Supplement Material

Materials and Methods

Isolation of Neonatal and Adult Mouse Cardiac Myocytes. Neonatal cardiac myocytes were isolated from new-born pups of different genotypes as previously described [1]. Adult mouse ventricular myocytes were isolated from 2 month old male hearts of different genotypes as previously described [2]. Myocytes were cultured in 35-mm dishes for protein phosphorylation and contraction rate assays or in coverslip chambers pre-coated with laminin for live imaging.

Neonatal Myocyte Contraction Rate Assay and Adult Myocyte Shortening Assay. Measurement of spontaneous contraction rate was carried out as described previously [1]. Measurement of adult myocyte shortening was carried out as described previously [3]. Responses in neonatal myocyte beating rate and adult myocyte shortening after drug treatments were analyzed by Metamorph software. Statistical analyses were performed using Prism (GraphPad Software, CA).

Cardiac Myocyte Culture and Adenovirus Infection. For neonatal cardiac myocytes, after isolation and plating by 24 hrs, cells were washed and infection with adenoviruses (moi 100) for expression of the PKA activity sensor (AKAR2.2 [4]) or the cAMP sensor (ICUE3 [5]), or expression of flag-tagged mouse β_2AR. Freshly isolated adult myocytes were infected with adenovirus (100 moi) as previously described for expression of AKAR2.2 [2].

Fluorescent Resonance Energy Transfer (FRET) Measurement. Myocytes were infected with adenoviruses to express AKAR2.2 or ICUE3 for 24 hours. Cells were rinsed and maintained in PBS with calcium for FRET recording. Cells were imaged on a Zeiss Axiovert 200M microscope with a 40×/1.3NA oil-immersion objective lens and a cooled CCD camera. Dual emission ratio imaging was acquired with a 420DF20 excitation filter, a 450DRLP diachronic mirror, and two emission filters (475DF40 for cyan and 535DF25 for yellow). The acquisition was set with 200 millisecond exposure in both channels and 20 second elapses. Images in both channels were subjected to background subtraction, and ratios of yellow-to-cyan color were calculated at different time points. After the PKA phosphorylation on the consensus site in AKAR2.2, the ratio YFP/CFP displayed increases. However, the binding cAMP to ICUE3 led to decreases in the ratio YFP/CFP [5], which were plotted with inverted y-axis (Online Figure III).

Drug treatments. Myocytes were stimulated with isoproterenol (ISO, 10μM, Sigma), forskolin (FSK, 10μM, Sigma) an adenylyl cyclase agonist, or 8-Bromo adenosine 3′, 5′-cyclic monophosphate (8-Br-cAMP, 10μM, Sigma) a cell-permeable cAMP analog, at indicated times. Cells were treated with the following inhibitors: adrenergic antagonist alprenolol (ALP, 100μM, Sigma), selective inhibitor of PKA N-[2-(p-Bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride (H-89, 10μM, Sigma), PDE4 inhibitor rolipram (ROL, 10μM, Calbiochem), PDE3 inhibitor cilostamide (CILO, 10μM, Calbiochem), and general PDE inhibitor 1-methyl-3-(2-methylpropyl)-7H-purine-2,6-dione (IBMX, 10μM, Sigma) at indicated times, or 10-15 minutes before protein phosphorylation assay. Pertussis toxin (PTX, 200-500ng/ml, Sigma, MO) a G

proteins (Gi/Go) inhibitor was preincubated for at least 2 hours as described previously [6].

Western Blot. Myocytes were stimulated with isoproterenol in the presence or absence of pretreatment of PDE4 and Gi inhibitors. In some experiments, forskolin was used to induce maximal increases in PKA-dependent phosphorylation. The samples were separated by SDS/PAGE for western blot with procedure previously described [7]. The PKA-mediated phosphorylation of flag-tagged mouse β_2AR, phospholamban, and AKAR2.2 were detected with phospho-Ser 345/6 of β_2AR (Santa Cruz Biotechnology, CA), phospho-Ser16 of phospholamban (p-PLB, Bradilla, UK), and PKA phospho-substrate specific antibody (Cell Signaling, MA), respectively. In addition, AKAR2.2 was probed with anti-GFP antibody (Invitrogen, CA); mouse β_2AR was probed with anti-flag M1 (Sigma, MO) or anti-mouse β_2AR antibody (Santa Cruz Biotechnology, CA); phospholamban was probed with anti-phospholamban antibody (anti-PLB, Bradilla, UK); internal control γ-tubulin was probed with anti-γ-tubulin antibody (Sigma, MO). Primary antibodies were visualized with IRDye 680CW goat-anti mouse or with IRDye 800CW goat-anti rabbit secondary antibodies using an Odyssey scanner (Li-cor biosciences, NE).

