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A Long Lasting β_1 Adrenergic Receptor Stimulation of cAMP/Protein Kinase A (PKA) Signal in Cardiac Myocytes*

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Background: Transient β_1AR activation remains at odds with long lasting cellular and physiological responses.
Results: The agonist-occupied β_1AR continuously signals to adenylyl cyclase (AC) to produce cAMP in both cardiac myocytes and neurons for more than 8 h, which is masked by receptor-associated PDE4D8.
Conclusion: Stimulation of β_1AR induces long-lasting cAMP production in the heart for ligand-induced physiological responses.
Significance: We show a novel mechanism to understand persistent β_1AR signaling in the heart.

Small-molecule, ligand-activated G protein-coupled receptors are generally thought to be rapidly desensitized within a period of minutes through receptor phosphorylation and internalization after repeated or prolonged stimulation. This transient G protein-coupled receptor activation remains at odds with many observed long-lasting cellular and physiological responses. Here, using live cell imaging of cAMP with a FRET-based biosensor and myocyte contraction assay, we show that the catecholamine-activated β_1 adrenergic receptor (β_1AR) continuously stimulates second messenger cAMP synthesis in primary cardiac myocytes and neurons, which lasts for more than 8 h (a decay $t_{1/2}$ of 3.9 h) in cardiac myocytes. However, the β_1AR-induced cAMP signal is counterbalanced and masked by the receptor-bound phosphodiesterase (PDE) 4D8-dependent cAMP hydrolysis. Inhibition of PDE4 activity recovers the receptor-induced cAMP signal and promotes contractile response in mouse hearts during extended periods of agonist stimulation. β_1AR associates with PDE4D8 through the receptor C-terminal PDZ motif-dependent binding to synaptic-associated protein 97 (SAP97). Knockdown of SAP97 or mutation of the β_1AR PDZ motif disrupts the complex and promotes sustained agonist-induced cAMP activity, PKA phosphorylation, and cardiac myocyte contraction response. Together, these findings unveil a long lasting adrenergic signal in neurons and myocytes under prolonged stimulation and an underappreciated role of PDE that is essential in classic receptor signaling desensitization and in maintaining a long lasting cAMP equilibrium for ligand-induced physiological response.

Activation of G protein-coupled receptors (GPCRs)[4] regulates a broad range of cellular responses. Following agonist binding, GPCRs couple to G protein to stimulate downstream effectors such as adenylyl cyclase (AC) and phospholipase to produce second messenger cAMP, diacylglycerol, and inositol 1,4,5-trisphosphate for diversified cellular responses (1–4). Many GPCRs are also desensitized through rapid phosphorylation by G protein-coupled receptor kinases within a period of less than 1 min. Subsequently, the phosphorylated GPCRs bind to β-arrestins (5) for endocytosis to further turn off receptor activation by repetitive stimulation or excessive agonists. After internalization, GPCRs are either dephosphorylated for recycling back to the cell surface or delivered to lysosomes for degradation (6). Interestingly, recent studies reveal that GPCRs activated by hormonal peptides such as relaxin and thyroid-stimulating hormone can promote a persistent cAMP signal at the cell surface and after internalization, respectively (7–9). This persistent signal is attributed to high-affinity binding of peptide agonists to their respective receptors. In contrast, the duration of GPCR activation by small-molecule ligands such as catecholamines is still generally considered to be transient (1–4).

Adrenoceptors (ARs) are activated by small-molecule catecholamines. Activation of βARs promotes cAMP-PKA activity for diversified physiological processes, from cardiac contraction and energy metabolism in the peripheral systems to learning and memory in the central nervous system (1, 10). Despite a transient feature of βAR signal in traditional biochemical studies in fibroblast cell lines (1–4), physiological responses induced by βAR activation suggest a persistent receptor signal in native tissues. Activation of cardiac βARs via the sympathetic nervous system promotes persistent contractile response in the heart during long periods of exercise, and

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[4] The abbreviations used are: GPCR, G protein-coupled receptor; AC, adenylyl cyclase; AR, adrenergic receptor; PDE, phosphodiesterase; LV, left ventricular; dp/dt, rate of pressure development; PBL, phospholamban; ANOVA, analysis of variance.

stimulation of βARs in neurons promotes long term potentiation that is critical for learning and memory. In agreement with these functional outputs, β_1ARs undergo minimal internalization in primary cardiac myocytes after agonist stimulation (11). Meanwhile, a recent study indicates that β_2AR is capable of maintaining ligand-induced active conformation after internalization and/or recycling (12). Together, these observations raise possibilities that small-molecule, ligand-activated βARs are capable of inducing a persistent signal in a physiological cellular context.

Here we provide evidence showing that activation of β_1AR is able to continuously signal for more than 8 h in primary cardiac myocytes. We further reveal that scaffold protein SAP97 connects β_1AR to the PDE4D8 isoform via the receptor C-terminal PDZ motif. PDE4D8-mediated cAMP hydrolysis shapes the persistent β_1AR signal for catecholamine-induced contractile responses in animal hearts.

MATERIALS AND METHODS

Langendorff Perfusion Heart Preparation—Animal experiments were performed following the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All procedures were approved by the Institutional Animal Care and Use Committee at the University of California at Davis. The isolated heart perfusion technique has been described previously (13). Hearts were excised from mice under anesthesia (sodium pentobarbital, 120 mg/kg body weight, intraperitoneally) and transferred rapidly to the Langendorff apparatus. After the heart was hung, it was perfused under constant pressure (80 mm Hg) with a solution containing 113.8 mM NaCl, 22 mM $NaHCO_3$, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.1 mM $MgSO_4$, 1.1 mM glucose, 2 mM $CaCl_2$, and 2 mM sodium pyruvate. A balloon made of plastic film was inserted into the left ventricular (LV) cavity and filled with water to set the diastolic pressure at 10 mm Hg of LV end-diastolic pressure. 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/min by pacing at the right ventricle with a Grass SD9 stimulator. The hearts became stabilized after pacing for 15 min. Drugs were applied through the perfusion solution. LV pressure, LV end-diastolic pressure, and the maximum rate of positive and negative change in LV pressure ($\pm$LV dp/dt) were recorded. Left ventricular developed pressure was calculated by subtracting the LV end-diastolic pressure from the LV systolic pressure. Data were analyzed offline with LabChart Pro-6.0.

Hemodynamic Study—Hemodynamic measurement was carried out with a pressure catheter (SPR-839, Millar Instruments) connected to an AD Instruments Power-Lab 4/30 (MPVS-300, ADInstruments) with Lab Chart Pro 6.0 software. In brief, mice were anesthetized with intraperitoneal injection of ketamine/xylazine (80 and 5 mg/kg, respectively). A midline neck incision was made, the carotid artery was separated from the vagus nerve, and a pressure catheter was inserted via the carotid artery tip into the left ventricle. After stabilization of the signal for 5–10 min, the baseline pressure was recorded, followed by intraperitoneal administration of isoproterenol (0.2 mg/kg) and rolipram (3 mg/kg), as indicated. Intra-LV blood pressure was recorded continuously to monitor the effects of the injected drugs. Data were analyzed offline with the blood pressure module in the LabChart 6.0 software.

