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TITLE PAGE

Equilibrium between adenylyl cyclase and phosphodiesterase patterns adrenergic agonist dose-dependent spatiotemporal cAMP/PKA activities in cardiomyocytes

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Running title: adenylyl cyclase and phosphodiesterase pattern cAMP signal

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Non-standard abbreviations: FRET, fluorescence resonance energy transfer; PKA, protein kinase A; cAMP, cyclic AMP; βAR, β adrenergic receptor; AC, adenylyl cyclase; PDE, phosphodiesterase; RyR, ryanodine receptor; PLB, phospholamban; TnI, troponin I; and TnT, troponin T.

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ABSTRACT

β adrenergic receptor (βAR) induces cAMP/Protein Kinase A (PKA) activation to regulate cardiac contraction. Using real-time, fluorescence resonance energy transfer (FRET) imaging for highly sensitive detection of cAMP and PKA activities, we show two distinct phases in isoproterenol dose-dependent responses in cardiomyocytes: a transient and dose-dependent increase in cAMP and PKA activities at lower concentrations from 10^{-12} M to 10^{-8} M; and a saturated initial increases at higher concentrations from 10^{-8} M to 10^{-5} M followed by a rapid decrease to different levels that were later sustained in a dose-dependent manner. The dose-dependent temporal responses are patterned by equilibrium between receptor-activated adenylyl cyclase (AC) and phosphodiesterase (PDE). At lower concentrations, cAMP is produced in an agonist dose-dependent manner with AC as a rate-limiting factor. However, the cAMP activities are confined within local domains for phosphorylation of PDE isoforms in the receptor complex, but not for phosphorylation of phospholamban and troponin I. At higher concentrations, isoproterenol promotes a dose-dependent selective dissociation of PDE4D, but not ACVI from the receptor complex, which shifts the equilibrium between AC and PDE. This shifted balance leads to sustained cAMP accumulation and diffusion for PKA phosphoylation of phospholamban and troponin I, and for myocyte contraction. Pharmacological inhibition or overexpression of either ACVI or PDE4D8 disrupts the balance, and shapes the temporal responses in cAMP accumulation. Together, our data reveals a new paradigm for adrenergic agonist dose-dependent cAMP/PKA activities for substrate-specific phosphorylation dictated by dual regulation of AC and PDE in cardiomyocytes.

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INTRODUCTION

Activation of adrenergic receptors (ARs) represents the primary mechanism to increase cardiac performance under stress. Activated ARs couple to Gs proteins, which leads to AC-dependent increases in secondary messenger cAMP to activate PKA (Lefkowitz, 2007). The increased PKA activities promote phosphorylation of diversified substrates ranging from the receptor and its associated partners, to ryanodine receptor (RyR), phospholamban (PLB), and contractile myofibril proteins such as troponin I (TnI) and troponin T (TnT), which eventually leads to increases in contractility and heart rate (Xiang and Kobilka, 2003; Xiao et al., 2006). While cAMP/PKA activation plays an essential role in controlling physiological responses, accumulating evidence indicates that changes in cAMP/PKA activities exert distinct cellular effects via substrate specificity in highly differentiated cardiomyocytes. For example, β_2AR displays a high sensitivity to PKA phosphorylation under stimulation with subnanomalor concentrations of isoproterenol in human embryonic kidney 293 (HEK 293) cells and mouse neonatal cardiomyocytes (Liu et al., 2009; Tran et al., 2004). In contrast, a minimal 1nM of isoproterenol is required to promote increases in myocyte contraction rate and contractility (De Arcangelis et al., 2008).

The concept of spatiotemporal regulation of cellular cAMP and PKA activities provides new insights into understanding how cAMP/PKA signaling is translated into physiological contraction response in highly organized muscle cells (Cooper, 2005; Zaccolo, 2006). PKA is anchored on distinct subcellular structures through a family of proteins named A-kinase anchoring proteins. In contrast, correlating to the distribution of most ACs, cellular cAMP is primarily confined along the plasma membrane under neurohormonal stimulation (Cooper, 2005). Despite being a diffusible small molecule, the distribution and diffusion of cAMP is rather limited due to cAMP degradation mediated by phosphodiesterases (PDEs) (Houslay et al., 2007; Mongillo and Zaccolo, 2006; Zaccolo, 2006). Under a specific hormonal stimulation, individual PKAs anchored at different subcellular compartments will be selectively activated to conduct phosphorylation of local proteins for specific cellular processes (Jarnaess and Tasken, 2007; McConnachie et al., 2006). A spatial distribution of cAMP/PKA signaling regulated by

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ACs and PDEs is therefore essential for selective phosphorylation of substrates for myocyte contraction.

Consistent with this notion, PDE 4D (PDE4D) has been shown to be significant in regulating the adrenergic receptor subtype-induced myocyte contraction rate response (Xiang et al., 2005). Recent evidence indicates that PDE4D splicing isoforms selectively bind β adrenergic receptors (De Arcangelis et al., 2008; Richter et al., 2008). Specifically, PDE4D8 binds to β_1AR in HEK293 cells, and dissociates from the receptor upon stimulation with incremental doses of agonist (Richter et al., 2008). In contrast, PDE4D9 and to a lesser extent PDE4D8 bind to β_2AR in cardiomyocytes (De Arcangelis et al., 2009). These receptor-associated PDE4Ds play critical roles in controlling cAMP/PKA activities in the vicinity of the receptors for differential cellular responses under stimulation (De Arcangelis et al., 2008; Richter et al., 2008; Xiang et al., 2005; Zaccolo and Pozzan, 2002). Inhibition of PDE4 significantly enhances propagation of cAMP/PKA activities for increasing PKA phosphorylation of PLB and myocyte contraction response under low doses of isoproterenol stimulation (De Arcangelis et al., 2008). This results in saturated responses, becoming equivalent to those induced by saturating doses of isoproterenol (De Arcangelis et al., 2008).

We hypothesized that cardiomyocyte cAMP/PKA signaling is differentially regulated through a balance between AC-dependent cAMP production and PDE-dependent cAMP degradation in an agonist dose-dependent manner. By employing high sensitive FRET-based biosensors for cAMP and PKA activities in living-cell imaging, we found that cAMP/PKA activities displayed two distinct phases in isoproterenol dose-dependent fashion: a transient and dose-dependent increase in FRET ratio at concentration from 10^{-12} M to 10^{-8} M, and a saturated initial increase in FRET ratio from 10^{-8} M to 10^{-5} M, which was followed by a rapid decrease to different levels that were later sustained in a dose-dependent manner. The transient and sustained cAMP/PKA signals are patterned by a shifting balance between AC-dependent cAMP production and PDE-dependent cAMP degradation at increasing concentration of isoproterenol, which also dictates substrate specificity for PKA phosphorylation and myocyte contraction responses.

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MATERIALS AND METHODS

Neonatal and adult cardiac myocyte contraction assays Neonatal and adult myocytes were isolated from newborn or 2-4 month old wild type FVB mice respectively. Spontaneously beating neonatal cardiac myocytes were prepared from newly born wild type or mice lacking β_1AR or β_2AR, or both genes as previously described (Devic et al., 2001). Measurement of spontaneous contraction rate was carried out as previously described (Devic et al., 2001). Adult myocytes were placed in a dish with HEPES buffer and electrically stimulated at 30 V/cm at 1 Hz at room temperature. Cell length was recorded with a charge-coupled device camera. Cell contraction shortening was analyzed by Metamorph software (Molecular Devices, Sunnyvale, CA) and normalized as the increase over the basal levels after being fitted to a sigmoidal curve. The maximal shortening was normalized to the baseline value or plotted as a percentage of the maximal response stimulated by 10 μM forskolin.

Drug treatment Neonatal myocytes were treated with rolipram (Rol, 10^{-5} M; Calbiochem), a PDE4 inhibitor for 10 min (Alvarez et al., 1995) or with 2',5' dideoxyadenosisne triphosphate (2',5'-DDA, 10^{-4} M, Sigma), a selective adenylyl cyclase inhibitor for 40 min before stimulation. Cells were stimulated with isoproterenol (Iso, 10^{-12} M to 10^{-5} M, Sigma) or an AC agonist forskolin (Forsk, 10^{-5} M, Sigma) (Wang et al., 2008).

Immunoprecipitation and Western blotting Neonatal cardiac myocytes from wild type or $\beta_1\beta_2$AR gene knockout (KO) pups were infected with recombinant adenovirus expressing HA-tagged mouse β_1AR, PDE4D8-mCherry, and/or ACVI as indicated. After Iso stimulation for 10 min at different concentrations, cells were rinsed in ice-cold PBS and lysed in co-immunoprecipitation buffer as described previously (De Arcangelis et al., 2009). Briefly, HA-β_1AR infected lysates were immunoprecipitated using anti-HA affinity matrix (Roche, IN). The total immunoprecipitated proteins and 5% of lysates were resolved by 4-20% Tris-HCl precast gel (Biorad, CA) and plotted with following antibodies: anti-HA (HA.11, BAbCO, CA), anti-ACV/VI (SCBT, CA), anti-pan PDE4 antibody (Abcam, MA), anti-RFP-mCherry (Rockland, PA).