Statistical Analysis. One or Two-way ANOVA and student *t*-test were performed using Prism (GraphPad Software, CA) accordingly.

Reference

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Online Figure Legends:

Online Figure I. Expression and characterization of AKAR2.2 in wild-type myocytes. (A) Efficiency and (B) expression levels of AKAR2.2 in myocytes using plasmid transfection and virus infection. FRET induced by isoproterenol (ISO, 10μM) in myocytes expressing AKAR2.2 are shown by fluorescence emission (C) in single channels or (D) in emission ratio. (E) Pseudocolor imaging of FRET ratio in a single cardiac myocyte before and after ISO stimulation at indicate times. (F) Addition of PKA inhibitor H-89 rapidly decreases the increases in PKA FRET ratio induced by forskolin (FSK). Representative curves of multiple independent experiments are displayed in figures C, D and F, respectively.

Online Figure II. Inhibition of PDEs affects PKA activities in cardiac myocytes upon βAR activation. Cardiac myocytes expressing AKAR2.2 are treated with or without PDE inhibitors followed by isoproterenol stimulation at indicated times. The initial and sustained increases in FRET ratio induced by 10 μM of isoproterenol are plotted in (A) wild-type, (C) β_1AR-KO, or (E) β_2AR-KO cardiac myocytes, respectively. The initial and sustained increases in FRET ratio induced by 1nM of isoproterenol are plotted in (B) wild-type, (D) β_1AR-KO, or (F) β_2AR-KO cardiac myocytes, respectively. * $p < 0.05$, ** $p<0.01$, *** $p<0.001$ when compared to control groups by one-way ANOVA. ns, not significant.

Online Figure III. Activation of βAR subtypes induces cAMP accumulation in cardiac myocytes. cAMP indicator ICUE3 is expressed in cardiac myocytes to monitor cAMP accumulation induced by activation of different βAR subtypes. Stimulation with 10 μM of isoproterenol induces significantly increases in ICUE3 FRET ratio in (A) β_1AR-KO,

(B) β_2AR-KO, and (C) wild-type cardiac myocytes. Pretreatment with PDE4 inhibitor rolipram (10 μM) significantly increased the levels of FRET ratio at rest state and enhanced the increases in FRET ratio after isoproterenol stimulation in all cell types. * $p < 0.05$, ** $p<0.01$, *** $p<0.001$ when compared to control groups by student *t*-test.

Online Figure IV. PKA activities induced by activation of overexpressed mouse β_2AR in β_1AR-KO myocytes. Mouse β_2ARs are overexpressed with different moi in β_1AR-KO myocytes together with AKAR2.2. (A) Activation of β_2ARs with 1nM of isoproterenol induces receptor-expression level-dependent increases in AKAR2.2 FRET ratio in β_1AR-KO myocytes. However, the increases are not sustained during extended stimulation. (B) Activation of β_2ARs with 10μM of isoproterenol induces similar increases in AKAR2.2 in β_1AR-KO myocytes with different levels of overexpressed β_2ARs. The increases are not sustained during extended stimulation. (C) Western blot shows different levels of β_2ARs (up to 3.4 folds of the levels of endogenous receptor) in β_1AR-KO myocytes after overexpression.

Online Figure V. Inhibition of PDEs affects PKA activities in cardiac myocytes at resting state. Myocytes expressing AKAR2.2 are treated with PDE inhibitors. (A) Neither rolipram (10 μM) nor cilostamide (10 μM) induces increases in baseline levels of PKA FRET ratio in β_1AR-KO myocytes. (B) Rolipram (10 μM), but not cilostamide (10μM) induces small but significant increases in baseline levels of PKA FRET ratio in β_2AR-KO myocytes. *** $p<0.001$ when comparing two groups by two-way ANOVA.