Cardiac Myocyte and Prefrontal Cortical Neuron Isolation and Adenovirus Infection—Neonatal cardiac myocytes and prefrontal cortical neurons were isolated from newborn pups lacking the β_2AR gene (β_2AR-KO), the β_1AR gene (β_1AR-KO), or lacking both the β_1 and β_2AR genes ($\beta_1\beta_2$AR-KO), as described previously (13, 14). Cells were cultured in coverslip chambers precoated with laminin for live cell imaging. After isolation and plating for 24 h, cells were washed and infected with adenoviruses for expression of the cytosolic cAMP sensor (ICUE3) (15) and PDE4D isoforms. The C-terminal tagged PDE4D isoforms containing the N-terminal regions were subcloned into the pEGFP-N2 plasmid with HindIII and EcoRI. The fusion protein was then shuttled with HindIII and XbaI into the viral vector for recombinant viruses. Wild-type, full-length PDE4D8 and the mutants PDE4D8-D498A and PDE4D9-D490A lacking enzymatic activity were subcloned into pcDNA3.1-mcherry with HindIII-EcoRI, and the fusion protein was shuttled with HindIII and XbaI into the viral vector for recombinant viruses. Adenovirus-expressing HA-tagged β_1AR has been described elsewhere (11).

Neonatal Myocyte Contraction Rate Assay—Measurement of spontaneous contraction rate was carried out as described previously (16). Changes in the neonatal myocyte beating rate after drug treatments were analyzed by Metamorph software (Molecular Devices).

Immunoprecipitation and Western Blot Analysis—β_1AR-KO myocytes expressing PDE4D isoforms, HA-tagged β_1AR, or both were stimulated with 1 nM of isoproterenol before being lysed in immunoprecipitation (IP) buffer. Alternatively, wild-type mouse hearts were lysed in IP buffer. The lysates were clarified by centrifugation before they were subjected to Western blot analysis or immunoprecipitation with anti-HA affinity resins (Roche) or anti-β_1AR antibody (SCBT). For time courses of PKA phosphorylation of PLB, β_1AR-KO myocytes expressing HA-tagged β_1AR were stimulated with 1 nM isoproterenol for the indicated times before washing out the drug. For the PKA phosphorylation of PDE4, wild-type myocytes were stimulated with 1 nM or 1 μM isoproterenol for 10 min before being lysed in the buffer. The lysates were clarified by centrifugation before being subjected to Western blot analysis. The cell lysates and immunoprecipitated proteins were resolved in SDS-PAGE for Western blot analysis with anti-β_1AR antibody (SCBT), total and phosphorylated PLB at serine 16 and threonine 17 (Badrilla), anti-phospho-PDE4D antibody (Fabgennix), anti-red fluorescence protein/mCherry antibody (Rockland), and anti-green fluorescence protein antibody (Invitrogen). The primary antibodies were revealed with fluorescent-conjugated secondary antibodies using a Licor scanner (Licor).

FRET Measurement—Cells were infected with adenoviruses to express ICUE3 for 24 h. Cells were rinsed and maintained in PBS with calcium for FRET recording. Cells were imaged on a Zeiss Axiovert 200 M microscope with a $\times$40/1.3 oil immersion

objective lens and a charge-coupled device 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 a 200-ms exposure in both channels and 20-s lapses. Images in both channels were subjected to background subtraction, and ratios of yellow to cyan color were calculated at different time points. The binding of cAMP to ICUE3 led to decreases in the ratio of YFP to cyan fluorescent protein that were plotted with an inverted *y* axis.

Drug Treatments—Cells were stimulated with isoproterenol (ISO, Sigma) at the indicated times. Cells were treated with the following inhibitors: the adrenergic antagonist alprenolol (ALP, 10 μM, Sigma), the selective AC inhibitor 2′,5′ dideoxyadenosine triphosphate, 100 μM, Sigma), and the PDE4 inhibitor rolipram (ROL, A.G. Scientific) at the indicated times and doses. Membrane-permeable tat-conjugated peptides containing either the N-terminal-specific 30 amino acids of PDE4D8 (MAFVWDPLGVTVPGPSPR TRTRLRFSKSYS$_{30}$) or the N-terminal-specific 22 amino acids of PDE4D9 (MSIIMK-PRSRSTSSLRTTEAVC$_{22}$) were synthesized as described previously (17), and 1 μM peptide was added to cells 20 min before stimulation with isoproterenol for the contraction rate assay.

Statistical Analysis—One-way and two-way ANOVA, followed by post hoc Tukey's test and Student's *t* test, were performed using Prism (GraphPad Software).