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Wild type neonatal cardiac myocytes were stimulated with Iso for 15min at different concentration (10^{-5} M or 10^{-9} M) as indicated. The lysates were separated by SDS/PAGE for Western blotting with antibodies against PLB, phosphoSer16-PLB (p-PLB), TnI, and phospho-TnI (Badrilla, UK), phospho-(Ser/Thr)-PKA substrate (Cell Signaling, MA) and anti γ-Tubulin (Sigma, MO). The primary antibodies were revealed with IRDye 680CW goat-anti mouse or IRDye 800CW goat-anti rabbit secondary antibodies using an Odyssey scanner (Li-cor biosciences).

Fluorescence resonance energy transfer (FRET) recording Neonatal cardiac myocytes from wild type or $\beta_1\beta_2$AR-KO pups were infected with viruses to express either A-kinase activity reporter AKAR3 (Allen and Zhang, 2006) or cAMP probe ICUE3 (Allen et al., 2006) as previously described (Soto et al., 2009). Living myocytes were imaged on a Zeiss Axiovert 200M microscope with a ×40/1.3 NA oil-immersion objective lens and a CCD camera, controlled by a Metafluor software (Molecular Devices, CA). Dual-emission recording of both Cyan direct (440/480 nm, ex./em.) and FRET (440/535 nm, ex./em.) was coordinated by Lambda 10-3 filter shutter controller (Sutter Instruments, CA). Exposure time was 100 ms, and recording interval was 20 s. 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 AKAR3, the ratio YFP/CFP displayed increases. However, the binding cAMP to ICUE3 led to decreases in the ratio YFP/CFP (Allen et al., 2006), which were plotted with inverted y-axis.

Statistical Analysis Curve-fitting and statistical analyses were performed using Prism (GraphPad Software, Inc, CA).

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RESULTS

FRET-based living cell imaging assays reveal distinct feature in initial and sustained responses in cAMP/PKA activities upon adrenergic stimulation in cardiac myocytes

To investigate mechanisms that control the spatiotemporal regulation of cAMP/PKA signaling for substrate phosphorylation and physiological myocyte contraction response, we explored real-time FRET-based living cell-imaging analysis of cAMP and PKA activities. Minimal increases in cAMP and PKA activities were detected at 10^{-10} M and 10^{-11} M of isoproterenol, respectively (Figure 1A and 1C), in contrast to a minimal 10^{-9} M of isoproterenol required for a detectable increase in myocyte contraction rate (De Arcangelis et al., 2008). From 10^{-12} M to 10^{-8} M, both cAMP and PKA activities displayed a transient and dose-dependent increase in FRET ratio (Figure 1A and 1C). From 10^{-8} M to 10^{-5} M, the initial peak increases in cAMP and PKA FRET ratios were equivalent but followed by a rapid decrease to different levels that were later sustained (Figure 1A and 1C). At the saturating dose of 10^{-5} M, the increase was sustained after reaching peak levels (Figure 1A and 1C). The EC_{50} of initial peak increases in cAMP and PKA FRET ratio were 6.86 x 10^{-10} M and 4.53 x 10^{-10} M, respectively (Figure 1B and 1D). In contrast, the sustained increases in cAMP/PKA FRET ratio have much higher EC50s (7.99 x 10^{-8} M for cAMP and 6.77 x 10^{-8} M for PKA, Figure 1B and 1D). The EC_{50} concentrations for sustained increases are in correlation with the constants of ligand binding (K_d) to βARs or the EC_{50} concentrations of the isoproterernol-induced increases in myocyte contraction rate reported previously (De Arcangelis et al., 2008; Insel et al., 1983; Pike and Lefkowitz, 1978).

Phosphorylation of phospholamban and troponin I, and myocyte contraction responses display agonist dose-dependent increases upon adrenergic stimulation

To understand how the temporal cAMP/PKA signal affects physiological function of cardiac myocytes, we examined PKA phosphorylation of PLB and TnI, and myocyte contraction rate response under stimulation with different doses of isoproterenol. At a saturating dose of 10^{-5} M, both PLB and TnI displayed rapid increases in PKA phosphorylation (Figure 2A). The increases were maintained during 30 minutes of stimulation (Figure 2A), consistent with the βAR-induced

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sustained increases in cAMP/PKA activities under the same stimulation condition (Figure 1A and 1C). At peak levels after 10 minutes of stimulation, the increases in PKA phosphorylation of PLB and TnI displayed an agonist dose-dependent manner (EC50, 3.38 x 10^{-8} for PLB and 7.41 x 10^{-9} for TnI, Figure 2B), which is consistent with the EC50 of sustained cAMP/PKA activities measured by FRET assays (Figure 1B and 1D). We then carried out neonatal myocyte contraction rate assay; a minimal 10^{-9} M of isoproterenol was required for a detectable increase in contraction rate (Figure 3A). While the baseline contraction rates were equivalent, upon increasing concentration of isoproterenol, the contraction rate displayed an agonist dose-dependent increase (EC50, 4.33 x 10^{-8}, Figure 3B and 3C). The contraction rate response also displays a high correlation with sustained cAMP and PKA activities (Figure 1).

We also examined the βAR-induced cAMP/PKA activities and contraction responses in more physiological relevant adult myocytes. In adult myocyte shortening assay, stimulation with isoproterenol, norepinephrine, or epinephrine all induced an agonist dose-dependent increases (Figure 3D). At 10^{-9} M of isoproterenol and epinephrine, minimal myocyte shortening was detected whereas at 10^{-9} M of norepinephrine, a small but significant myocyte shortening was detected. At 10^{-5} M, all three drugs induced similar maximal shortening in adult myocytes. We then examined cAMP activities in adult myocytes with ICUE3 FRET assay. Stimulation of myocyte with isoproterenol induced a dose-dependent ICUE3 FRET response (Figure 3E). Similar to those observed in neonatal myocytes, the responses were transient at submaxial doses, but sustained at saturated dose. The EC50 for the initial peak increase and the sustained increase were 7.46 X 10^{-10} M and 9.04 X 10^{-8} M, respectively (Figure 3F).

ACs controls the initial peak of cAMP/PKA activities under adrenergic stimulation in cardiomyocytes

To understand the relative contribution of β_1AR and β_2AR, the two major subtypes expressed in cardiac myocytes, we used myocytes isolated from mice lacking either β_1AR or β_2AR gene (β_1AR-KO and β_2AR-KO, respectively). Stimulation of endogenous β_1AR with isoproterenol in β_2AR-KO myocytes induced responses similar to those in WT myocytes, a transient increase at 10^{-8} M and a sustained increase at 10^{-5} M of isoproterenol, respectively (Figure S1A and S1B).

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Inhibition of PDE4 with rolipram enhanced the initial peak increases, which also became sustained at both concentrations (Figure S1A and S1B). In contrast, stimulation of endogenous β_2AR with isoproterenol in β_1AR-KO myocytes induced smaller and transient responses at both 10^{-8} M and 10^{-5} M of isoproterenol. Inhibition of PDE4 with rolipram enhanced the initial peak increases, which also became sustained at both concentrations (Figure S1C and S1D). We then aimed to determine the roles of two key components in the system, ACs and PDEs, in controlling the temporal cAMP/PKA activities induced by adrenergic stimulation. ACs have been implicated as a rate-limiting factor in the βAR/Gs/AC signaling pathway (Ostrom et al., 2000). Inhibition of AC with 2', 5'-DDA, a selective AC inhibitor, significantly reduced the cAMP FRET response induced by 10^{-9} M of isoproterenol (Figure 4A and 4D). ACV and ACVI are highly expressed in cardiac muscle cells; overexpression of ACVI alone significantly enhanced the increases of cAMP activity upon stimulation with either minimal 10^{-9} M or saturating 10^{-5} M of isoproterenol (Figure 4A-4D). Inhibition of PDE4 with rolipram further enhanced the initial peak increases induced by 10^{-9} M of isoproterenol (Figure 4A, 4C and 4D). In contrast, overexpression of β_1AR, the major adrenergic subtype to stimulate cardiac contraction, failed to promote higher initial peak increase in cAMP FRET ratio than those by endogenous βARs in wild type myocytes (Figure 4C and 4D). This data suggest that AC is the determining factor for the initial peak increase in cAMP induced by adrenergic stimulation.

