kDa). Total PLN was used for the analysis.



Online Figure V: Increased levels of phoshorylation of PKA-related protein targets in PDE3A$^{-/-}$ hearts. A) Western Blots of WT and PDE3$^{-/-}$ heart lysates (40 µg) showing phosphorylation level***s*** of CREB, and other PKA substrates. Phospho-PKA Substrate antibody (RRXS*/T****,*** Cell Signaling Technology) detects phospho-Ser/Thr residue***s*** with arginine at the -3 and -2 positions. Phospho-CREB antibody (Millipore) detects CREB when phosphorylated at serine 133. This blot is representative of 3 independent experiments, each with duplicate samples from 3 WT and 3 PDE3A$^{-/-}$ heart lysates. B) Western Blot of WT and PDE3$^{-/-}$ heart lysates illustrating the effect of PDE3A ablation on the protein expression levels of SERCA2a and RyR2. This blot is representative of 2 independent experiments, each with duplicate samples from 3 WT and 3 PDE3A$^{-/-}$ heart lysates. The bar graphs summarize fold-changes in SERCA2a and RyR2 expression for 6 WT and 6 PDE3A$^{-/-}$ hearts. * p< 0.01 versus WT. C) SERCA activity in WT and PDE3A$^{-/-}$ SR vesicles (~10 µg) was assessed as described in methods. All media contained 1 mmol/L EGTA and the concentration of free Ca^{2+} was varied from 0-6.2 µmol/L as indicated. Data (triplicate assays) are representative of 2 experiments, each with SR fractionsfrom 4 WT and 4 PDE3$^{-/-}$ hearts. D) Ca^{2+} uptake in SR vesicles (~20 µg) from WT and PDE3A$^{-/-}$ hearts was measured at the indicated times as described in methods. *p<0.05, ***p<0.001 (n=4).

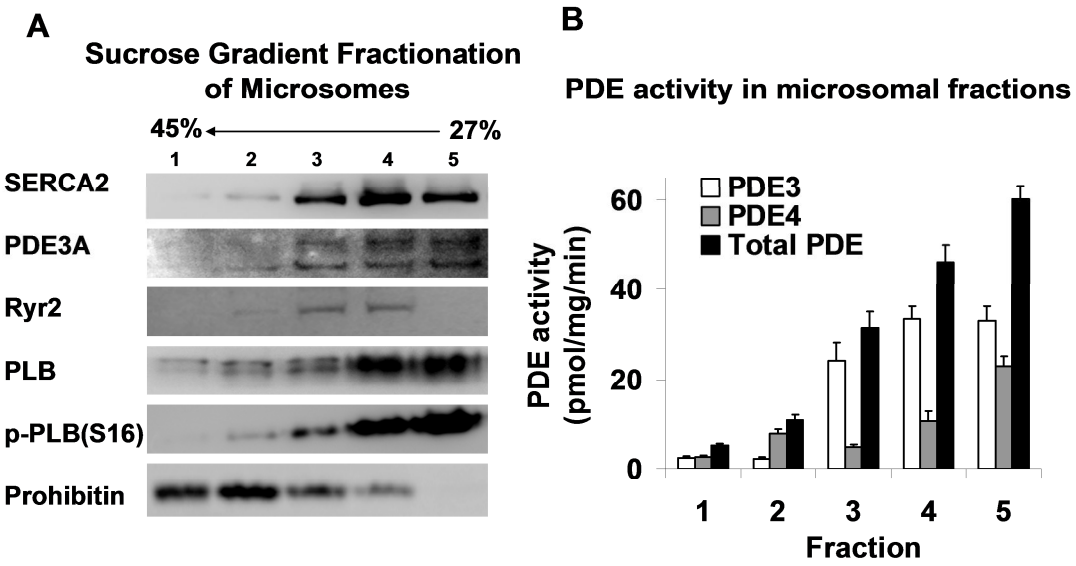
A



B **LC MS/MS Peptide Reuslts**

Protein	Sequence
ATPase, Ca^{2+} transporting	
	IRDEMVATEQER
	GAPEGVIDR
	VDQSILTGESVSVIK
	NAENAIEALKEYEPEMGK
	TVEEVLGHFGVNESTGLSLEQVKK
	AKDIVPGDIVEIAVGDKVPADIR
	VSFYQLSHFLQCK
	CHQYDGLVELATICALCNDSALDYNEAK
	DIVPGDIVEIAVGDKVPADIR
	TASEM*VLADDNFSTIVAAVEEGR
	IVEFLQSFDEITAM*TGDGVNDAPALK
	ISLPVILMDETLK
phosphodiesterase 3A	
	LADINGPAK
	ILSQVSYR
	HFDFVAK
	VSSTWTTTTSATGLPTLEPAPVRR
	AVSNLLSTQLTFQAIHKPR
	TNAFLVATSAPQAVLYNDR
	LFEDM*GLFEAFK