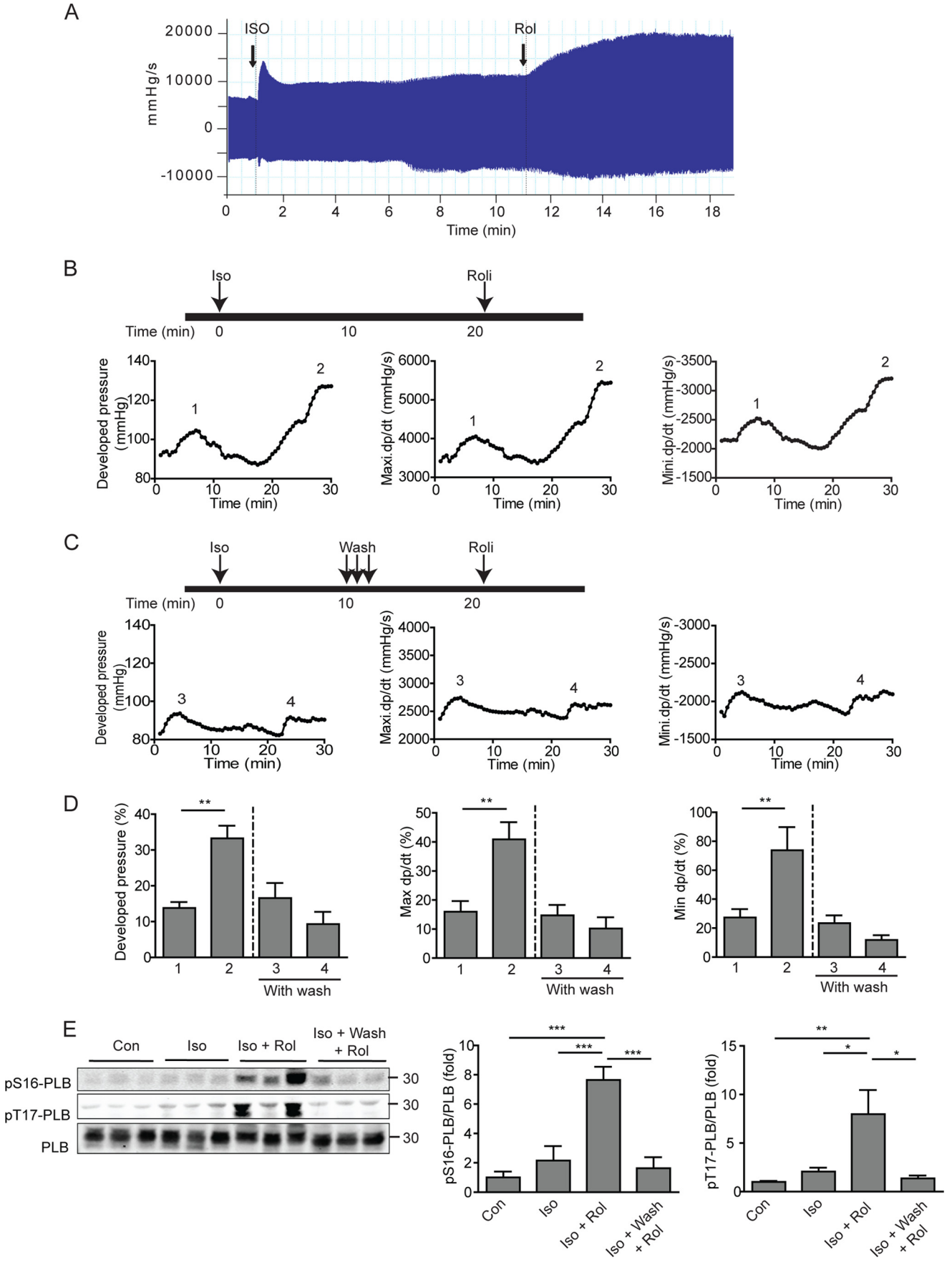
RESULTS

Recent studies indicate that PDE isoforms associate with βARs and other GPCRs and shape agonist-induced, dose-dependent spatiotemporal response of cAMP in different cells (7, 18, 19). We hypothesize that stimulation of βAR promotes a persistent intracellular signal that is masked by the receptor-associated PDEs. We examined the effect of βAR stimulation on contractile function of mouse hearts *in vivo* with a hemodynamics study. Intraperitoneal injection of isoproterenol (0.2 mg/kg) induced a transient cardiac contractile response in mouse hearts *in vivo*. Addition of rolipram after the transient response recovered a much stronger, second contractility response (Fig. 1*A*). We then used Langendorff-perfused hearts to directly access βAR stimulation on contractile function in mouse myocardium *ex vivo*. Perfusion of wild-type mouse hearts with 1 nM of the βAR agonist isoproterenol induced small and transient cardiac contractile response, including increases in developing pressure, maximal dp/dt, and minimal dp/dt (Fig. 1, *B* and *D*). Addition of PDE4 inhibitor rolipram at 20 min of isoproterenol perfusion induced a much stronger second contractile response (Fig. 1, *B* and *D*). However, removal of isoproterenol before addition of rolipram abolished the rolipram-induced second response in cardiac contractility (Fig. 1, *C* and *D*). Biochemical assays confirmed that addition of rolipram after isoproterenol stimulation induced a strong PKA phosphorylation of phospholamban (PLB), a critical protein in the regulation of calcium signaling for the cardiac contractile response (Fig. 1*E*). However, this increase in PKA phosphorylation of PLB was absent in the hearts when isoproterenol was washed away before the addition of rolipram (Fig. 1*E*).

Among cardiac βARs, β_1AR is the primary subtype responsible for contractility. Therefore, we used primary cardiac myocytes lacking the β_2AR gene (β_2AR-KO) to analyze the β_1AR-induced cAMP-PKA signal in the hearts. The cAMP FRET biosensor ICUE3 (15) expressed in primary cardiac myocytes displayed a transient response to stimulation with unsaturated 1 nM isoproterenol, which returned to the baselines in several minutes (Fig. 2, *A* and *B*). However, pretreatment with the PDE4 inhibitor rolipram promoted a higher and sustained cAMP FRET response after stimulation with 1 nM isoproterenol (Fig. 2*A*). These data support that β_1ARs continuously signal to AC to produce cAMP under persistent agonist stimulation. However, the receptor-induced cAMP signal was masked by PDE4-mediated cAMP hydrolysis. To test this idea, we applied the PDE4 inhibitor rolipram after the transient cAMP FRET response induced by 1 nM isoproterenol. The addition of rolipram after the first transient peak recovered a second strong and sustained cAMP FRET response in primary cardiac myocytes (Fig. 2*B*). Meanwhile, we observed similar responses in primary β_2AR-KO prefrontal cortical neurons (Fig. 2*C*), suggesting a conserved mechanism on persistent β_1AR signaling in both brain and heart tissues.

This rolipram-induced second cAMP signal was abolished by either adding a competitive βAR antagonist, alprenolol, by washing away isoproterenol with PBS (Fig. 2, *D* and *E*), or by adding the AC inhibitor 2′, 5′ dideoxyadenosine triphosphate before inhibition of PDE4 (Fig. 2*F*). In agreement, stimulation of cardiac myocytes with 1 nM isoproterenol induced small increases in the spontaneous contraction rate (Fig. 3, *A* and *B*). Addition of the PDE4 inhibitor rolipram recovered much higher and sustained second contraction rate responses (Fig. 3*A*). However, the βAR antagonist alprenolol blocked the rolipram-induced secondary increases in contraction rate (Fig. 3*B*). Moreover, the rolipram-induced second increases in cAMP FRET and contraction rate were attenuated rapidly to the baseline levels after addition of alprenolol (Fig. 3, *C* and *D*). As a control, rolipram alone did not affect the cAMP FRET and contraction rate at the baseline level (Fig. 3, *E* and *F*). Together, these data suggest that β_1ARs continuously signal through G_s and AC for cAMP synthesis, which is also dependent on agonist occupation of the receptors.

We hypothesized that β_1AR forms a stable complex with both the cAMP-stimulatory components G_s and AC and the cAMP degradation component PDE to maintain long acting receptor signaling under stimulation with 1 nM isoproterenol. Among the PDE genes, PDE4D is implicated in association with βARs in cardiac myocytes (18). We successfully pulled down PDE4D with endogenous β_1AR together with the scaffold protein SAP97 and AKAP150 in mouse hearts (Fig. 4*A*). In addition, we pulled down the positive stimulatory components G_s and PKA, the negative regulatory components PDE4D and PP2A, as well as scaffold proteins SAP97 and AKAP150 together with FLAG-β_1AR expressed in cardiac myocytes (Fig. 4*B*). This complex was stable under stimulation with 1 nM isoproterenol (Fig. 4*B*). This is in contrast to agonist-induced, selective dissociation of PDE4 from the complex under stimulation with a saturated concentration of 10 μM isoproterenol (19, 20). Moreover, PDE4 was also phosphorylated by PKA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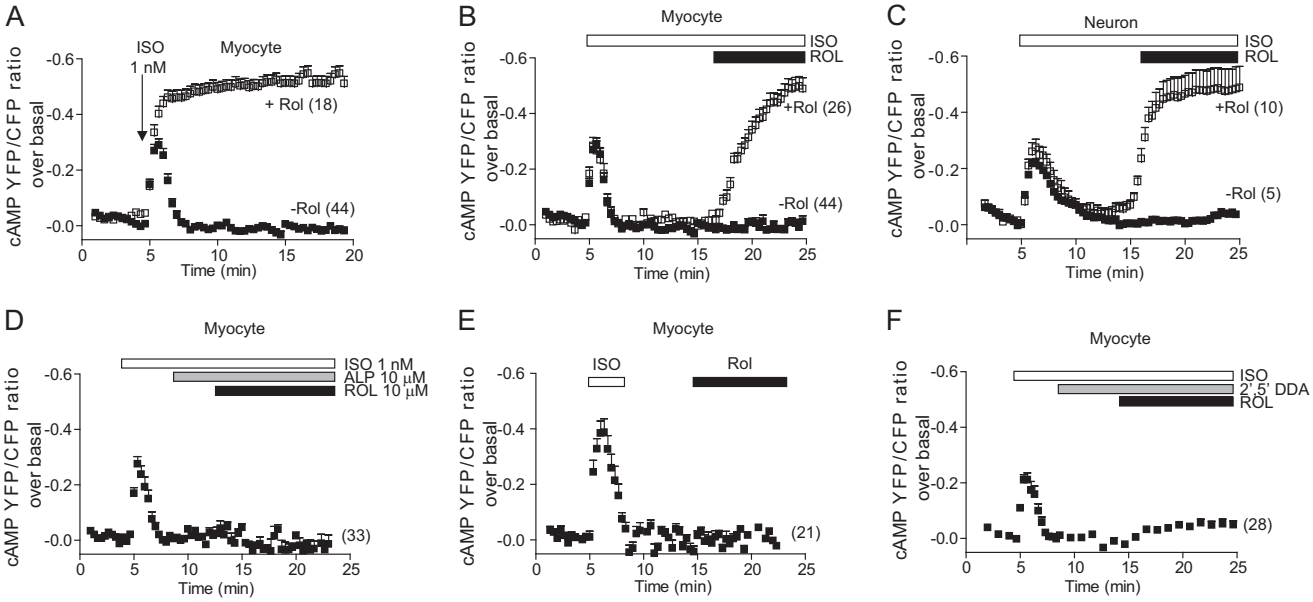

