Supporting Information

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SI Materials and Methods

Cell Culture, Adenovirus Infection, and Plasmid Transfection. Neonatal myocytes were isolated from wild-type, β_1AR-KO, and β_2AR-KO mouse pups. Cells were infected with adenovirus expressing SR-AKAR3 for FRET study as described in *Material and Methods*. Cells were infected with adenoviruses expressing hemagglutinin (HA)-β_1AR or Flag- β_2AR together with PDE4D8 isoform for coimmunoprecipitation. HEK293 cells were transfected with Flag-EP4, Flag- β_2AR, HA-β_2AR, and HA-β_1AR as indicated. Cells were stimulated with ISO (10 μM; 10 min) in the presence or absence of pretreatment with PGE2 (1 μM; 5 min). Cells were then lysed for immunoprecipitation with anti-Flag M2 affinity beads (Sigma) or anti-HA affinity beads (Roche) as indicated. The pull-down proteins were detected with anti-PGE4, anti-HA, and anti-Flag antibodies.

Drug Treatments. Myocytes were stimulated with dopamine receptor agonist dopamine (100 μM), urocortin receptor agonist urocortin (100 nM), relaxin receptor relaxin (20 ng/mL), or together with ROL (10 μM) as indicated. Alternatively, cells were stimulated with these drugs for 5 min before FSK (an adenylyl cyclase agonist; 10 μM; Sigma) or ISO (Sigma) at indicated doses and times.

Membrane and Sarcoplasmic Reticulum Fraction. Briefly, myocytes were harvested in cold 1× PBS buffer followed by washing. Cells were centrifuged three times (1,000 × *g*; 5 min each time; 4 °C), suspended in 1 mL of ice-cold 0.5 M sodium carbonate (pH 11), sonicated on ice for 30 s, and then homogenized with a loose-fitting Dounce homogenizer followed by a Polytron tissue grinder at 4 °C. Cell lysates were centrifuged at 16,000 × *g* for 30 min, SR was located in the pellets, and the supernatant was loaded on the bottom of an ultracentrifuge tube and spun in an SW40 rotor at 100,000 rpm at 4 °C for 1h. The membrane fraction was collected in the bottom pellets, and supernatant was cytosolic fraction. Membrane fraction pellets were solubilized by PBS contained 1% Triton X-100, and centrifuged at 16,000 × *g* rpm at 4 °C for 30 min. Protein from each fraction was solubilized in 1× SDS/PAGE sample buffer. The precipitated proteins in the fraction were separated on 10% SDS/PAGE gel for Western blot with antibody against PLB (Badrilla), GFP, and Na-K-ATPase (SCBT).

Langendorff Perfusion Heart Preparation. Animal experiments were performed following the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*, and all procedures were approved by the Institutional Animal Care and Use Committee at the University of Illinois at Urbana and Temple University. The isolated heart perfusion technique was described previously (24). Hearts were excised from mice under anesthesia (sodium pentobarbital; 120 mg/kg body weight; i.p.) and rapidly transferred to the Langendorff apparatus. Once the heart was hung, it was perfused under constant pressure (80 mmHg) with a solution containing (in mM): NaCl 13.8, NaHCO3 22, KCl 4.7, KH_2PO4 1.2, $MgSO_4$ 1.1, glucose 1.1, $CaCl_2$ 2, Na-pyruvate 2. A balloon made of plastic film was inserted into the left ventricular (LV) cavity and then was filled with water to set the diastolic pressure at 10 mmHg of LV end-diastolic pressure (LVEDP). The balloon was connected to a Millar blood pressure system (Millar Instruments), and the pressure was measured with a pressure catheter (SPR-671; Millar Instruments) connected to an ADInstruments PowerLab 16/30 with LabChart Pro-6.0 (ADInstruments). All hearts were immersed in a water-jacketed organ chamber to maintain a temperature of 37 °C. The heart rate was maintained at 480 beats per minute by pacing at the right ventricle with a Grass SD9 Stimulator. The hearts become stabilized after pacing for 15 min. Drugs were applied through the perfusion solution. Once a stable effect of the previous dose (~5 min) had taken place, the next dose was applied. LV pressure (LVP), LVEDP, and the maximum rate of positive and negative change in LV pressure (±LV dP/dt) were recorded. LVDP was calculated by subtracting the LVEDP from the LV systolic pressure. Data were analyzed with LabChart Pro-6.0 offline.