Online Figure VI: LC-MS/MS analysis of PDE3A immunoprecipitates. A) In-Gel digestion: As described above, nonimmune IgG (3 µg) andPDE3A immunoprecipitates (3 µg) were separated by SDS-PAGE. Gels were stained by Coomassie® G-250, and then cut according to molecular weight as indicated**,** and followed by trypsin (12.5 ng/µl) digestion overnight at 37^{o}C respectively. Peptides from fractions 1~7 were extracted and subjected to LC-MS/MS analysis. B) LC-MS/MS analysis**:** PDE3A peptides and SERCA2 peptides were identified in fraction 3 in PDE3A, but not in nonimmune IgG imunoprecipitates (n=2 experiments)



Online Figure VII: PDE3A co-fractionated with SERCA2a and phospholamban during discontinuous sucrose gradient centrifugation of WT mouse cardiac membranes. A) Cardiac microsomes (2mg) from 8-16 week old male mice were separated on discontinuous sucrose gradients as described. Fraction 1 is collected from 40% gradient; fraction 2 from 38-40% gradient interphase; fraction 3 from 34-38% gradient interphase; fraction 4 from 32-34% gradient interphase; fraction 5 from 37-32% gradient interphase. Equal volumes of each interphase were subjected to SDS-PAGE followed by Western Blotting. PDE3A cofractionated with SERCA2a and phosholamban. One representative blot is shown (n=4). B) PDE3, PDE4 and total PDE activities were quantified, showing that PDE3A activity is the highest in fractions 3-5. Data is presented as Mean ± S.D. (n=3).

Online Table I: Comparison of contractile function in WT and PDE3A$^{-/-}$ hearts at the baseline and 8Hz, before and after specific PDE3 inhibition with 10μmol/L milrinone.

LVP indicates left ventricular pressure; HR, heart rate; dP/dt_{max}, maximum first derivative of the change in left ventricular pressure; dP/dt_{min}, minimum first derivative of the change in left ventricular pressure; EDP, end-diastolic pressure; LVDP, left ventricular developed pressure. Values are mean± SEM; *p <0.05 vs. control in the same group; †p<0.05 between groups.

	PDE3A$^{+/+}$ (n=5)			PDE3A$^{-/-}$ (n=5)		
	baseline	paced	paced+Mil	baseline	paced	paced+Mil
iHR (bpm)	335.9± 29.7			396.1± 27.2		
LVP (mmHg)	92.0± 4.9	91.6± 8.17*	107.0± 7.6*	106.5± 2.6†	95.8± 2.7	97.9± 1.3
dP/dt_{max} (mmHg/s)	2498.6± 101.3	2804.7± 367.4*	3479.3± 415.9*	3080.8± 193.2†	2768.1± 66.2	2899.4± 129.1
dP/dt_{min} (mmHg/s)	-1841.3± 97.0	-2184.3± 305.6*	-2720.0± 337.6*	-2236.3± 221.8	-1960.3± 56.4	-2056.5± 102.4
EDP (mmHg)	5.4± 0.3	5.4± 0.9	3.0± 2.3	6.8± 0.5	9.8± 1.7	8.5± 3.4
LVDP(mmHg)	86.6± 4.9	86.2± 8.9*	104.1± 7.9*	99.7± 2.5†	86.0± 1.3	89.4± 3.7

Online Table II: Mean Ca^{2+} transient and I_{CaL} amplitudes, I_{NCX} and kinetics measured at 0mV in WT and PDE3A $^{-/-}$ cardiomyocytes at baseline, and also in response to specific PKA inhibition (100μM Rp-cAMPS) and specific PDE3 inhibition (10μM milrinone).

	WT			PDE3A$^{-/-}$		
	Control	+Mil (n=16)	+Rp-cAMP (n=7)	Control	+Mil (n=9)	+Rp-cAMP (n=7)
Ca^{2+} transient ($\Delta F/Fo$)	2.5 ± 0.2	3.2 ± 0.4*	2.1 ± 0.1	3.6 ± 0.6†	3.2 ± 0.4	2.0 ± 0.2*
τ_{decay}	158 ± 9.7	139 ± 7.5*	162 ± 8.7	142 ± 4.4	131 ± 8.5	160 ± 10.2*
$I_{Ca,L}$ (pA/pF)	-7.4 ± 0.5	-6.8 ± 0.5	-6.9 ± 0.6	-7.1 ± 0.5	-6.3 ± 0.6	-7.3 ± 0.4
G_{max}	229 ± 25	224 ± 19	154 ± 16	199 ± 22	170 ± 19	159 ± 19
$V_{1/2}$	-9.2 ± 1.4	-11.5 ± 1.1	-9.6 ± 3.6	-11.6 ± 1.0	-12.1 ± 1.6	-9.2 ± 3.0
$\int I_{NCX}$ (pA/pF·ms)	-0.7 ± 0.1	-1.0 ± 0.03*		-1.0 ± 0.01†	-1.0 ± 0.04	

Tau decay (τ_{decay}) was determined by fitting the decay of the Ca^{2+} transient with a mono exponential function; tau fast (τ_{fast}) and tau slow (τ_{slow}) were obtained by fitting the decay of I_{CaL} with a biexponential function. Ca^{2+} release from the SR ($\Delta F/Fo$), accompanied by I_{NCX} was induced by application of 20mM caffeine. Only one condition was tested in each cell. There were no appreciable differences in control parameters within the WT and PDE3A $^{-/-}$ groups. Data shown as mean ± SEM; *p<0.05 versus control within same group; †p<0.05 versus WT control.

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