FIGURE 2. **PDE4 masks the cAMP signal induced by β_1AR in primary cardiac myocytes and prefrontal cortical neurons under stimulation with isoproterenol.** Cardiac myocytes or prefrontal cortical neurons from β_2AR-KO mice expressing the cAMP biosensor ICUE3 were stimulated with isoproterenol as indicated. *A*, cells were stimulated with 1 nM isoproterenol (*ISO*) in the absence or presence of pretreatment with the PDE4 inhibitor rolipram (*Rol*, 10 μM). *CFP*, cyan fluorescent protein. Cardiac myocytes (*B*) and prefrontal cortical neurons (*C*) were stimulated with 1 nM isoproterenol. After the transient initial increase in FRET ratio, cells were treated with and without the PDE4 inhibitor rolipram (10 μM). *D–F*, cardiac myocytes were stimulated with 1 nM isoproterenol. After the transient initial increase in FRET ratio, cells were treated with addition of the βAR antagonist alprenolol (*ALP*, 10 μM) (*D*), washing with PBS to remove the agonist (*E*), or with addition of the AC inhibitor 2′,5′ dideoxyadenosine triphosphate (2′,5′ *DDA*, 100 μM) (*F*) before addition of the PDE4 inhibitor rolipram (10 μM). The changes in FRET ratio were recorded.

under stimulation with 1 nM isoproterenol (Fig. 4*C*), which can enhance enzymatic activity for cAMP degradation (21). Among PDE4D isoforms, we identified that only the PDE4D8 isoform displayed a significant association with β_1AR in cardiac myocytes (Fig. 4, *D* and *E*).

PDE4D isoforms differ at the N-terminal regions with specific sequences important for subcellular localization and function. The N-terminal regions can act as dominant negatives by displacing the endogenous isoforms from the proper subcellular location (17). Overexpression of the N terminus of PDE4D8, but not other PDE4D isoforms, in β_2AR-KO myocytes enhanced contraction rate response induced by 1 nM isoproterenol (Fig. 4*F*). Introducing a single amino acid mutation in the catalytic domains abolishes the enzymatic activity of the PDE4D isoforms (22). Overexpression of catalytically inactive PDE4D8-D498A, but not PDE4D9-D490A, enhanced the maximal cAMP FRET response induced by 1 nM isoproterenol in β_2AR-KO myocytes (Fig. 4, *G* and *H*). Consequently, overexpression of catalytically inactive PDE4D8-D498A enhanced the myocyte contraction rate increases induced by 1 nM isoproterenol, whereas wild-type PDE4D8 did not (Fig. 4*I*). Further analysis revealed that scaffold protein SAP97 is critical to connect PDE4D8 to β_1AR. Knockdown of SAP97 with shRNA disrupted the association between β_1AR and PDE4D8 (Fig. 5*A*) and enhanced both the magnitude and duration of cAMP FRET response in wild-type cardiac myocytes induced by 1 nM isoproterenol (Fig. 5*B*). These data indicate a critical role of PDE4D8 association with β_1AR for tuning cAMP amplitude and duration after isoproterenol stimulation.

To assess the duration of the β_1AR-induced cAMP signal, we added rolipram at different durations after the first transient cAMP FRET response induced by 1 nM isoproterenol in β_2AR-KO myocytes. Strikingly, the rolipram-induced second cAMP FRET response was evident after 8 h of stimulation with a decay $t_{1/2}$ of 3.9 h (Fig. 6, *A* and *C*). In agreement, the addition of rolipram induced secondary increases in the myocyte contraction rate with a decay $t_{1/2}$ of 3.4 h (Fig. 6, *B* and *D*). Meanwhile, we also examined whether we could detect similar response in wild-type myocytes. 1 nM isoproterenol induced a transient response in cAMP FRET. The rolipram-induced secondary response in the cAMP FRET ratio were also observed in wild-type cardiac myocytes (Fig. 7*A*). After 4 or 8 h of stimulation with 1 nM isoproterenol, rolipram still induced significant second response in cAMP FRET (Fig. 7, *B–D*). To determine whether agonist occupation was necessary for the second responses after extended periods of stimulation, we washed

FIGURE 1. **Inhibition of PDE4 unveils the βAR-induced persistent signal for cardiac contractility in mouse hearts.** *A*, hemodynamics were measured in wild-type mouse hearts after intraperitoneal injection of the β adrenergic agonist isoproterenol (*ISO*, 0.2 mg/kg), followed by injection of rolipram (*Rol*, 3 mg/kg). Data show a representative curve of maximal dp/dt and minimal dp/dt. *B–E*, wild-type mouse hearts were used in Langendorff perfusion with isoproterenol as indicated. Time courses of developing pressure, maximal dp/dt, and minimal dp/dt were recorded. *B*, hearts were perfused with 1 nM isoproterenol (*Iso*) for 20 min, followed by addition of the PDE4 inhibitor rolipram (*Roli*, 10 nM). *C*, hearts were perfused with 1 nM isoproterenol for 10 min, followed by perfusion with saline to wash away the agonist for 10 min before addition of the PDE4 inhibitor rolipram (10 nM). *D*, the peak changes in developing pressure, maximal (*Max*) dp/dt, and minimal (*Min*) dp/dt from different time points in *A* and *B*. *E*, heart tissues were lysed, and the PKA phosphorylation of PLB at serine 16 and calmodulin-dependent kinase II phosphorylation at threonine 17 were examined by Western blot analyses and normalized against the levels of total PLB. *Con*, control. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$ between the indicated groups by one-way ANOVA.