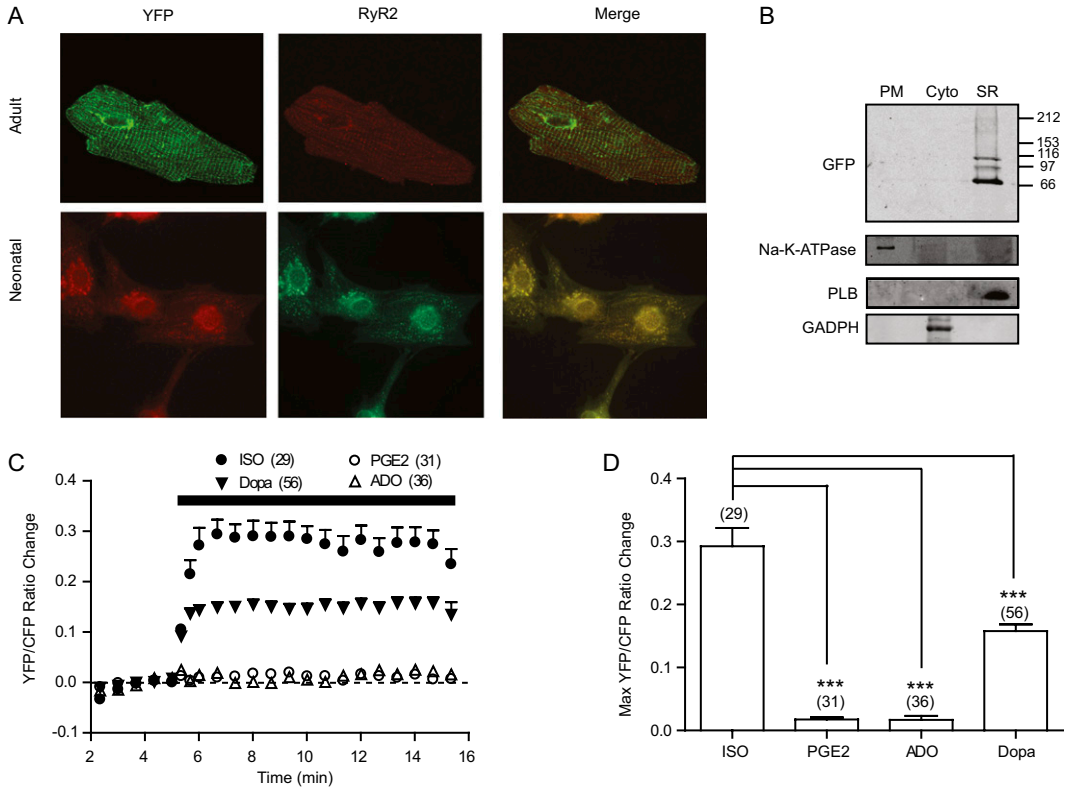

Fig. S1. Characterization of SR-AKAR3 in neonatal and adult cardiomyocytes. (*A*) SR-AKAR3 was expressed in both neonatal and adult cardiomyocytes, the distribution of SR-AKAR3 was imaged together with the SR marker ryanodine receptor 2 (SCBT). (*B*) Neonatal cardiomyocytes expressing SR-AKAR3 were lysed and subjected to membrane fractionation to collect the SR, the PM, and the cytosolic fractions. The fractions were blotted with antibodies against GFP, the PM marker Na-K-ATPase, the SR marker PLB, and the cytosolic marker GAPDH, respectively. (*C*) Neonatal cardiomyocytes expressing SR-AKAR3 were stimulated with dopamine (Dopa) (10 μM), adenosine (ADO) (100 μM), FSK (10 μM), or ISO (10 μM), and the changes in SR-AKAR3 FRET ratio were recorded. (*D*) The maximal changes in the SR-AKAR3 FRET ratio were plotted.