PDEs dissociate with βAR and control the duration of cAMP/PKA activities in cardiomyocytes

In contrast, when the concentration of isoproterenol was increased from the nanomalor to the micromolar range, the βAR-induced initial increases in cAMP FRET ratio were saturated (Figure 1A). However, these cAMP signals underwent rapid attenuation to different levels, which were later sustained in a dose-dependent fashion (Figure 1A). Recent studies show that PDE4D is the major PDE gene that associates with adrenergic stimulation for cardiac myocyte contraction responses (Xiang et al., 2005), and PDE4D isoforms display preferential association with βAR subtypes (De Arcangelis et al., 2009; Richter et al., 2008). In agreement, inhibition of PDE4 with specific inhibitor rolipram significantly increased both initial and sustained responses in cAMP

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FRET ratios induced by 10^{-9} M of isoproterenol, but was less effective in enhancing the initial and sustained increases in cAMP induced by 10^{-5} M of isoproterenol (Fig. 5A and 5B). After inhibition of PDE4, the responses induced by isoproterenol at both doses were equivalent (Figure 5C and 5D). As a control, inhibition of PDE4 alone did not affect the basal cAMP levels in myocytes (Figure 5E). PDE4D can be activated through PKA phosphorylation to act as a negative feedback mechanism that attenuates cAMP signal upon receptor activation (Alvarez et al., 1995; Baillie et al., 2001). Indeed, endogenous PDE4D was phosphorylated by receptor-induced PKA activity at both 10^{-9} M and 10^{-5} M of isoproterenol (Figure 6A). These data support the role of PDE4D activity in shaping the dose-dependent sustained increases in cAMP/PKA activities in myocytes.

Since β_1AR is the major βAR subtype responsible for adrenergic stimulation of cardiac contraction, we then examined the association between PDE4D isoforms and β_1AR upon adrenergic stimulation. Endogenous PDE4D8 bound the β_1AR in cardiac myocytes at resting state (Figure 6B), consistent with the binding of PDE4D8 to β_1AR in HEK293 cells (Richter et al., 2008). Moreover, the PDE4D8/β_1AR complex was stable under stimulation with 10^{-9} M of isoproterenol; however the enzyme was dissociated from the receptor under stimulation with a saturating 10^{-5} M of isoproterenol (Figure 6B). We also examined the association between individual PDE4D isoforms and β_1AR upon adrenergic stimulation. PDE4D8, but not a closely related PDE4D9, bound the β_1AR in cardiac myocytes at resting state, but selectively dissociated from the receptor upon stimulation with a saturating 10^{-5} M of isoproterenol (Figure 6C). Further examination showed that PDE4D8 displayed an agonist-dependent dissociation from the receptor (Figure 6D). In contrast, ACVI remained in the receptor complex under stimulation with increasing concentrations of isoproterenol (Figure 6D). These data indicate that selective dissociation of PDE4D8 shifts the balance between AC-dependent cAMP production and PDE-dependent cAMP degradation at increasing concentrations of isoproterenol, and patterns the agonist dose-dependent temporal responses in cAMP/PKA activities in cardiomyocytes.

We further probed the role of PDE4D8 in shaping the sustained responses induced by βAR activation. Overexpression of PDE4D8 completely blocked the increases in cAMP FRET ratio

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induced by activation of βAR at a saturating concentration 10^{-5} M of isoproterenol or by activation of ACs with 10^{-5} M forskolin (Figure 7A-7C). However, the inhibitory effect of PDE4D8 was readily released by pretreatment with rolipram (Figure 7A) or by addition of rolipram (Figure 7C and 7B), which promoted the saturated increases in the cAMP FRET ratio. With overexpressed PDE4D8, even activation of overexpressed β_1AR failed to induce any significant increase in cAMP FRET ratio at 10^{-9} M of isoproterenol. However, additional inhibition of PDE4 with rolipram led to the saturated increases in cAMP FRET ratio (Figure 7D). In addition, we further dissected the role of individual PDE4D isoforms with overexpression of dominant negative PDE4Ds containing a mutation destroying catalytic activities. The overexpressed dominant negative inhibits the endogenous PDE4D isoforms by displacing them from the correct association with receptor complexes. Overexpression of PDE4D8 dominant negative selectively enhanced the cAMP increase induced by of 10^{-9} M isoproterenol, but did not change the transient feature of cAMP response (Figure 7E). In contrast, overexpression of dominant negative PDE4D9, an isoform that does not bind to β_1AR did not affect the maximal increase in cAMP FRET ratio, but slightly delayed the decrease of cAMP FRET responses (Figure 7E). These data solidify the functional association of a dominant PDE4D8 activity with the β_1AR for tuning the cAMP equilibrium upon isoproterenol stimulation in cardiomyocytes.

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DISCUSSION

A typical monoexponential dose-dependent cellular response has been a widely accepted pharmacological principle for most GPCR actions. However, there is lack of correlation between cAMP/PKA activities and myocyte contraction responses under same stimulation condition (De Arcangelis et al., 2008; Zhu et al., 2005). In this study, we have used sensitive FRET-based living-cell imaging to analyze cellular cAMP/PKA signals induced by adrenergic agonist isoproterenol. Our data indicates that isoproterenol induced two distinct phases in dose-dependent responses in cAMP/PKA activities in cardiac myocytes: a transient and dose-dependent increase of initial response under concentrations from 10^{-12} M to 10^{-8} M of isoproterenol, and a saturated initial increase under concentration from 10^{-8} M to 10^{-5} M of isoproterenol followed by a dose-dependent decrease to different levels which are later sustained (Figure 1 and 8). The sustained, but not the initial cAMP/PKA activities display a high correlation to the substrate phosphorylation and myocyte contraction rate response. Moreover, the agonist dose-dependent temporal increases in cAMP/PKA activities are patterned by a shifting equilibrium between two distinct mechanisms, AC-dependent cAMP production and PDE-dependent cAMP degradation due to the selective dissociation of PDE, but not AC from the activated receptor at higher concentrations. This shifting equilibrium allows cAMP accumulation and propagation in cardiomyocytes, which dictates PKA substrate specificity and cardiac contraction response (Figure 8).

Among βAR subtypes, the β_1AR is the major subtype expressed in myocytes and induces stronger cAMP/PKA activities in comparison to those by the β_2AR, consistent with our previous studies showing the β_1AR signaling induces stronger contraction rate responses than that induced by the β_2AR in cardiac myocytes (Devic et al., 2001). In addition, a minor role of β_3AR in negatively controlling cAMP/PKA activities and myocyte contraction rate has been detected previously (Devic et al., 2001; Mongillo et al., 2006). However, due to the minimal expression of this subtype in cardiac myocytes, it should have no effect on the cAMP/PKA activities at low doses of isoproterenol, and probably limited effect to modify the cAMP signaling at high doses of isoproterenol. The transient initial increases of cAMP/PKA activities display a high sensitivity

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to isoproterenol stimulation at low concentrations, which has a very low EC_{50} in comparison to the binding constants (k_d) (EC_{50} is not a binding constant, though it is usually proportional to it) of isoproterenol to βARs (Insel et al., 1983; Pike and Lefkowitz, 1978). Since receptors are more abundant than G proteins and ACs (Gao et al., 1998; Ostrom et al., 2001), activation of a small number of receptors may be sufficient in evoking the receptor/Gs/AC system for cAMP production. Thus, the initial peak increases may be a reflection of available pool of ACs activated in the receptor/G protein/AC system, supporting the notion that the quantity of ACs is the rate-limiting factor in producing cellular cAMP (Gao et al., 1998; Ostrom et al., 2001). In agreement, overexpression of ACVI, but not β_1ARs significantly enhances the maximal increases in cAMP accumulation (Figure 4).

Alternatively, it has been reported that adrenergic receptors can form precoupled complexes with Gs proteins, which display a much higher binding affinity to isoproterenol (Green et al., 1992). At concentrations from 10^{-12} M to 10^{-8} M, the dose-dependent maximal increases of cAMP/PKA activities may be influenced by binding of isoproterenol to the high affinity sites of the precoupled receptors. In this scenario, the maximal responses are likely due to agonist occupancy at the precoupled receptors, which appears to be sufficient to promote the maximal cAMP production via receptor/Gs/AC axis (Figure 1 and 8). However, under these low concentrations, the agonist-induced cAMP are rapidly degraded by the PKA-activated PDE4D within receptor complexes, a negative feedback mechanism to attenuate cAMP/PKA signaling (Leroy et al., 2008; Mongillo et al., 2004; Willoughby et al., 2006). The equilibrium between AC-dependent cAMP production and PDE-dependent cAMP degradation is dominated by the powerful PDE activities, which also functions as a "gating/braking" mechanism to ensure cAMP activities restricted within the receptor complex or the vicinity for local activation of PKA. Such PKA activation can only access to the activated receptors (Liu et al., 2009; Tran et al., 2004), and receptor-associated downstream signaling components such as PDE4D (Figure 6A), but not to the substrate in distance, such as phospholamban and troponin I for cardiac contraction.

This scenario is totally different when the concentration of isoproterenol is increased from 10^{-8} M to 10^{-5} M. At these concentrations, the AC-mediated cAMP production appears to be maximized. The saturation of cAMP production can be due to many factors, including activation

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of AC by either Gαs or Gβγ subunits inside or outside of caveolae, receptor desensitization, G protein hydrolysis, and negative regulation of AC activities by either kinases or Gi proteins (Dessauer, 2009; Hanoune and Defer, 2001; Sadana and Dessauer, 2009; Violin et al., 2008). In contrast, the receptor-associated PDE4D isoforms display an agonist dose-dependent dissociation from the receptor complex, which results into a shifting equilibrium between AC-dependent cAMP production and PDE-dependent cAMP degradation and promotes sustained increases in cAMP in a dose-dependent manner. The dissociation of PDE4D isoforms also functions as releasing the "gate/brake" to allow propagation of cAMP signal to potentiate PKA phosphorylation of phospholamban and troponin I, and cardiac contraction. Perturbation of the balance by altering the expression levels of either AC or PDE, or by inhibition of either of them drastically changes the temporal profiles of cAMP activities (Figure 4, 5, and 7), dictating the substrate specificity by PKA (De Arcangelis et al., 2008). Therefore, at higher concentrations, while the AC-dependent cAMP production remains constant within receptor complexes, the dissociation of PDE4D from the receptor appears to open the gate/release the brake for cAMP diffusion, and plays a critical role in shaping the dose-dependent cAMP signaling propagation for myocyte contraction (De Arcangelis et al., 2008).