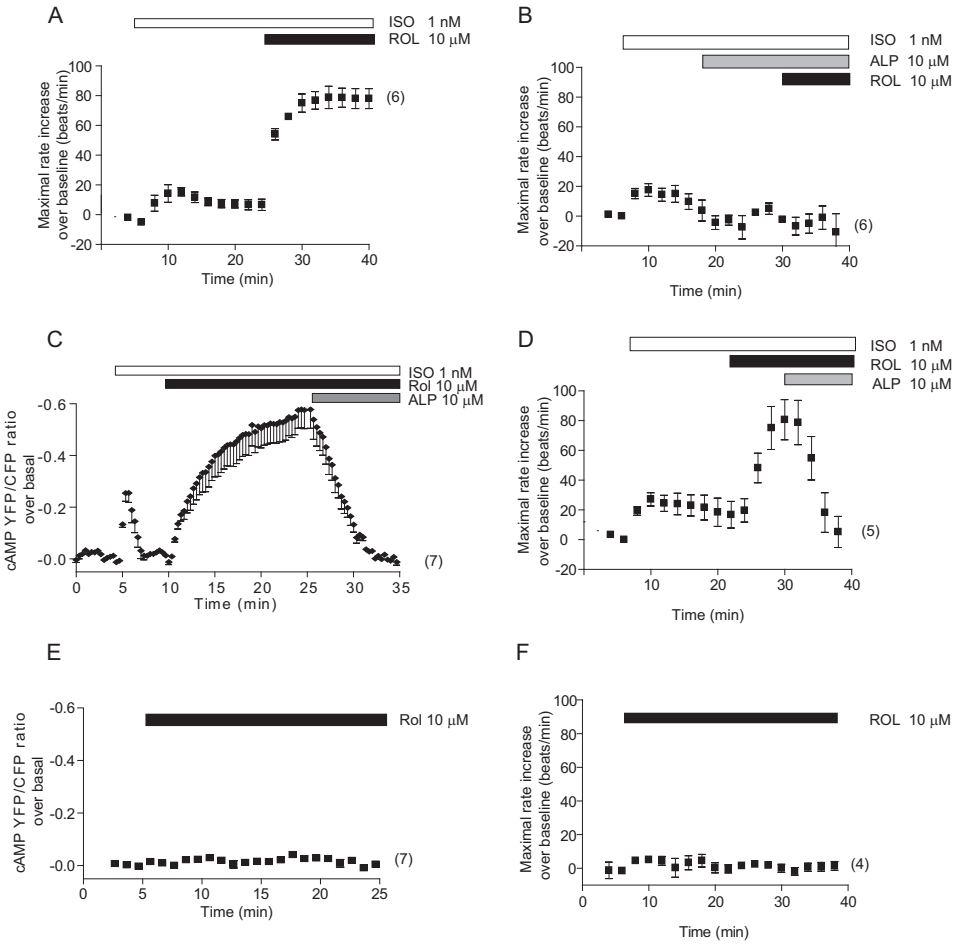

FIGURE 3. **Activation of β_1AR induces sustained contraction rate response under stimulation with 1 nM isoproterenol.** *A*, in β_2AR-KO myocytes, stimulation of β_1AR with 1 nM isoproterenol (*ISO*) induced a small and transient contraction rate response. The addition of rolipram (*ROL*) promoted a higher and sustained contraction rate response. *B*, addition of the βAR antagonist alprenolol (*ALP*) before rolipram attenuated the myocyte contraction rate induced by 1 nM isoproterenol and completely abolished the higher contraction rate response promoted by rolipram. *C* and *D*, addition of βAR antagonist alprenolol after rolipram reversed the second cAMP FRET and myocyte contraction rate responses back to the baseline levels. *CFP*, cyan fluorescent protein. *E* and *F*, rolipram did not significantly affect the baseline cAMP FRET ratio and myocyte contraction rate.

cells with PBS to remove the agonist 30 min prior to the administration of rolipram. Washing away the agonist with PBS abolished the rolipram-induced second response in cAMP FRET. However, additional direct stimulation of AC with forskolin induced a strong response in the cAMP FRET ratio, suggesting that AC was still active in these cells (Fig. 7, *B* and *C*). In contrast, washing cells with PBS containing 1 nM isoproterenol did not affect the rolipram-induced second responses in cAMP FRET when compared with those without washing (Fig. 7, *B–D*). In agreement, rolipram induced significant increases in the contraction rate after 4 and 8 h of stimulation with 1 nM isoproterenol (Fig. 7*E*). These increases were abolished by washing with PBS 30 min before the addition of rolipram but not affected by washing with PBS containing 1 nM isoproterenol. Together, these data suggest that the βAR-induced cAMP signal is constantly masked by receptor-associated PDE4 for extended periods and that only inhibition of the enzyme is able to reveal the cAMP signal and contractile response in cardiac myocytes.

To further illustrate the long action of β_1AR in cardiac myocytes, we introduced a mutation to the β_1AR C-terminal PDZ motif that selectively disrupted the receptor binding to SAP97 and PDE4D8 but did not affect the receptor association with G_s (Fig. 8*A*). When introduced into β_1AR-KO cardiac myocytes, the mutant β_1AR induced much stronger and sustained cAMP FRET responses than the wild-type receptor (Fig. 8*B*) and promoted sustained increases in contraction rate during 8-hour stimulation (Fig. 8*C*). In contrast, wild-type β_1AR induced transient increases in contraction rate that returned to baseline level. Accordingly, stimulation of wild-type β_1AR induced transient PKA phosphorylation of PLB in cardiac myocytes, whereas the mutant β_1AR promoted stronger and sustained PKA phosphorylation of PLB during 4-h stimulation (Fig. 8, *D* and *E*).

DISCUSSION

Small-molecule, ligand-induced GPCR activation is thought to be transient because of receptor phosphorylation and internalization and because of receptor dissociation from the agonist. However, this notion remains at odds with many GPCR-dependent, long lasting cellular and physiological response *in vitro* and *in vivo*. Here we found that ligand-occupied β_1ARs continuously stimulated AC to produce cAMP in primary cardiac myocytes and prefrontal cortical neurons (Fig. 2, *B*