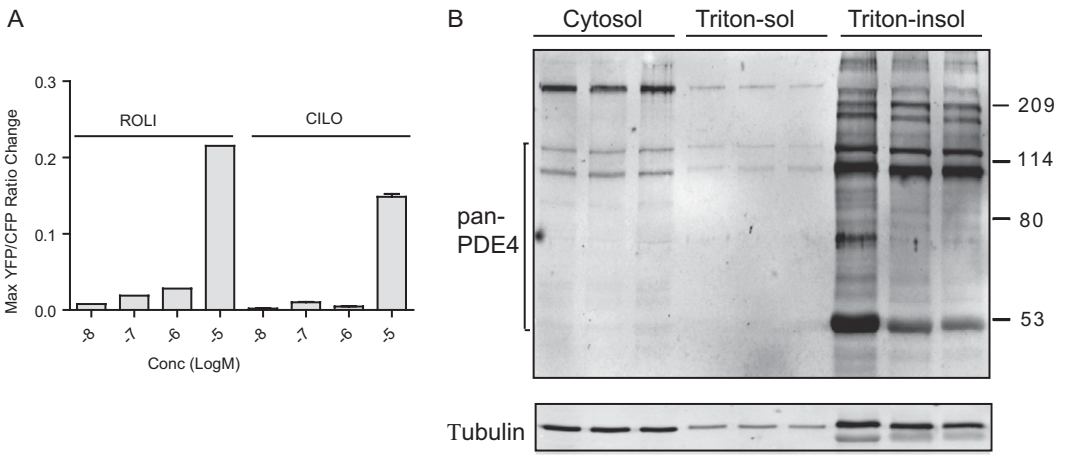

Fig. S2. Characterization of PDE4 distribution and activities in cardiomyocytes. (*A*) Neonatal cardiomyocytes expressing SR-AKAR3 were treated with either PDE4 inhibitor ROL or PDE3 inhibitor CILO at different concentration; the changes in SR-AKAR3 FRET ratio were recoded and the maximal changes were plotted. (*B*) Neonatal cardiomyocytes were subjected to solubilization with 1% Triton-X 100 at 4 °C for 2 h, the soluble fractionation and insoluble fraction were collected, and the distribution of PDE4 was probed with antibody against PDE4.

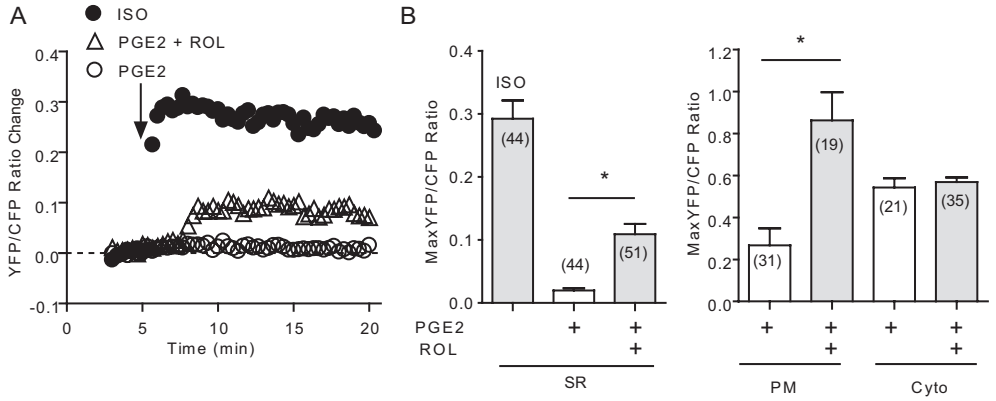

Fig. S3. Inhibition of PDE4 partially rescues the cAMP signaling at the SR in neonatal cardiomyocytes under PGE stimulation. Neonatal cardiac myocytes expressing the SR-targeted (*A*) and the PM-targeted or general cytoplasmic (*B*) PKA biosensors were stimulated with PGE2 together with PDE4 inhibitor ROL. The changes in the PKA FRET ratio were recorded.