Together, using real-time FRET-based biosensors we have revealed biphasic dose-dependent cAMP and PKA activities under adrenergic stimulation in cardiomyocytes: a transient and dose-dependent increase in initial peak responses at picomolar doses, and saturated initial increases followed by dose-dependent sustained increases at nanomolar doses. These data underscores an elegant integration of dual mechanistic regulation of cAMP/PKA activities by βAR-associated AC and PDE in an agonist-dose dependent manner, which shapes the temporal responses in cAMP/PKA activities for substrate specificity and physiological myocyte contraction rate responses. Our data provides a new paradigm for further investigation of cAMP/PKA signaling for cardiac responses under different physiological and clinical conditions.

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ACKNOWLEDGEMENT

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FOOTNOTES

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FIGURE LEGENDS

Figure 1 Activation of βARs induces a dose-dependent increase in cAMP ICUE3 and PKA AKAR3 FRET ratio in cardiomyocyte. *(A and B)* The cAMP biosensor ICUE3 was expressed in wild type myocytes. Cells were treated with isoproterenol at different concentrations. Changes in cAMP ICUE3 FRET ratio (an indication of cAMP activity) were measured. *(A)* Time courses of changes in cAMP FRET ratio were calculated and normalized against the baseline levels. (*B)* The initial peak increases (EC50 6.86 x 10^{-10} M) and the sustained increases (EC50 7.99 x 10^{-8} M) in cAMP FRET ratio were plotted. *(C-D)* The PKA biosensor AKAR3 was expressed in wild type myocytes. Cells were treated with isoproterenol at different concentrations. Changes in PKA AKAR3 FRET ratio (an indication of PKA activity) were calculated and normalized against the baseline levels. *(C)* Time courses of changes in PKA FRET ratio were plotted. *(D)* The initial peak increases (EC50 4.53 x 10^{-10} M) and the sustained increases (EC50 6.77 x 10^{-8} M*)* in PKA FRET ratio were plotted.

Figure 2 Activation of βARs induces a dose-dependent increase in PKA phosphorylation and myocyte contraction rate increases in cardiomyocyte. *(A)* Wild type myocyte were stimulated with isoproterenol for different time as indicated. Time courses of PKA phosphorylation of phospholamban (PLB, left) and troponin I (right) induced by isoproterenol (10^{-5}M), * < 0.05 and ** $p < 0.01$ by one-way ANOVA in comparison to unstimulated controls. *(B)* At 10 minutes of stimulation, isoproterenol dose-dependent increases in PKA phosphorylation of PLB (left, EC50 3.38 x 10^{-8}) and troponin I (righ, EC50 7.41 x 10^{-9}) were plotted. The intensity of each band was quantified and normalized against the total PLB or TnI in the same sample, and plotted in the bar graph.

Figure 3 Activation of βARs induces a dose-dependent increase in myocyte contraction in both neonatal and adult cardiomyocytes. *(A)* Upon isoproterenol stimulation, Time courses of changes in spontaneous myocyte contraction rate over baseline level were calculated and plotted. The

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baseline contraction rate (*B*) and the maximal increase (*C*, EC50 4.33 x 10^{-8} M) in contraction rate upon stimulation with isoproterenol were plotted. *(D)* Wild type adult myocytes were paced at 1Hz, and stimulated with forskolin, isoproterenol, norepinephrine, or epinephrine as indicated. The maximal myocyte shortening were normalized against the baseline, and plotted a percentage to those induced by forkolin. *(E)* The cAMP biosensor ICUE3 was expressed in wild type adult myocytes. Cells were treated with isoproterenol at different concentrations. Changes in cAMP ICUE3 FRET ratio (an indication of cAMP activity) were calculated and normalized against the baseline levels. *(F)* The initial peak increases (EC50 7.46 X 10^{-10} M) and the sustained increases (EC50 9.04 X 10^{-8} M*)* in PKA FRET ratio were plotted.

Figure 4 AC determines initial peak increases in cAMP FRET ratio upon adrenergic stimulation in cardiomyocytes. ACVI or HA-β_1AR was expressed together with the cAMP biosensor ICUE3 in wild type cardiomyocytes. *(A)* Effects of inhibition of AC with 2', 5' DDA (10^{-4} M) or overexpression of ACVI on increases in cAMP FRET ratio induced by 10^{-9} M of isoproterenol alone or by 10^{-9} M of isoproterenol with additional inhibition of PDE4 with inhibitor rolipram (10^{-6} M). *(B)* Effects of overexpression of ACVI on increases in cAMP FRET ratio induced by 10^{-5} M of isoproterenol. *(C)* The maximal increases in cAMP FRET ratio in panel *A-B* as well as the maximal increase in cAMP FRET ratio induced by 10^{-9} M of isoproterenol on $\beta_1\beta_2$AR-KO myocytes with HA-β_1AR overexpression were plotted. * < 0.05 and ** $p < 0.01$ by one-way ANOVA in comparison to controls stimulated with same concentration of isoproterenol. *(D)* The expression of ACVI, HA-β_1AR, and ICUE3 was detected in western blot.

Figure 5 Inhibition of PDE4 enhances increases in cAMP FRET ratio induced by submaximal dose of isoproterenol. The cAMP biosensor ICUE3 was expressed in wild type cardiomyocytes. Cells were treated with isoproterenol in the presence of absence of PDE4 selective inhibitor rolipram (10^{-6} M). Effects of inhibition of PDE4 with rolipram on increases in cAMP FRET ratio induced by isoproterenol at 10^{-9} M *(A)* or 10^{-5} M *(B)*. The initial increases and the sustained increases

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induced by isoproterenol at 10^{-9} M *(C)* or 10^{-5} M *(D)* were plotted. *(E)* Effect of inhibition of PDE4 with rolipram (10^{-6} M) alone on cAMP FRET ratio. *****, $P<0.05$ by one way ANOVA.

Figure 6 Agonist dose-dependent dissociations of PDE4D8, but not ACVI from β_1AR under stimulation of isoproterenol. *(A)* β_2AR-KO cardiomyocytes were stimulated with isoproterenol at either 10^{-9} M or 10^{-5} M for 10 minutes. The endogenous PDE4 proteins were immunoisolated with anti-PDE antibody, and the isoproterenol-induced PKA phosphorylation of PDE4 proteins were detected in Western blotting. *(B)* $\beta_1\beta_2$AR-KO cardiomyocytes expressing HA-β_1AR were stimulated with isoproterenol at either 10^{-9} M or 10^{-5} M for 10 minutes. The endogenous PDE4 proteins were co-immunoprecipitated with anti-HA affinity beads before Western blotting. *(C)* $\beta_1\beta_2$AR-KO cardiomyocytes expressing HA-β_1AR together with either PDE4D8-GFP or PDE4D9-GFP were stimulated with either 10^{-9} M or 10^{-5} M of isoproterenol for 10 minutes. The receptor-associated PDE4D isoforms was immunoprecipitated with anti-HA affinity beads before Western blotting. *(D)* $\beta_1\beta_2$AR-KO cardiomyocytes expressing HA-β_1AR, ACVI, and PDE4D8-RFP were stimulated with isoproterenol at different concentrations for 10 minutes. The receptor-associated ACVI and PDE4D8 were immunoprecipitated with anti-HA affinity beads before western blotting. * < 0.05 and ** p < 0.01 by one-way ANOVA in comparison to unstimulated controls.

Figure 7 Overexpression of PDE4D8 blocks the cAMP FRET responses induced by either isoproterenol or forskolin. (*A*-*C*) PDE4D8 and the cAMP biosensor ICUE3 were coexpressed in wild type cardiomyocytes. *(A)* Changes in cAMP ICUE3 FRET ratio were measured after inhibition of PDE4 with rolipram (10^{-6} M) followed by additional stimulation with isoproterenol (10^{-9} M). *(B)* Changes in cAMP ICUE3 FRET ratio were measured after stimulation with isoproterenol (10^{-9} M) followed by additional inhibition of PDE4 with rolipram (10^{-6} M). *(C)* Changes in cAMP ICUE3 FRET ratio were measured after stimulation with isoproterenol (10^{-9} M), followed by additional stimulation with forskolin (10^{-5} M) before additional inhibition of PDE4

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with rolipram (10^{-6} M). *(D)* PDE4D8, HA-β_1AR, and ICUE3 were coexpressed in $\beta_1\beta_2$AR-KO cardiomyocytes. Changes in cAMP ICUE3 FRET ratio were measured after stimulation with isoproterenol (10^{-9} M) followed by additional inhibition of PDE4 with rolipram (10^{-6} M). *(E)* Dominant negative PDE4D8 (498A) and PDE4D9 (490A) were coexpressed together with ICUE in β_2AR-KO cardiomyocytes. Changes in cAMP ICUE3 FRET ratio were measured after stimulation with isoproterenol (10^{-9} M). The expression of dominant negative PDE4D isoforms was detected in Western blot. $^{\#} < 0.05$ by two-way ANOVA in comparison to the control.