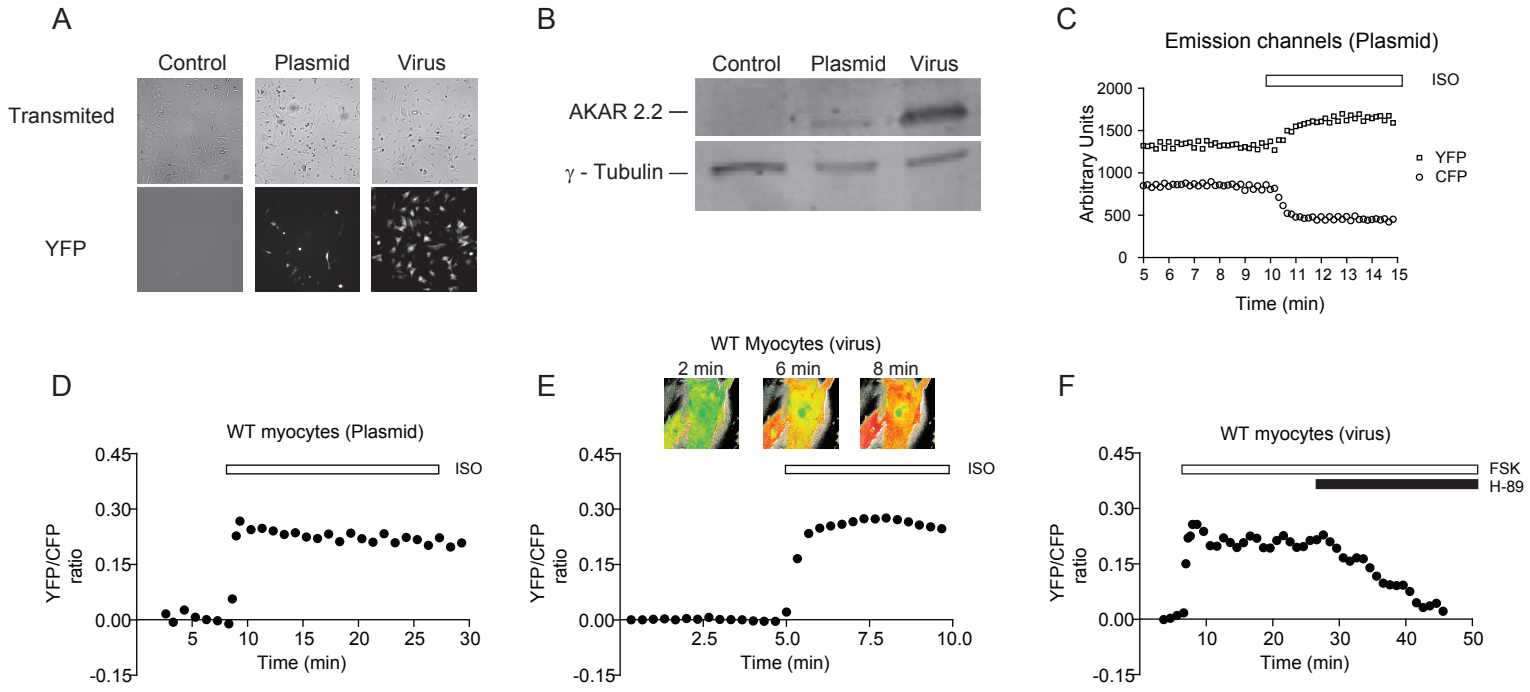
Online Figure VI. β_2AR-induces PKA phosphorylation in different substrates in β_1AR-KO cardiac myocytes. (A) Flag-mouse β_2ARs (flag-mβ_2ARs) are overexpressed in β_1AR-

KO cardiac myocyte with adenovirus infection. Upon stimulation with 10 μM of isoproterenol, phosphorylation in serine 345 and 346 of flag-mβ_2AR is significantly increased with peak levels at 3 minutes. (B) In β_1AR-KO cardiac myocytes, stimulation with 10 μM of isoproterenol does not increase PKA phosphorylation in serine 16 of phospholamban. However, pretreatment with rolipram significantly promotes PKA phosphorylation in serine 16 of phospholamban after isoproterenol stimulation. (C) AKAR2.2 is overexpressed in β_1AR-KO cardiac myocytes with adenovirus infection. Phosphorylation in the PKA site of AKAR2.2 is significantly increased upon stimulation with 10 μM of isoproterenol. # Time-course curves are significantly different between the rolipram and the control groups by two-way ANOVA, * $p < 0.05$, when compared to control groups by student *t*-test.