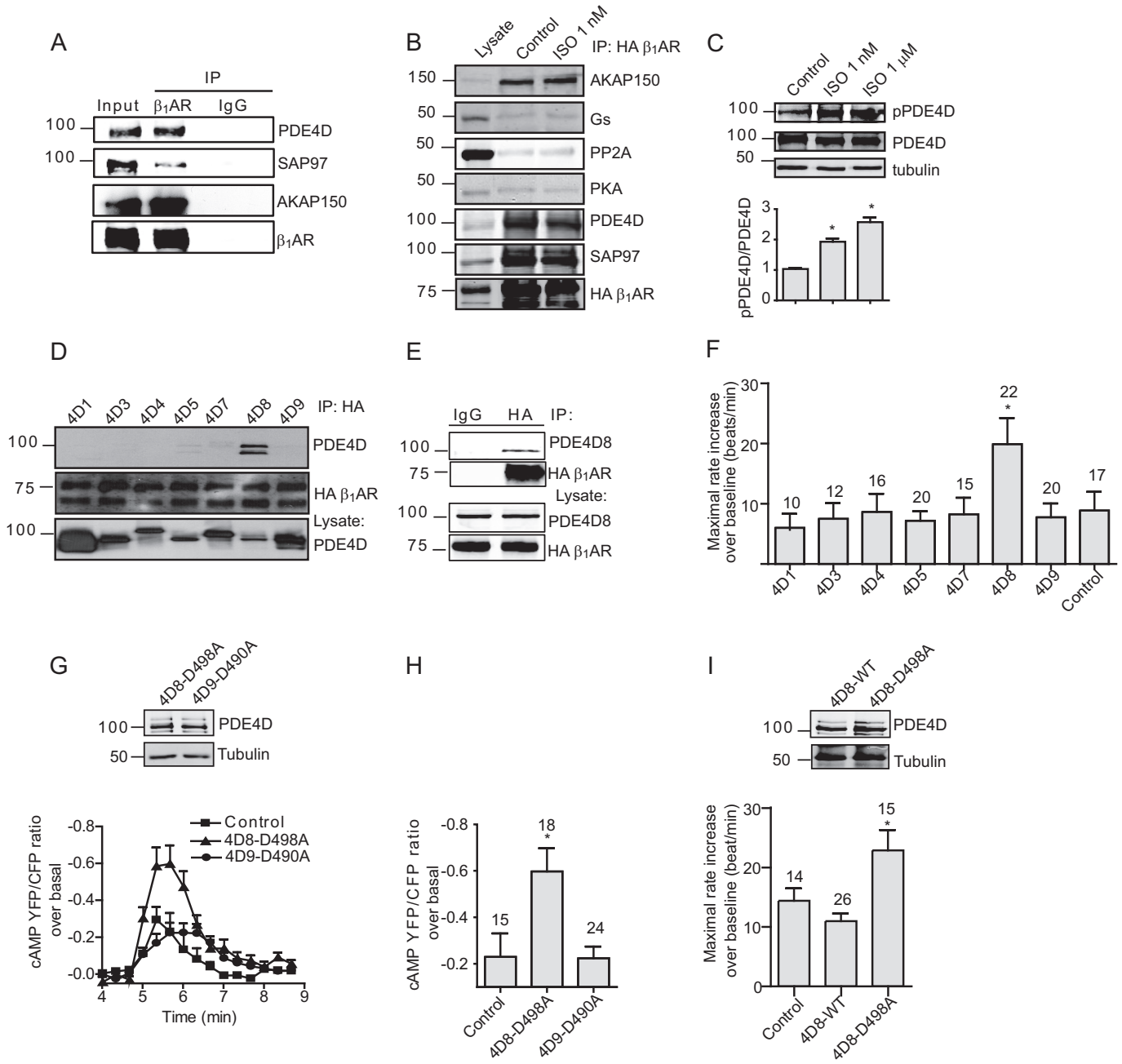

FIGURE 4. **β_1AR forms a macrocomplex with both G_s and PDE4D8 to tune the receptor-induced cAMP activity and spontaneous contraction rate response.** *A*, wild-type mouse hearts were lysed before being pulled down with anti-β_1AR antibody. The precipitates were examined in Western blot analyses with the indicated antibodies. *IP*, immunoprecipitation. *B*, β_1AR-KO cardiac myocytes expressing HA-β_1AR were stimulated with 1 nM isoproterenol (*ISO*). Cell lysates were subjected to immunoprecipitation with anti-HA antibody. The proteins that were pulled down were detected in Western blot analyses with the indicated antibodies. *C*, wild-type cardiac myocytes were stimulated with 1 nM or 1 μM isoproterenol. PKA phosphorylation of PDE4D was detected with anti-phospho-PDE4D-specific antibody and normalized against total PDE4D. *D* and *E*, β_1AR-KO cardiac myocytes expressed HA-β_1AR together with the PDE4D isoforms. Cell lysates were subjected to immunoprecipitation with anti-HA antibody. The β_1AR and PDE4D isoforms that were pulled down were detected in Western blot analyses. *F*, β_2AR-KO cardiac myocytes expressing the N-terminal domains of different PDE4D isoforms were stimulated with 1 nM isoproterenol. The maximal increases in the myocyte contraction rate were measured. *G* and *H*, β_2AR-KO cardiac myocytes expressing the cAMP biosensor ICUE3 together with catalytically inactive PDE4D8 or PDE4D9 were stimulated with 1 nM isoproterenol. The time courses and the maximal changes of the cAMP FRET ratio were measured. The expression of catalytically inactive PDE4D8 and PDE4D9 were shown in Western blot analyses. *CFP*, cyan fluorescent protein. *I*, β_2AR-KO cardiac myocytes expressing wild-type PDE4D8 or catalytically inactive PDE4D8 were stimulated with 1 nM isoproterenol. The maximal increases in myocyte contraction rate were measured. The expression of wild-type and catalytically inactive PDE4D8 were shown in Western blot analyses. *, $p < 0.05$ in comparison with the control by one-way ANOVA.

and *C*). However, this signal was suppressed by the receptor-associated PDE4D8 and could be revealed by inhibition of PDE4 activity (Fig. 2, *B* and *C*). Therefore, in this signaling paradigm, equilibrium is maintained between agonist-dependent continuous stimulation of AC for cAMP production and PDE-mediated cAMP degradation. This agonist-dependent equilibrium lasts for more than 8 h (a decay $t_{1/2}$ of 3.9 h, Fig. 6), challenging the classic dogma of rapid receptor desensitization occuring within several minutes of agonist stimulation.

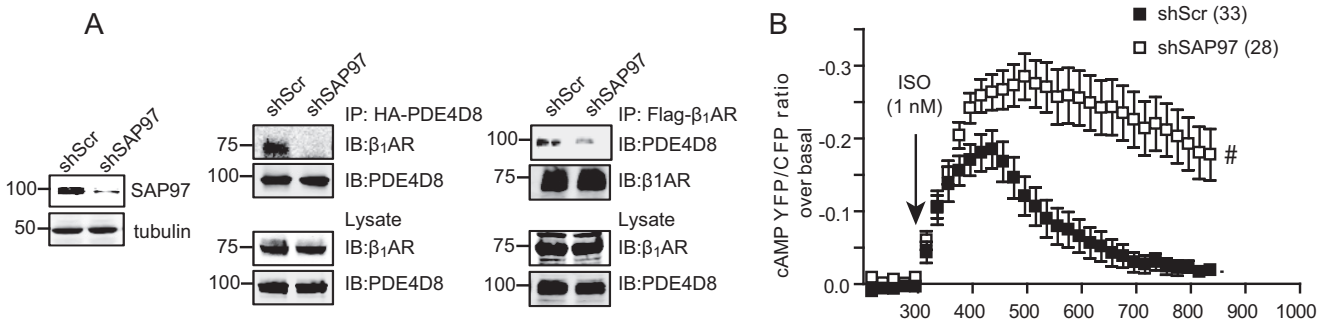

FIGURE 5. **SAP97 scaffolds PDE4D8 to the β_1AR to tune the receptor-induced cAMP activity and spontaneous contraction rate responses.** *A*, HEK293 cells stably expressing scrambled shRNA (*shScr*) or shRNA against SAP97 (*shSAP97*) were transfected with HA-PDE4D8 and FLAG-β_1AR. Cell lysates were subjected to immunoprecipitation (*IP*) with the indicated antibody. The β_1AR and PDE4D8 that were pulled down were detected in Western blot analyses (*IB*). *B*, β_2AR-KO cardiac myocytes expressing the cAMP biosensor ICUE3 together with either scramble or SAP97 shRNA were stimulated with 1 nM isoproterenol (*ISO*). The changes in cAMP FRET ratio were recorded. *CFP*, cyan fluorescent protein. #, $p < 0.05$ in comparison with the scrambled group by two-way ANOVA.