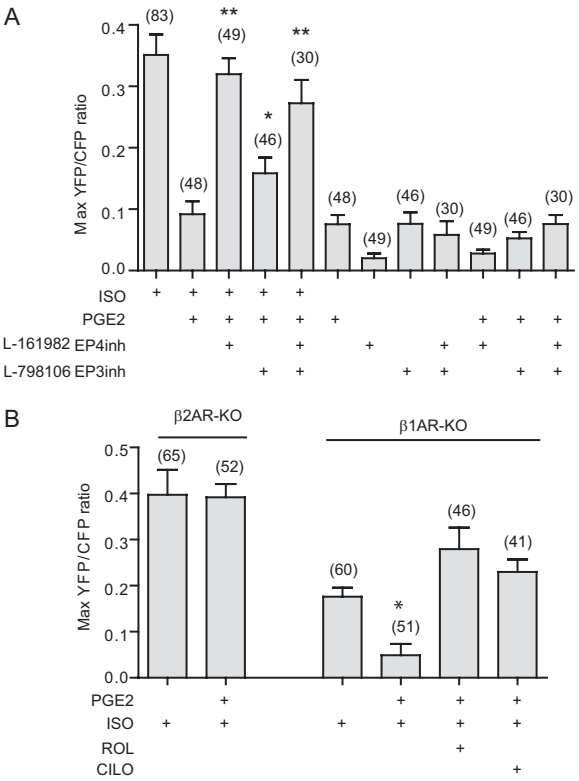

Fig. S4. The inhibitory effect of PGE2 on adrenergic signaling is mediated via EP4 and β_2AR in cardiomyocytes. (*A*) Wild-type neonatal cardiomyocytes expressing the SR-targeted PKA biosensors were stimulated with PGE2 (1 μM) or ISO (10 μM) in the presence or absence of EP3-selective antagonist L-798106 (20 nM) and EP4-selective antagonist L-161982 (200 nM) as indicated. **P* < 0.05 and ***P* < 0.01 by one-way ANOVA in comparison with PGE2-ISO group. (*B*) β_2AR-KO and β_1AR-KO cardiomyocytes expressing the SR-targeted PKA biosensors were stimulated with PGE2 (1 μM), ISO (10 μM), and ROL (10 μM) as indicated. The changes in the PKA FRET ratio were recorded, and the maximal responses were plotted. **P* < 0.05 by one-way ANOVA in comparison with ISO group.

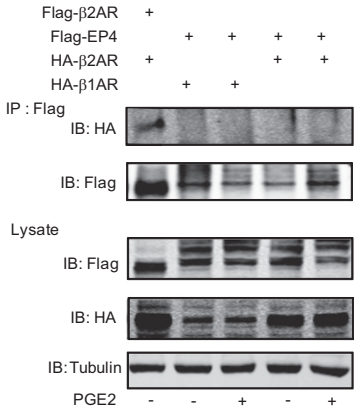

Fig. S5. EP4 does not associate with βAR in HEK293 cells. Flag-EP4 or Flag-β_2AR was coexpressed with HA-β_2AR or HA-β_1AR in HEK293 cells. Cells were stimulated with PGE2 (1 μM; 10 min) before being harvested for immunoprecipitation with anti-Flag M2 beads. The pull-down proteins were detected with antibodies indicated.