Online Figure VII. Overexpression of AKAR2.2 does not affect myocyte contraction responses induced by β_2AR in β_1AR-KO cardiac myocytes. (A) Overexpression of AKAR2.2 does not affect myocyte contraction rates at rest state in β_1AR-KO cardiac myocytes, nor does it affect maximal contraction rate increases induced by activation of β_2AR with 10 μM of isoproterenol. (B) Western blot shows the expression of AKAR2.2 in β_1AR-KO cardiac myocytes. ns, not significant.

Online Figure VIII. βAR subtype induces increases in adult myocyte shortening. Adult myocytes were stimulated with 1nM or 10μM of isoproterenol, and the maximal increases in myocyte shortening are plotted. *** $p<0.001$ when compared to the baseline levels by student *t*-test.

Online Figure I



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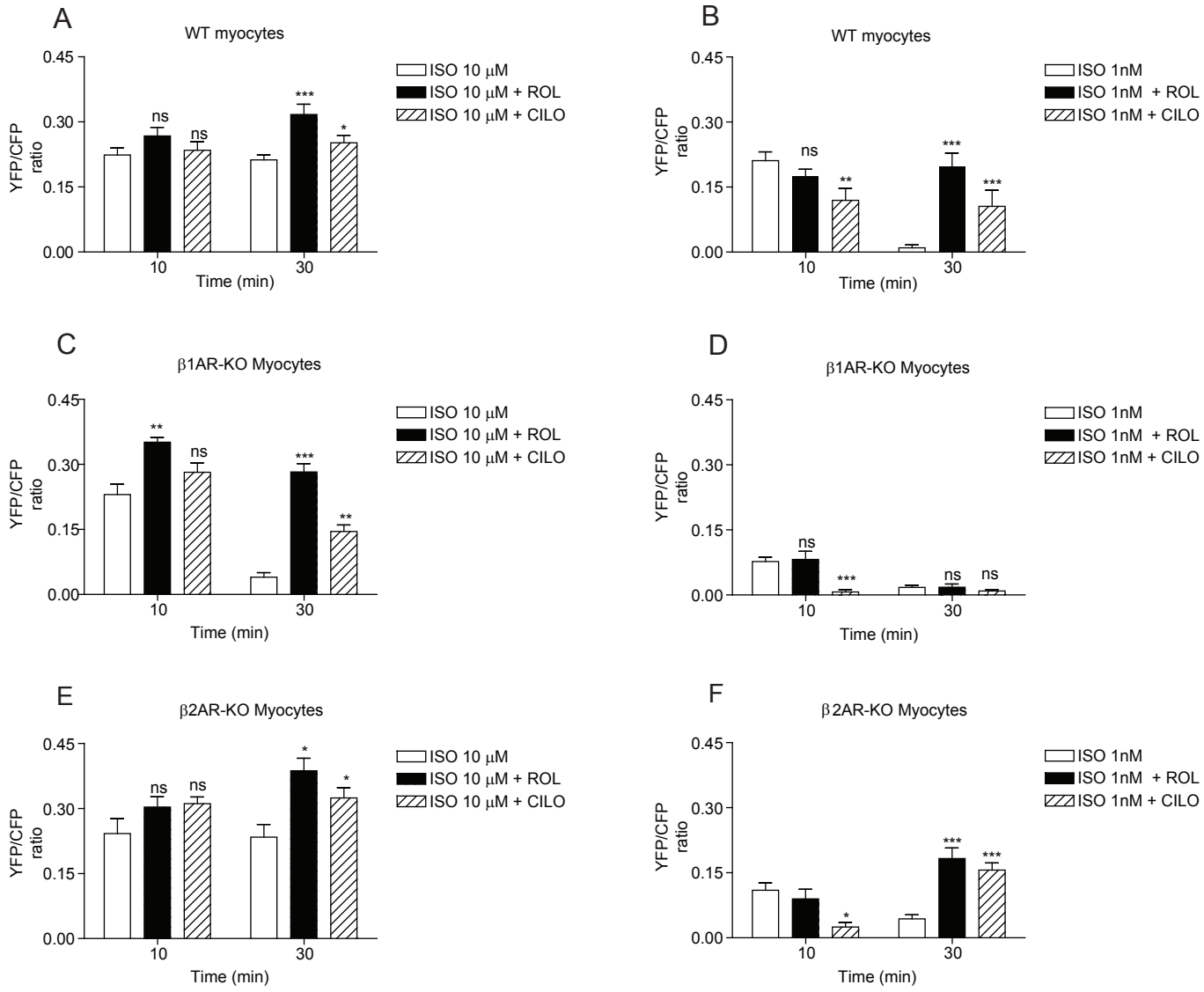