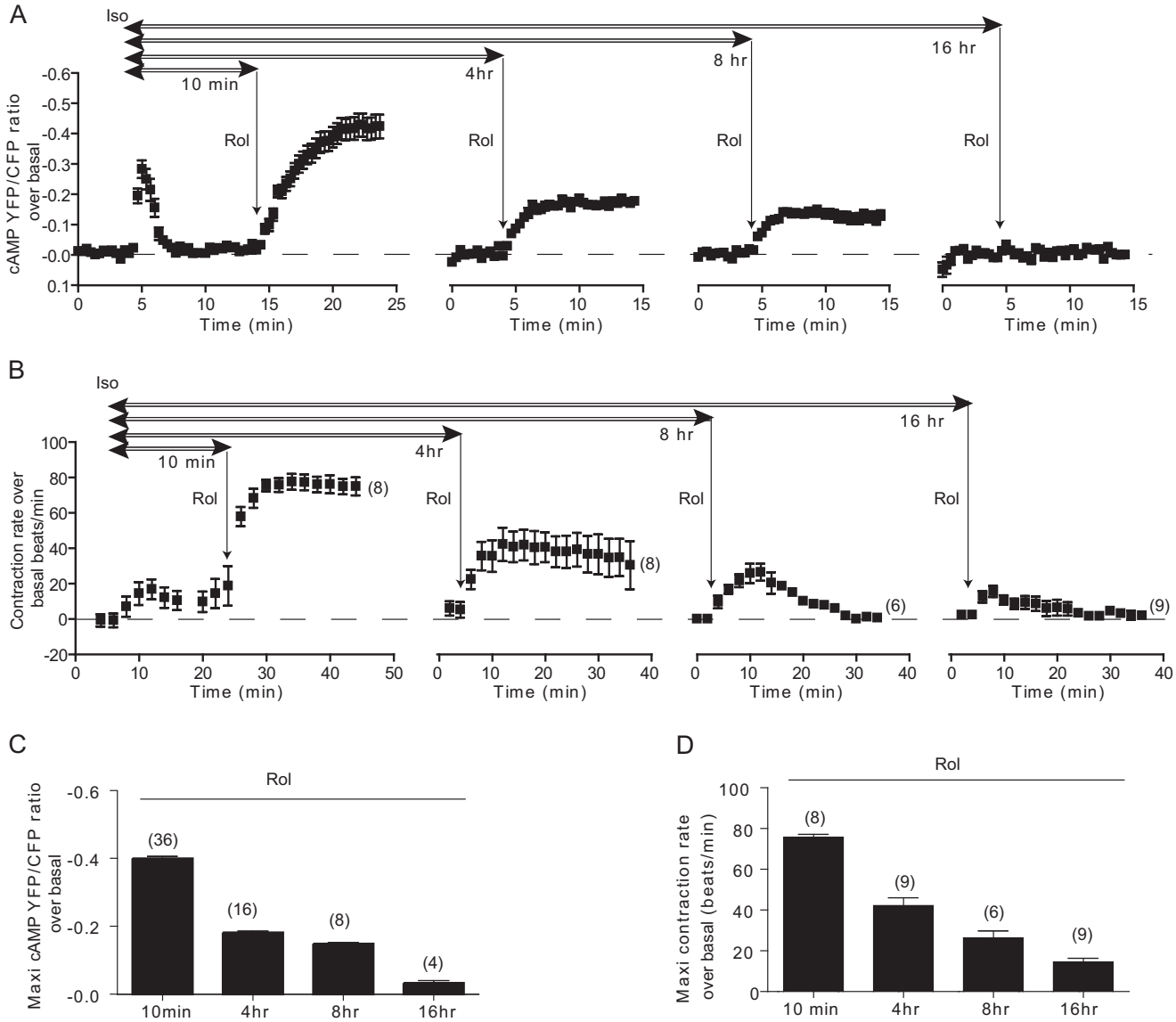

FIGURE 6. **Stimulation of β_1AR promotes a long-acting equilibrium between cAMP synthesis and degradation.** β_2AR-KO cardiac myocytes with (*A*) or without (*B*) expression of the cAMP biosensor ICUE3 were stimulated with isoproterenol (*Iso*) for the indicated periods before addition of the PDE4 inhibitor rolipram (*Rol*, 10 μM). The changes in FRET ratio (*A*) or contraction rate (*B*) were recorded. *CFP*, cyan fluorescent protein. *C* and *D*, the peak levels of the rolipram-induced second responses in FRET ratio (*A*) or contraction rate (*B*) were plotted in *bar graphs*.

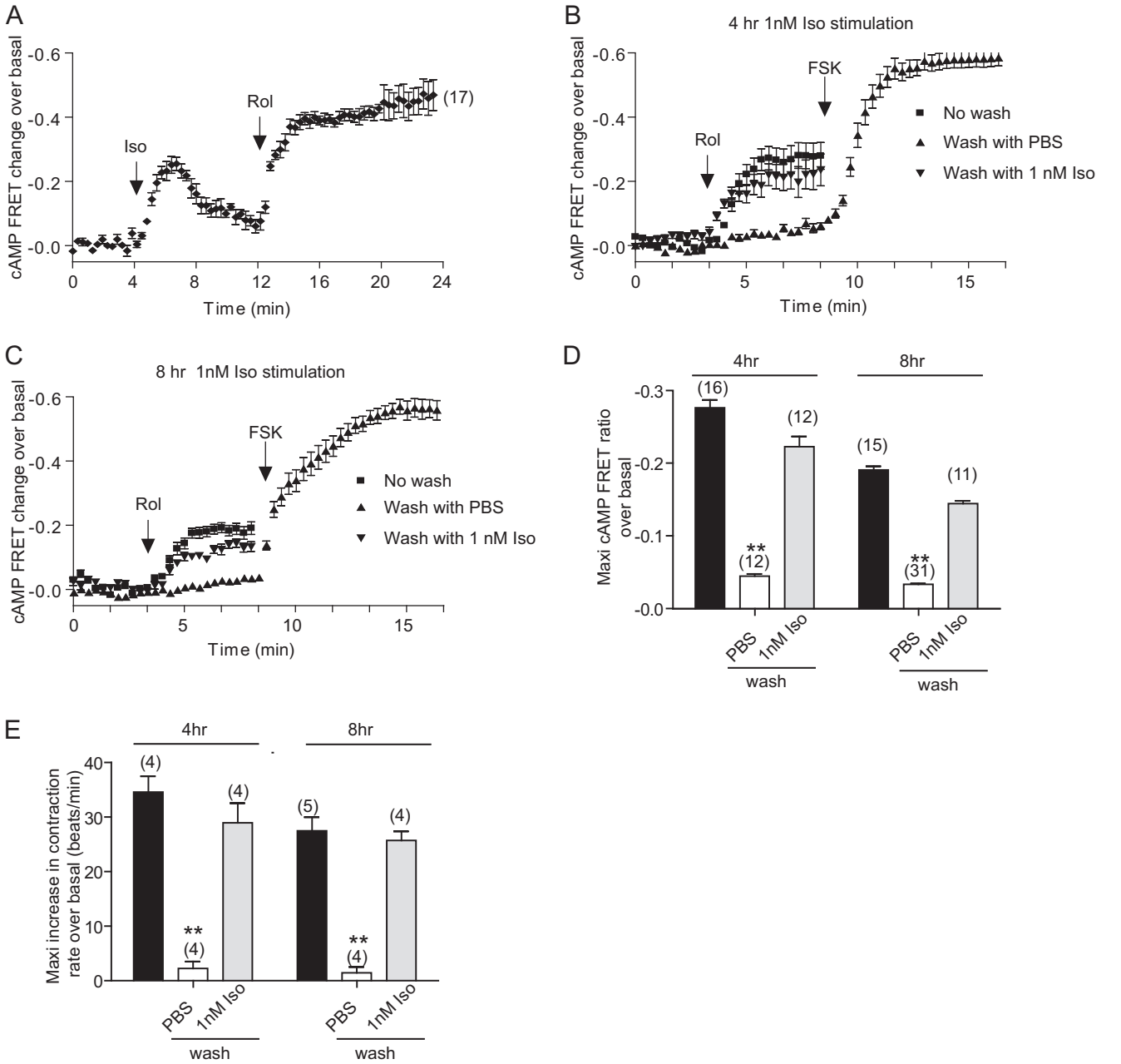

FIGURE 7. **Agonist occupation of βAR is necessary for long acting stimulation of cAMP and contractile response in cardiac myocytes.** *A*, wild-type cardiac myocytes expressing the cAMP biosensor ICUE3 were stimulated with 1 nM isoproterenol (*Iso*) before addition of the PDE4 inhibitor rolipram (*Rol*, 10 μM). The changes in FRET ratio were recorded. *B* and *C*, wild-type cardiac myocytes expressing the cAMP biosensor ICUE3 were stimulated with 1 nM isoproterenol for the indicated periods before addition of the PDE4 inhibitor rolipram (10 μM) and forskolin (*FSK*, 10 μM), as indicated. Alternatively, cells were washed with PBS alone or PBS containing 1 nM isoproterenol 30 min before the addition of rolipram. The changes in FRET ratio were recorded after addition of rolipram or forskolin. *D*, the maximal changes in the FRET ratio in *B* and *C*. *E*, wild-type myocytes were treated with drugs under the same conditions as in *B* and *C*, the changes in myocyte contraction rate after addition of rolipram were recorded, and the maximal changes were plotted. **, $p < 0.01$ in comparison with the unwashed groups by one-way ANOVA.