Figure 8 Model of dual mechanistic regulation of cAMP/PKA activities by AC and PDE4D under different doses of adrenergic stimulation. At 10^{-9} M of isoproterenol, the βAR-activated AC induces significant production of cAMP (the gas pedal is on), which is transient and restricted at the vicinity of the receptor for local PKA activation. The activated PKA has access to the receptor and receptor associated PDE that negatively feedback to confine and attenuate cAMP signaling at local domains (the brake is still on). At 10^{-5} M of isoproterenol, the AC-produced cAMP (the gas pedal is on) can propagate to access to PKA in different subcellular compartments due to dissociation of PDE4D from the activated receptors (the brake is off). The activated PKA phosphorylates both local (near the receptor) and distant substrates such as phospholamban (PLB) and troponin I (TnI) for myocyte contraction responses.

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Figure 1

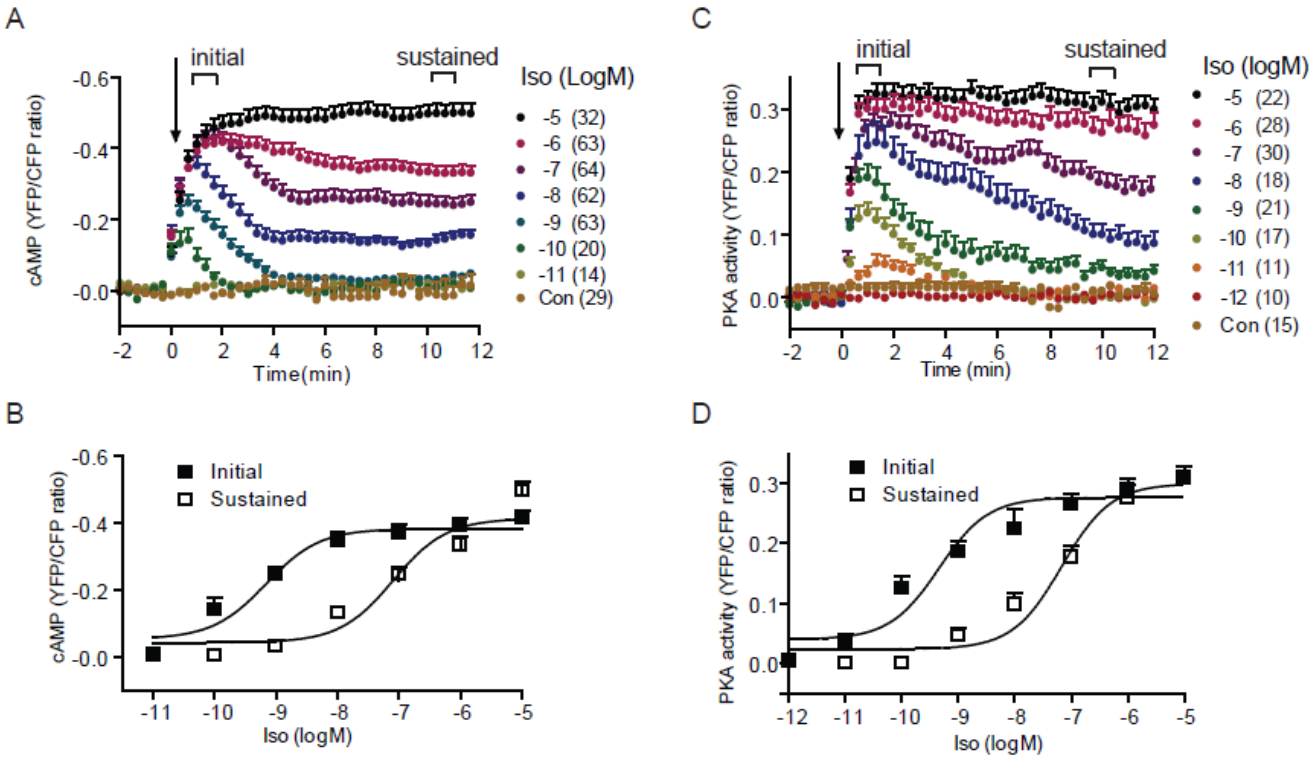

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Figure 2

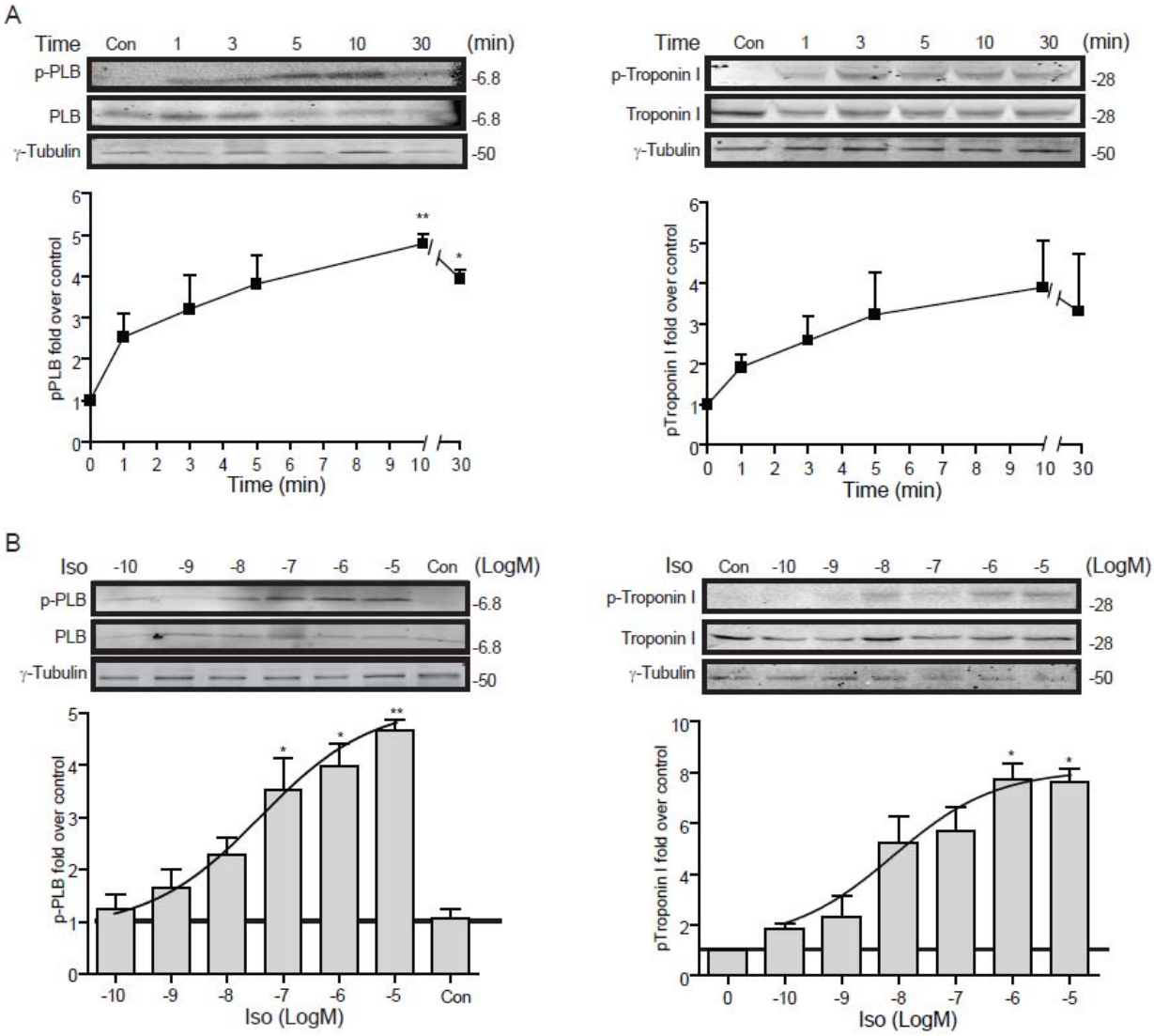

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Figure 3

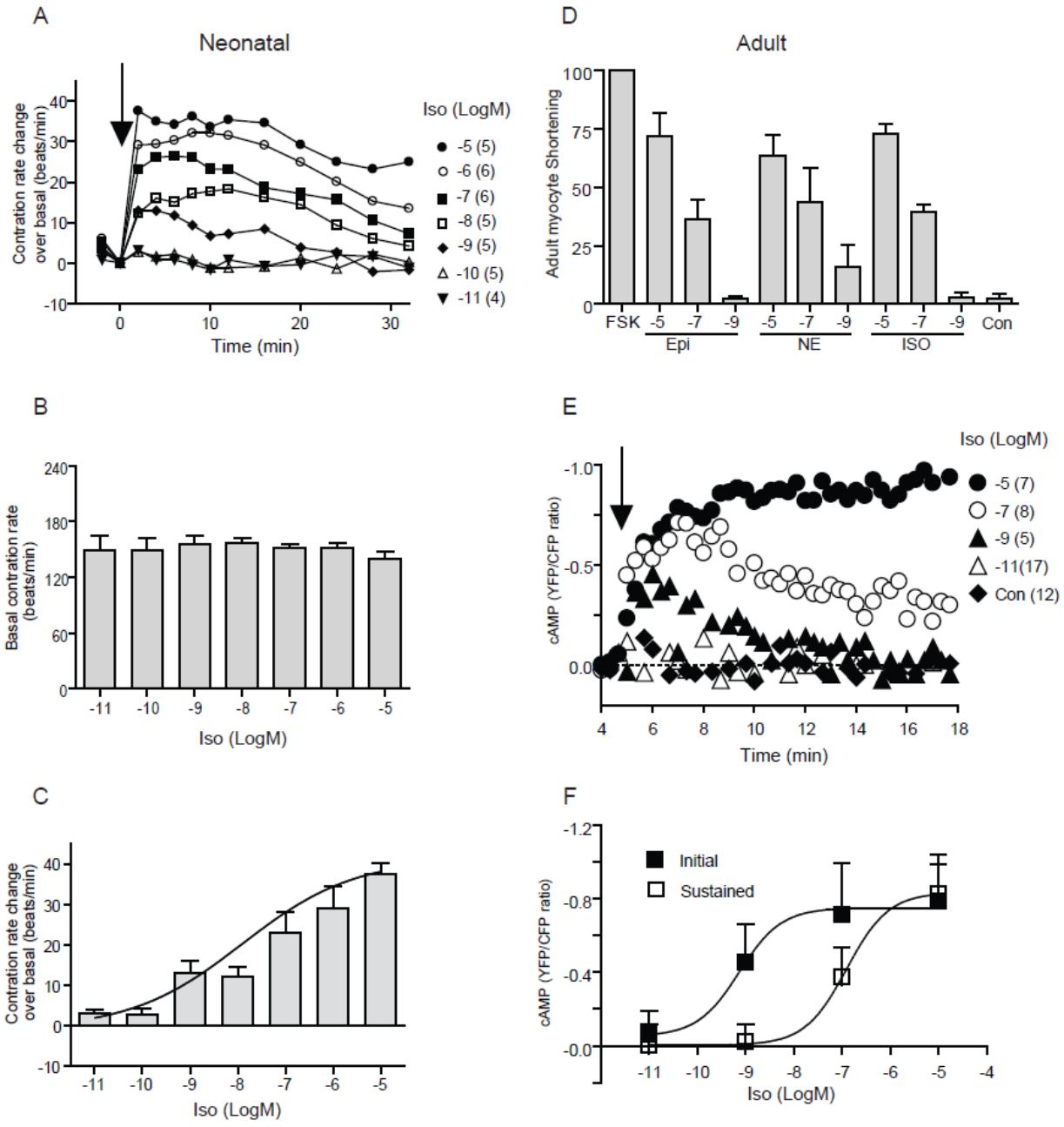

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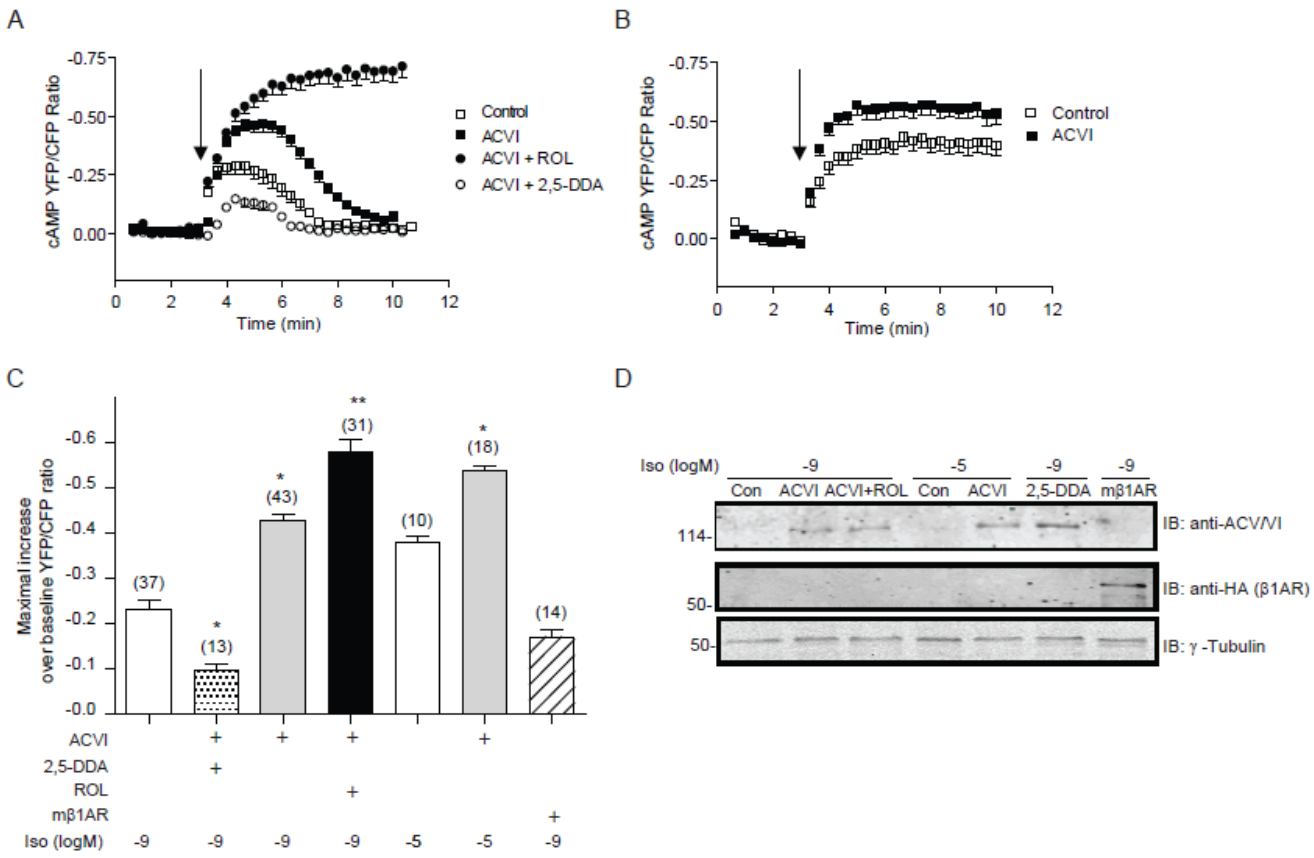
Figure 4



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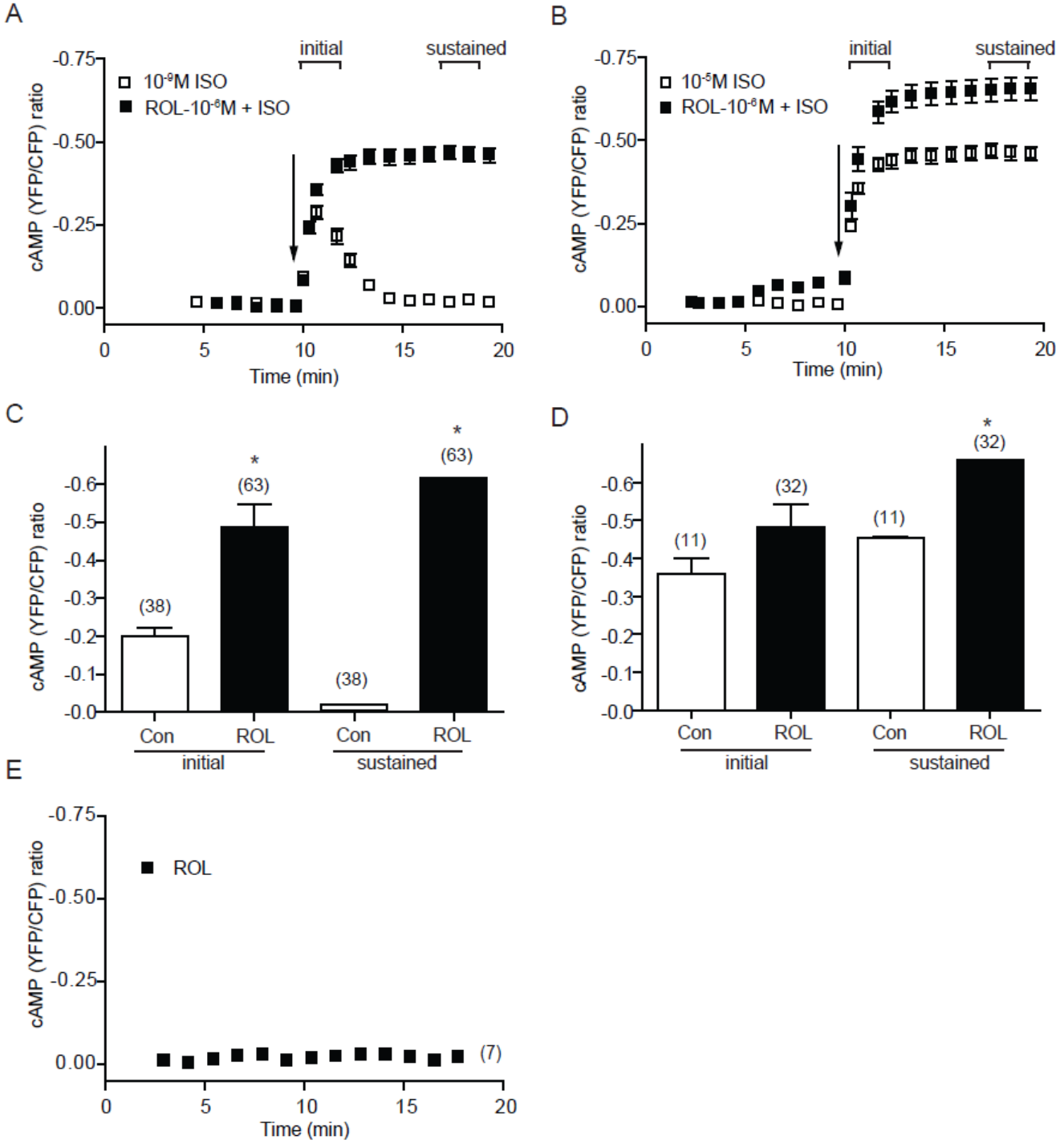