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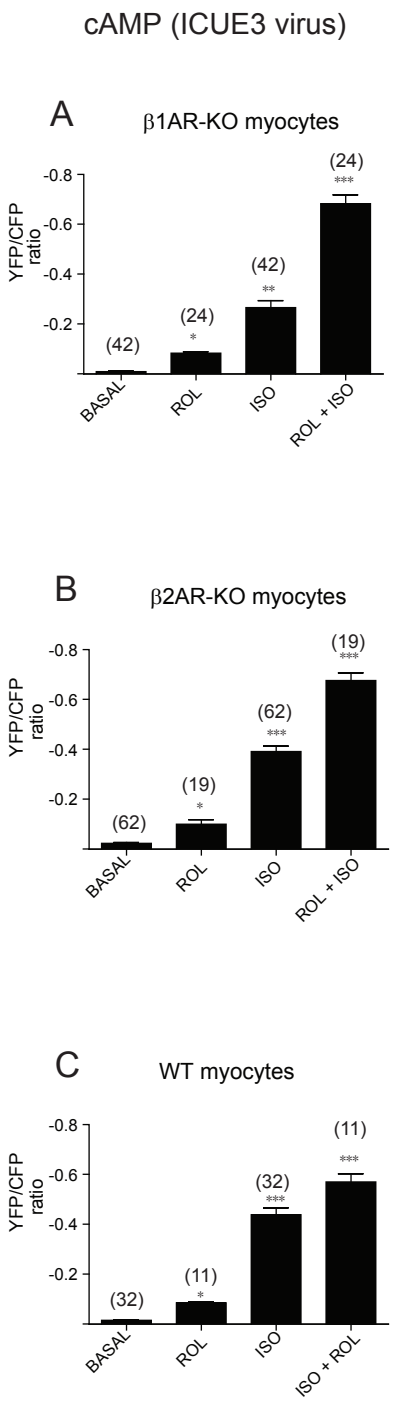

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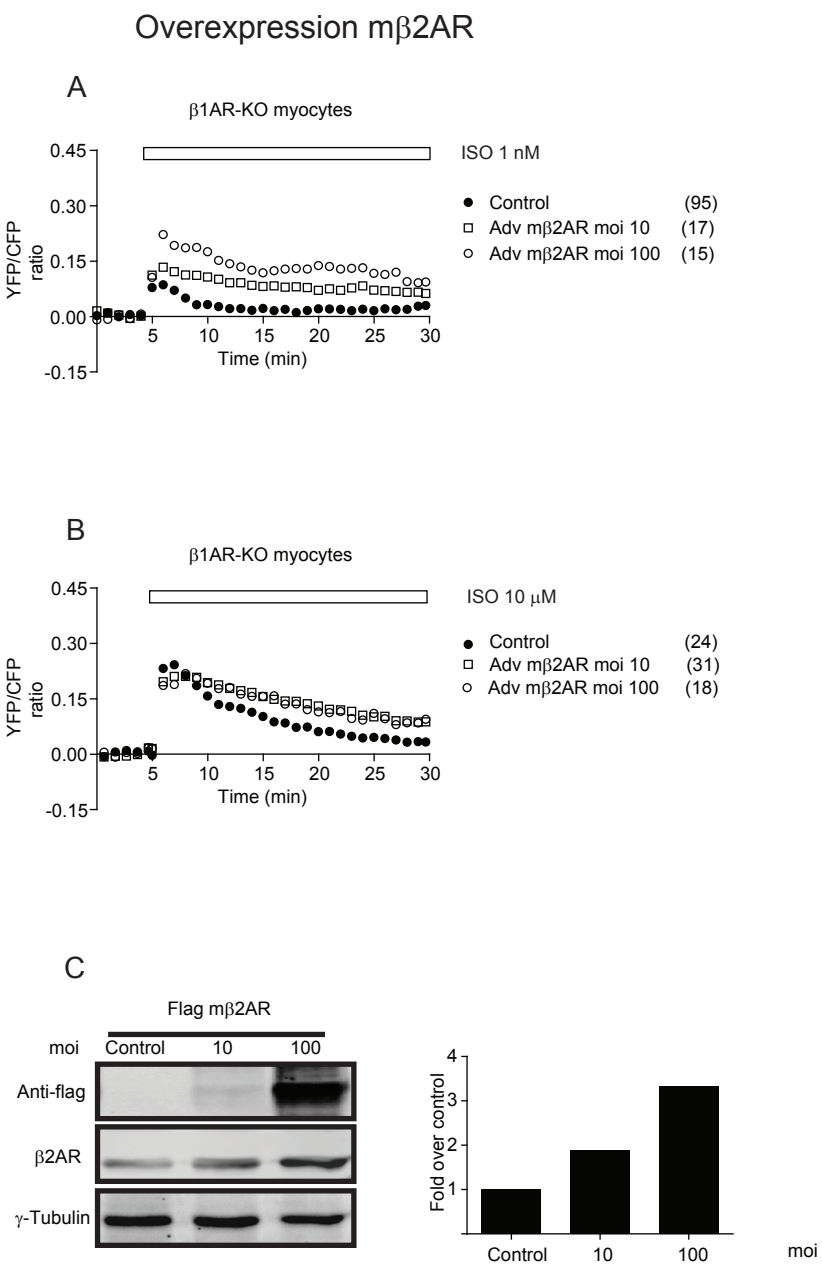