In a classic paradigm, stimulation of βAR promotes receptor phosphorylation by G protein-coupled receptor kinases in an agonist dose-dependent fashion, which leads to receptor desensitization and internalization to terminate receptor signaling. This paradigm was challenged by recent studies showing that, under saturated agonist stimulation, β_2AR is capable of maintaining an agonist-induced active conformation after internalization and/or recycling (12), which is thought to allow the receptor to send a second wave of signals into cells (23). In comparison with β_2AR, β_1AR displays minimal internalization in cardiac myocytes because of its low-affinity binding to arrestin and its tethering to scaffold proteins (*e.g.* SAP97) at the sarcolemma (11, 24). Even after internalization, SAP97 works together with AKAP150/79 to facilitate β_1AR recycling to the cell surface by PKA-mediated phosphorylation of the receptor at serine 312 (25–27). Together, the processes enable β_1ARs to access available agonists at the extracellular space and signal to G protein. Still, one must assume that either in the media, isoproterenol is more stable than measured previously or that the complex can continue to signal in the absence of ligand but

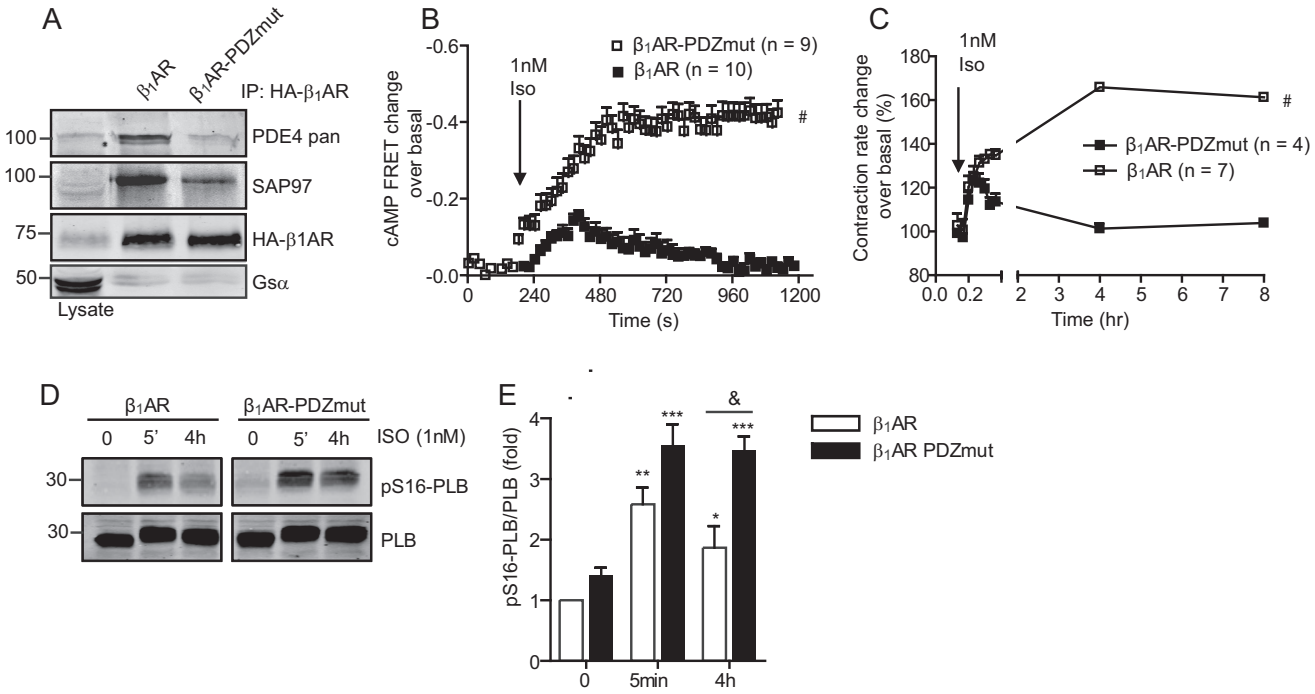

FIGURE 8. **Disruption of the β_1AR-PDE complex promotes sustained cAMP activity, PKA phosphorylation, and myocyte contraction rate response.** *A*, cardiac myocytes from β_1AR-KO were infected with adenoviruses to express either wild-type β_1AR or mutant β_1AR lacking the PDZ-domain binding motif (*PDZmut*). β_1AR was immunoprecipitated (*IP*) with anti-HA antibody. The proteins that were pulled down were detected in a Western blot analysis. *B*, β_1AR-KO cardiac myocytes expressing the cAMP biosensor ICUE3 together with wild-type or mutant β_1AR were stimulated with 1 nM isoproterenol (*Iso*). The changes in cAMP FRET ratio were recorded. *C*, β_1AR-KO cardiac myocytes expressing wild-type or mutant β_1AR were stimulated with 1 nM isoproterenol for up to 8 h. The changes in myocyte contraction rate were recorded. *D*, β_1AR-KO cardiac myocytes expressing wild-type or mutant β_1AR were stimulated with 1 nM isoproterenol for the indicated times. The PKA phosphorylation of phospholamban from cell lysates was examined using anti-phospho-PLB antibody. *E*, the phosphorylation levels in *D* were normalized against the total protein, respectively. Data represent the mean from four to six individual experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ by one-way ANOVA in comparison with the controls; &, < 0.05 by Student's *t* test between paired groups; #, $p < 0.05$ in comparison with the wild-type control by two-way ANOVA.

clearly not with binding of antagonist. The mechanism underlying the observed long-lasting signaling of β_1AR under stimulation with 1 nM isoproterenol is unclear at this point.

Interestingly, this persistent β_1AR signal is not evident because of an effective culling of cAMP by the receptor-associated PDE4D8, which is phosphorylated and activated by PKA and serves as a negative mechanism to keep cAMP levels in check. However, despite the transient cAMP detected by the FRET-based biosensor in living cells, the physiological responses are maintained for long periods of time. This apparent difference is not yet well understood and could be, in part, due to the limited sensitivity of the ICUE3 biosensor (28) and/or the low amount of cAMP in local compartments. We speculate that the initial cAMP spike promotes binding to PKA, which could have a much more stable interaction than appreciated previously (29, 30), and sustained PKA activation to maintain long term physiological response. Alternatively, cAMP produced by AC could supply enough nucleotides to maintain the equilibrium of the binding between cAMP and PKA within local complexes for extended periods. Meanwhile, the increased PDE4D8 activity under PKA phosphorylation could ensure limited or no cAMP to escape from the complexes. The mechanism of whether and how PDE prevents cAMP diffusion is still poorly understood. In one hypothetical scenario, an agonist-activated receptor complex could undergo rearrangement to allow PDE4D8 positioning right toward the catalytic domain of AC to degrade the newly synthesized cAMP and effectively prevent cAMP escaping from the complex. Physiologically, the PDE4D8-maintained local cAMP/PKA activities could lead to activation of hyperpolarization-activated cyclic nucleotide-gated channels (31, 32) and L-type calcium channel (33) within local compartments. Activation of hyperpolarization-activated cyclic nucleotide-gated ion channel is essential for heart rate regulation, whereas activation of L-type calcium channel can promote calcium transient and excitation-contraction coupling in ventricular myocytes to enhance contractility.

The PDE4D8-β_1AR complex is organized by scaffold protein SAP97. SAP97 displays an agonist-induced and G protein-coupled receptor kinase-mediated dissociation from β_1AR (24). Consequently, PDE4D8 also displays agonist dose-dependent dissociation from β_1AR. However, the cAMP-producing enzyme AC does not dissociate from the receptor (19, 20). Therefore, under increasing doses of agonist, the AC-mediated cAMP production is uncoupled from the PDE4D-mediated cAMP degradation in an agonist dose-dependent fashion that allows cAMP equilibrium to shift to higher levels over the baselines (20). As a result, at the minimal concentration of agonist, cAMP detected by the biosensor ICUE3 displays a transient spike after stimulation. Only after a higher concentration of agonist stimulation, PDE dissociates from the receptor complex, permitting cAMP diffusion in the cytoplasm, which displays a sustained increase detected by the biosensor ICUE3 (20). When the PDE activity is inhibited by pharmacological

inhibitor(s) or by disrupting the association with β_1AR, the receptor is able to promote a persistent cAMP signal in cardiac myocytes and prefrontal cortical neurons. Together, our data reveal a persistent β_1AR signal in physiologically relevant myocytes and neurons and an underappreciated role of receptor-associated PDE in maintaining the cAMP equilibrium under prolonged agonist stimulation. These results also argue that the PDE-mediated degradation of the second messenger plays an essential role in classic receptor signaling desensitization.

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