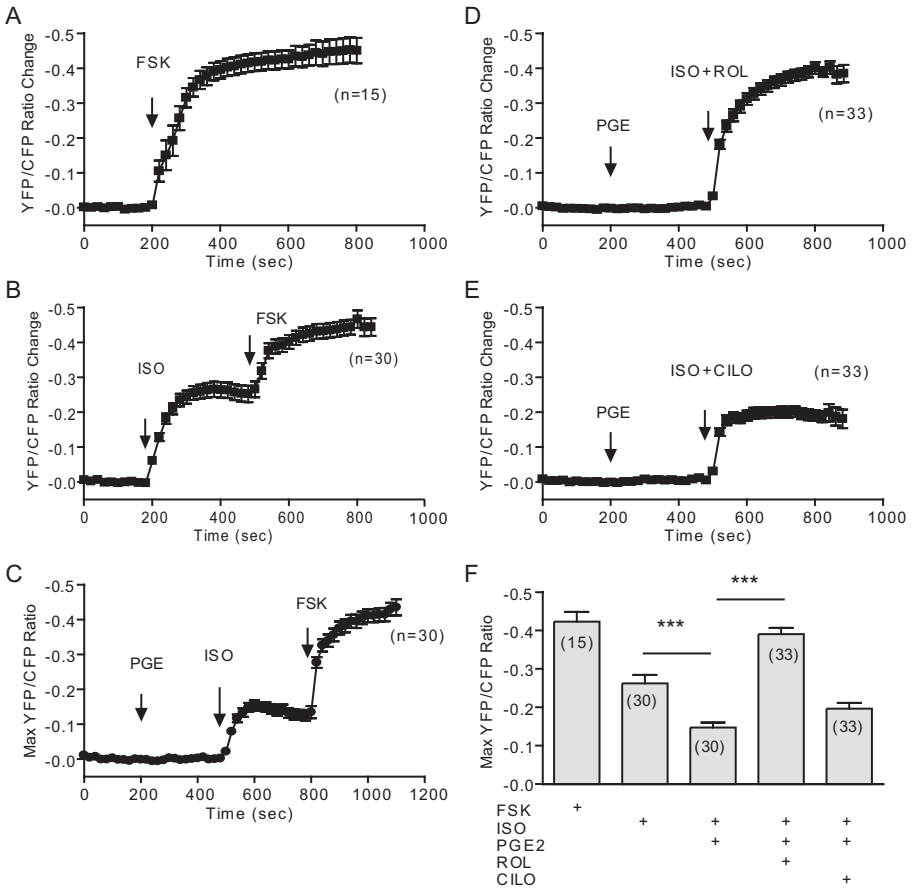

Fig. S6. PGE2 inhibits cAMP signal in the nucleus induced by adrenergic stimulation in MEFs. MEFs expressing the nuclear-targeted cAMP biosensor were stimulated with PGE2 (1 μM), ISO (10 μM), FSK (10 μM), ROL (1 μM), and/or CILO (10 μM) as indicated. The changes in FRET ratio of the PKA biosensors were recorded (*A*–*E*). The maximal increases in the cAMP FRET ratio were plotted (*F*). ***$P < 0.001$ by one-way ANOVA with post hoc test for multigroup comparison.