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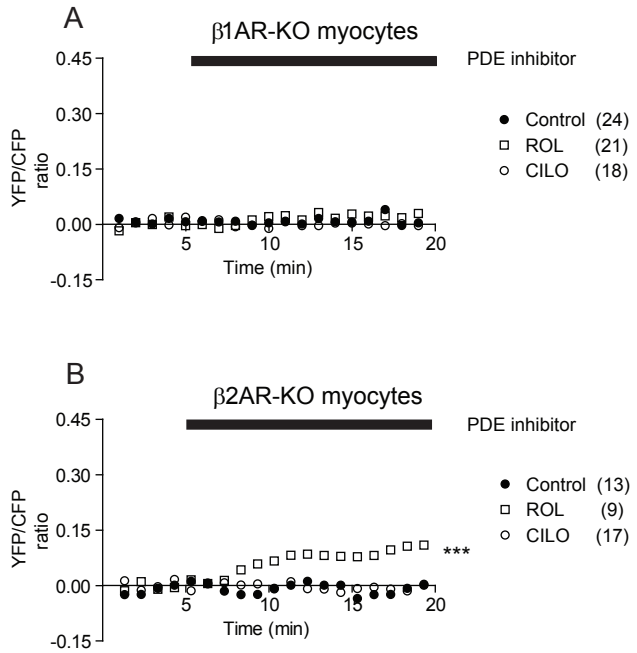