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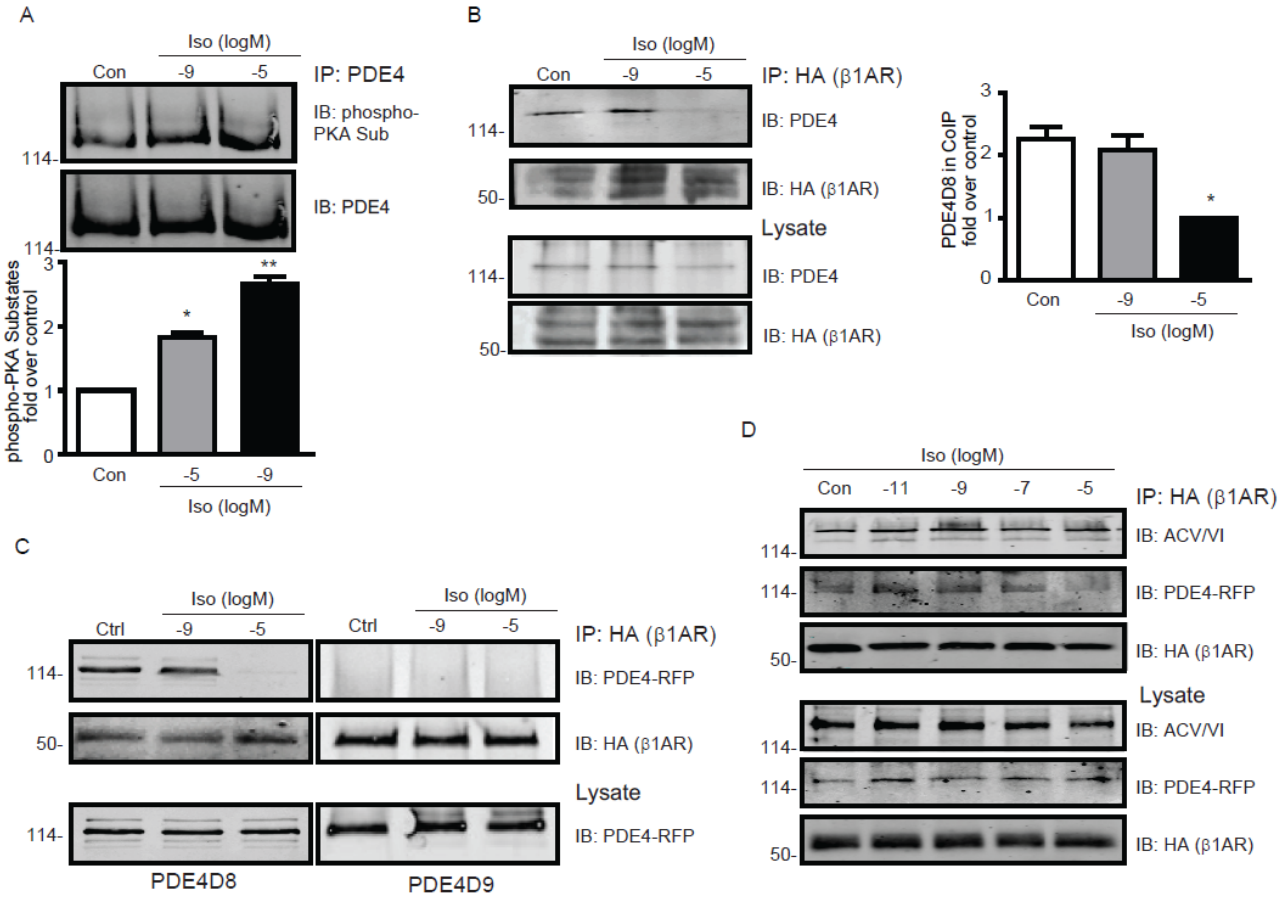

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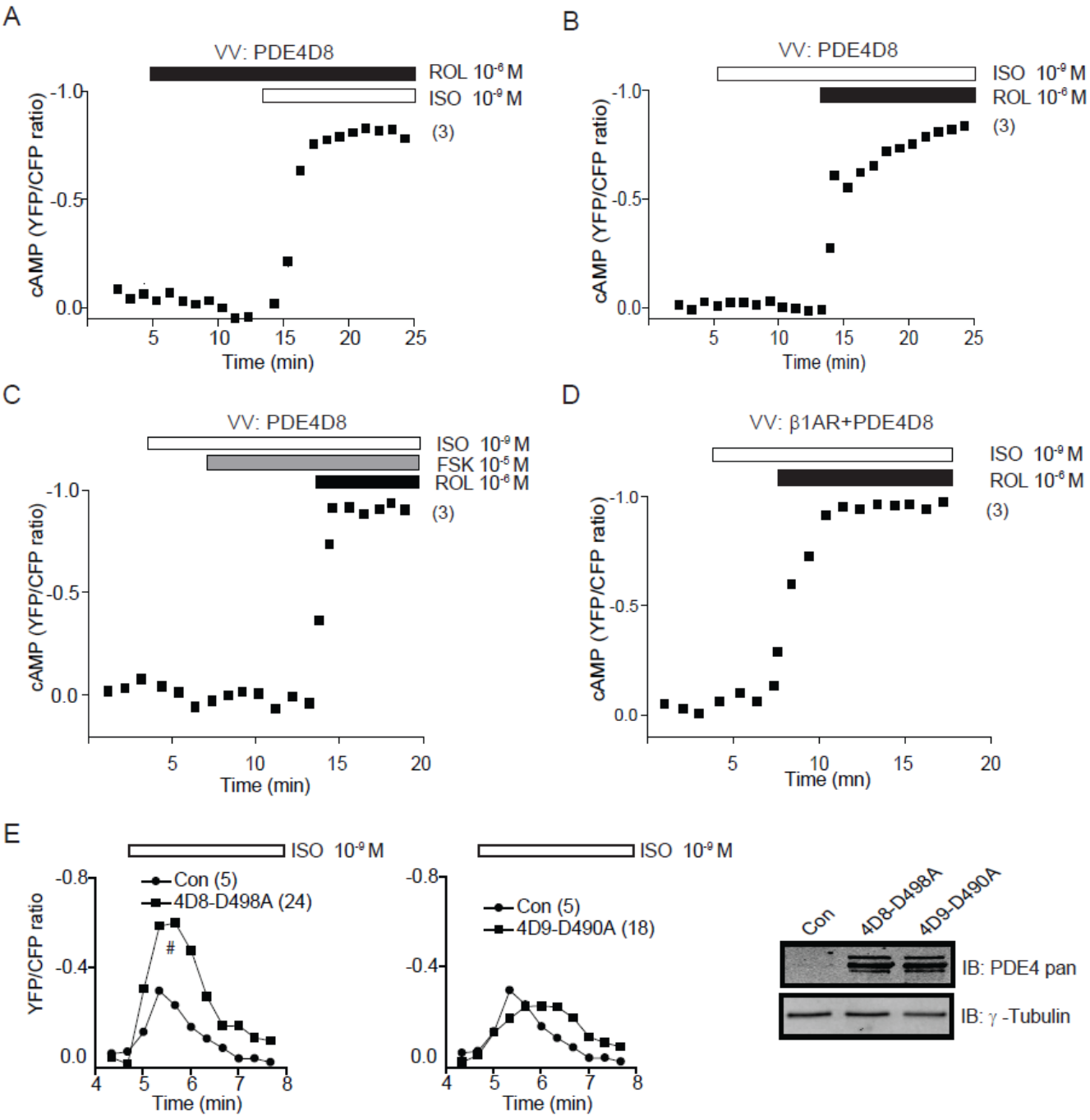
Figure 7