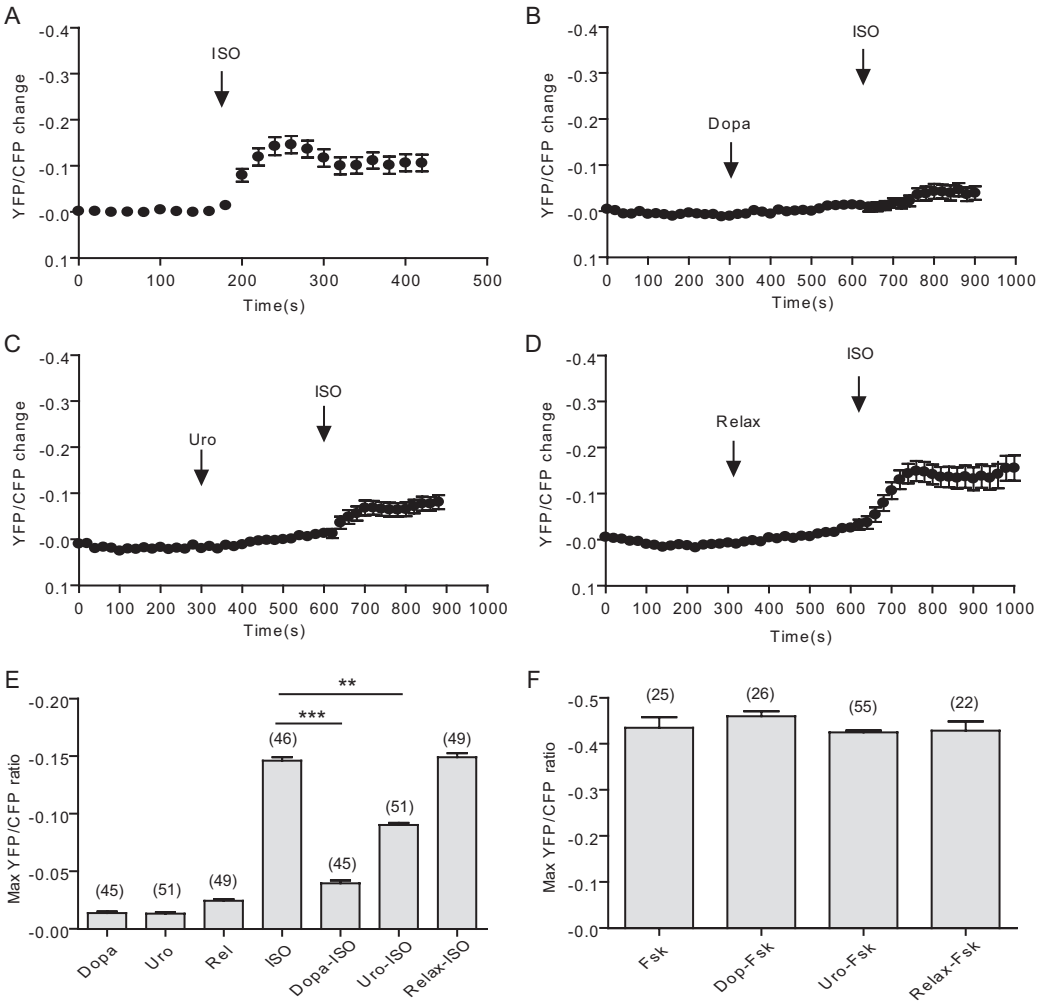

Fig. S7. Activation of Gs-coupled GPCRs affects adrenergic signaling in MEF cells. (*A–D*) Wild-type MEFs expressing the nuclear-targeted PKA biosensors were stimulated with dopamine (100 μM), urocortin (100 nM), relaxin (20 ng/mL), ISO (10 μM), FSK (10 μM), and ROL (1 μM) as indicated. ISO and FSK were added 5 min after pretreatment with other GPCR agonists. (*E–F*) The maximal responses after stimulation were plotted. $^{**}P < 0.05$ and $^{***}P < 0.01$ by one-way ANOVA with post hoc test for multigroup comparison.

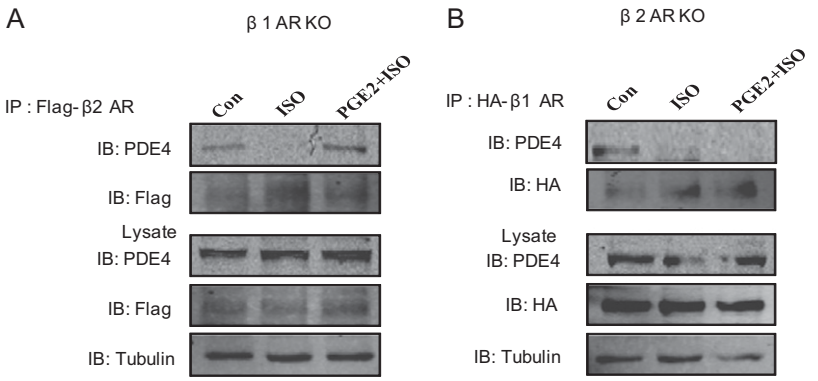

Fig. S8. PGE2 inhibits the dissociation of PDE4 isoform from βARs induced by ISO. Flag-β_2AR (*A*) or HA-β_1AR (*B*) was coexpressed with PDE4D8 in β_1AR-KO or β_2AR-KO myocytes as indicated. Cells were stimulated with ISO (10 μM; 10 min) in the presence or absence of pretreatment with PGE2 (1 μM; 5 min). Cells were lysed for immunoprecipitation with anti-HA or anti-Flag affinity beads. The pull-down proteins were detected with antibodies indicated.