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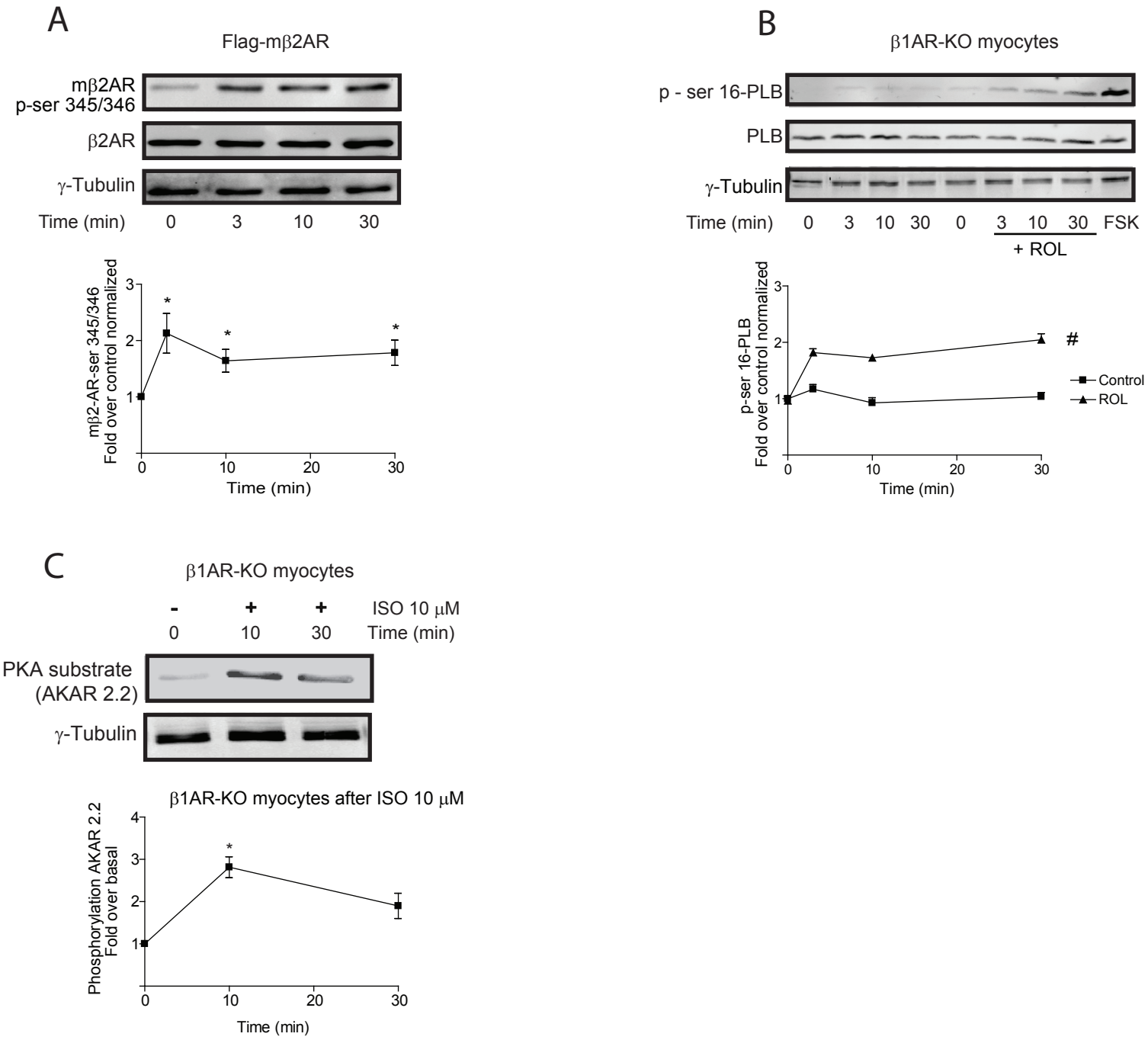

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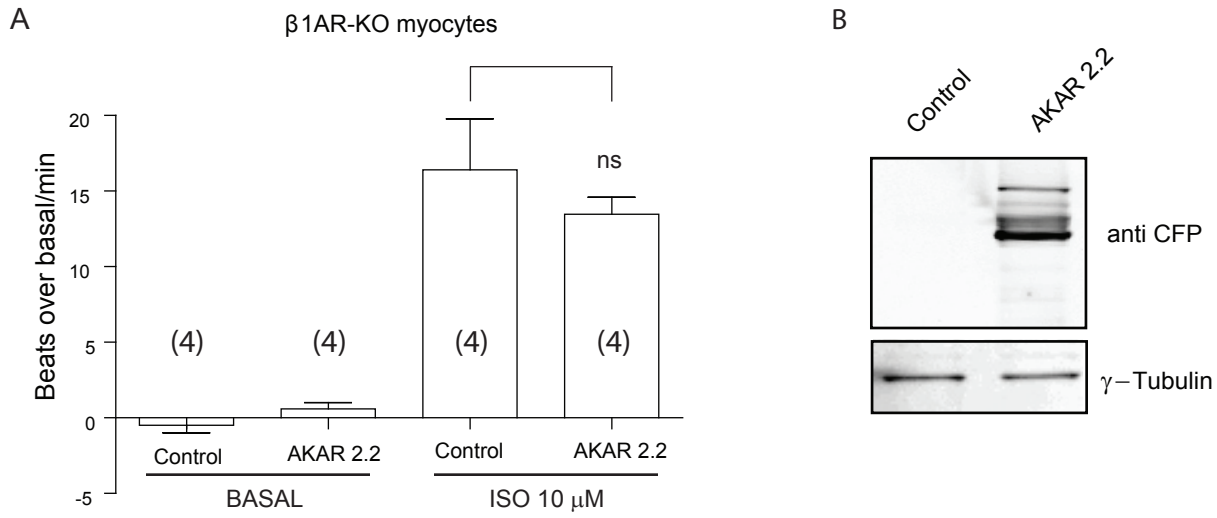

Online Figure VIII