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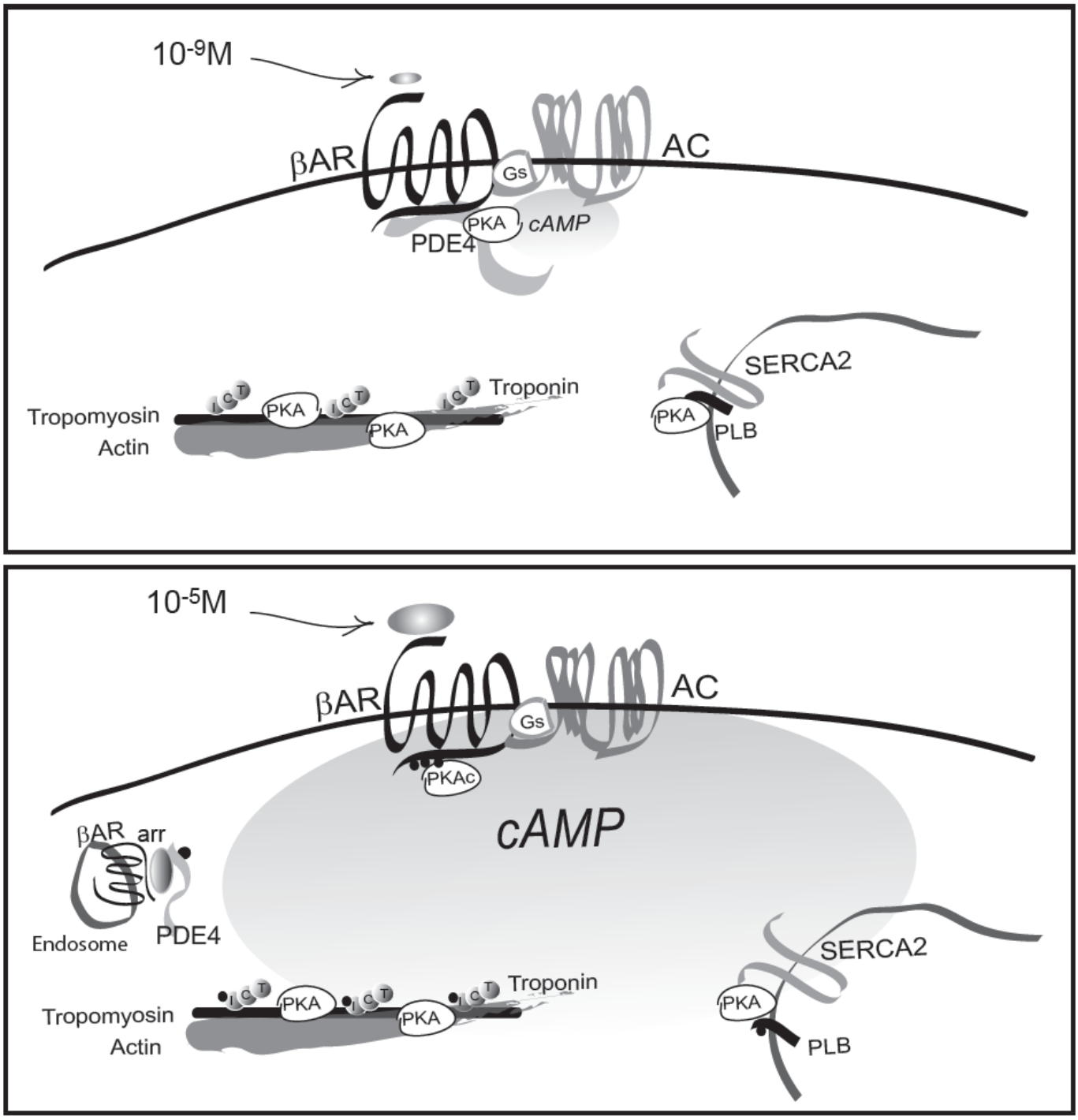

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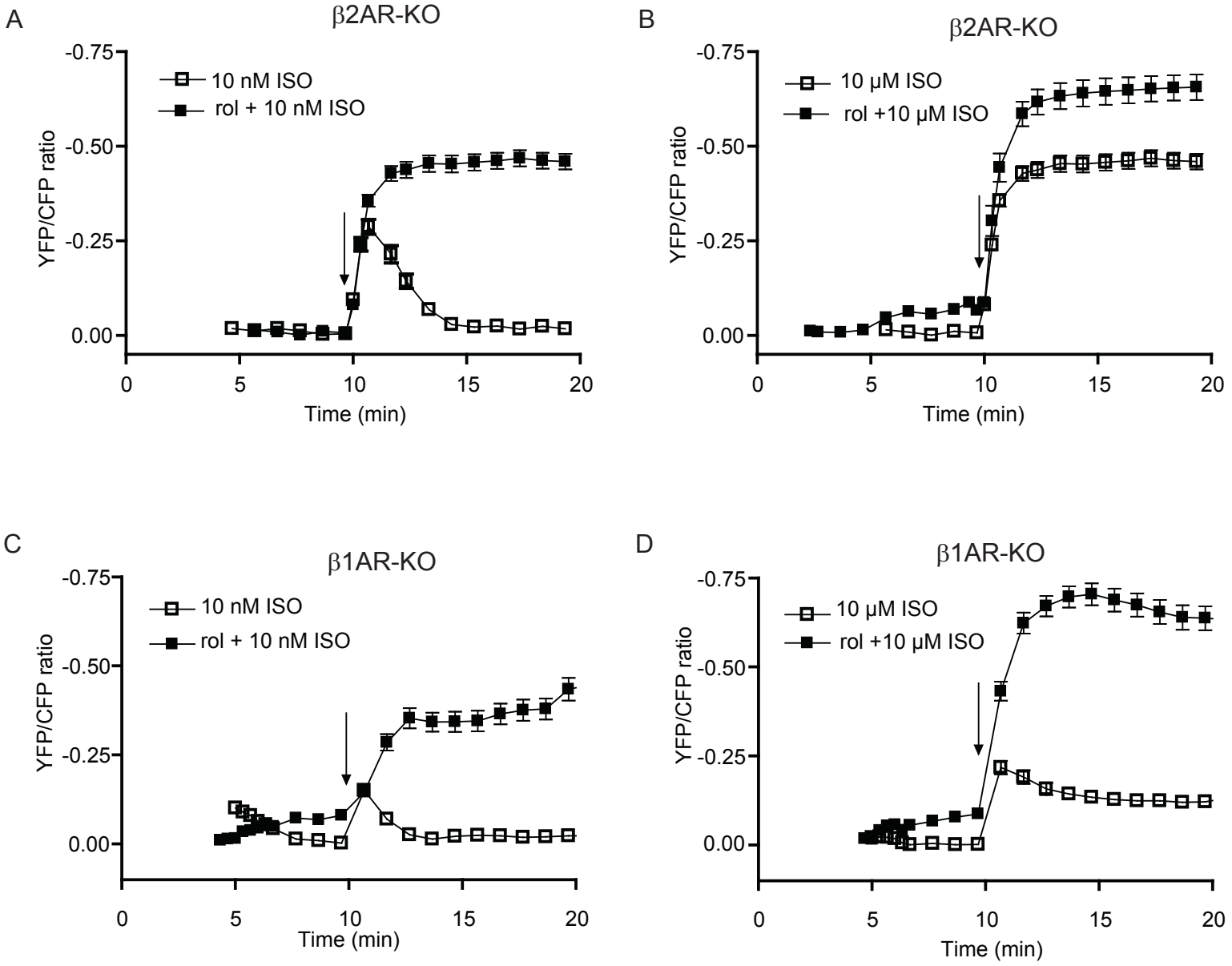
Journal: Molecular Pharmacology
Title: Equilibrium between adenylyl cyclase and phosphodiesterase patterns adrenergic agonist dose-dependent spatiotemporal cAMP/PKA activities in cardiomyocytes
Author: Vania De Arcangelis, Shubai Liu, Dawen Zhang, Dagoberto Soto, and Yang K. Xiang

Online supplementary Figure 1. Agonist dose-dependent cAMP activities induced by either β_1AR or β_2AR in cardiac myocytes. *(A and B)* β_2AR-KO myocytes were infected with adenovirus expressing cAMP probe ICUE3. The cells were stimulated with either 10^{-9} or 10^{-5} M of isoproterenol in the absence or presence of 10^{-5} M of PDE4 inhibitor rolipram (rol). The time course of changes in ICUE3 cAMP FRET ratio was recorded before and after stimulation. *(C and D)* β_1AR-KO myocytes were infected with adenovirus expressing cAMP probe ICUE3. The cells were stimulated with either 10^{-9} or 10^{-5} M of isoproterenol in the absence or presence of 10^{-5} M of PDE4 inhibitor rolipram (rol). The time course of changes in ICUE3 cAMP FRET ratio was recorded before and after stimulation.

Figure S1


A
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YFP/CFP ratio
Time (min)
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C
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rol + 10 nM ISO
YFP/CFP ratio
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