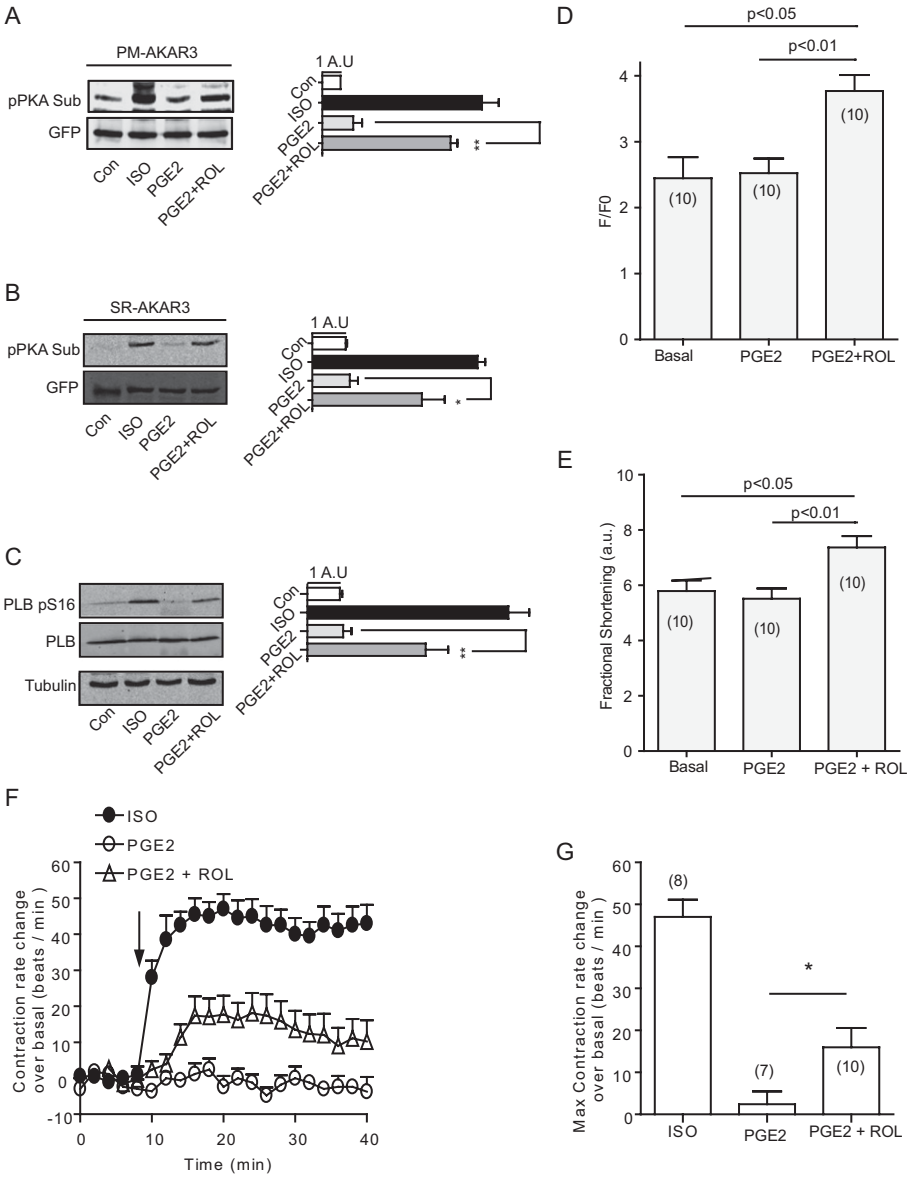

Fig. S9. PGE2 and ISO induce distinct PKA phosphorylation of substrates at subcellular organelles and myocyte contractile responses. (*A*–*C*) Neonatal cardiomyocytes expressing the SR- or PM-targeted PKA biosensors were stimulated with PGE2 (1 μM), ISO (10 μM), or PGE2 and ROL (1 μM). The phosphorylation of SR-AKAR3 and PM-AKAR3 was probed with either anti-phospho-PKA substrate, and the phosphorylation of endogenous PLB was probed with anti-phospho serine 16 antibodies, respectively. Adult cardiomyocytes were loaded with calcium dye Fluo4-AM and stimulated with PGE2 (1 μM) in the absence and presence of PDE4 inhibitor ROL (1 μM). The changes in myocyte calcium transients (*D*) and fractional shortening (*E*) were rescored. (*F*) Neonatal cardiomyocytes were stimulated with PGE2 (1 μM) with or without ROL (1 μM), and the increases in the spontaneous contraction rate were recorded. (*G*) The maximal increases were plotted. $*P < 0.05$ and $**P < 0.01$ by one-way ANOVA with post hoc test for multigroup comparison.