A Long Lasting $\beta_1$ Adrenergic Receptor Stimulation of cAMP/Protein Kinase A (PKA) Signal in Cardiac Myocytes*

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Background: Transient $\beta_1$AR activation remains at odds with long-lasting cellular and physiological responses.

Results: The agonist-occupied $\beta_1$AR 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 $\beta_1$AR induces long-lasting cAMP production in the heart for ligand-induced physiological responses.

Significance: We show a novel mechanism to understand persistent $\beta_1$AR 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 $\beta_1$ adrenergic receptor ($\beta_1$AR) 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 $\beta_1$AR-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. $\beta_1$AR 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 $\beta_1$AR 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 $\beta$-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 $\beta$ARs 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 $\beta$AR signal in traditional biochemical studies in fibroblast cell lines (1–4), physiological responses induced by $\beta$AR activation suggest a persistent receptor signal in native tissues. Activation of cardiac $\beta$ARs 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, βARs undergo minimal internalization in primary cardiac myocytes after agonist stimulation (11). Meanwhile, a recent study indicates that β2AR 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 β1AR is able to continuously signal for more than 8 h in primary cardiac myocytes. We further reveal that scaffold protein SAP97 connects β2AR to the PDE4D8 isoform via the receptor C-terminal PDZ motif. PDE4D8-mediated cAMP hydrolysis shapes the persistent β2AR 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 NaHCO3, 4.7 mM KCl, 1.2 mM KH2PO4, 1.1 mM MgSO4, 11 mM glucose, 2 mM CaCl2, 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 (±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 (SJR-832, 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 β2AR gene (β2AR-KO), the β1AR gene (β1AR-KO), or lacking both the β1 and β2AR genes (β1β2AR-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 and XbaI, and the fusion protein was shuttled with HindIII and XbaI into the viral vector for recombinant viruses. Adenovirus-expressing HA-tagged β1AR 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**—β1AR-KO myocytes expressing PDE4D isoforms, HA-tagged β1AR, 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-β1AR antibody (SCBT). For time courses of PKA phosphorylation of PLB, β1AR-KO myocytes expressing HA-tagged β1AR 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-β1AR antibody (SCBT), total and phosphorylated PLB at serine 16 and threonine 17 (Badrilla), anti-phospho-PDE4D antibody (Fabgenix), 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 Axiosvert 200 m microscope with a Xenon/1.3 oil immersion lens.
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 TRTRLRFSKSYS30) or the N-terminal-specific 22 amino acids of PDE4D9 (MSIIMKPRSRSTSSLRTEAVCA22) 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 contraction rate (Fig. 1A). 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. 1E). However, this increase in PKA phosphorylation of PLB was absent in the hearts when isoproterenol was washed away before the addition of rolipram (Fig. 1E).

Among cardiac βARs, β1AR is the primary subtype responsible for contractility. Therefore, we used primary cardiac myocytes lacking the β2AR gene (β2AR-KO) to analyze the β1AR-induced cAMP-PKA signal in the hearts. The cAMP 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. 2A). These data suggest that β1 ARs 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. 2B). Meanwhile, we observed similar responses in primary β2AR-KO prefrontal cortical neurons (Fig. 2C), suggesting a conserved mechanism on persistent β1AR 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. 2F). 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. 3A). However, the βAR antagonist alprenolol blocked the rolipram-induced secondary increases in contraction rate (Fig. 3B). 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 β1ARs continuously signal through GS and AC for cAMP synthesis, which is also dependent on agonist occupation of the receptors.

We hypothesized that β1AR forms a stable complex with both the cAMP-stimulatory components GS 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 β1AR together with the scaffold protein SAP97 and AKAP150 in mouse hearts (Fig. 4A). In addition, we pulled down the positive stimulatory components GS and PKA, the negative regulatory components PDE4D and PP2A, as well as scaffold proteins SAP97 and AKAP150 together with FLAG-β1AR expressed in cardiac myocytes (Fig. 4B). This complex was stable under stimulation with 1 nm isoproterenol (Fig. 4B). 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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A

![Graph showing changes in developed pressure with ISO and Rol](image)

B

![Graphs showing developed pressure, Max dp/dt, and Min dp/dt with Iso and Rol](image)

C

![Graphs showing developed pressure, Max dp/dt, and Min dp/dt with Iso, Wash, and Rol](image)

D

![Bars showing developed pressure, Max dp/dt, and Min dp/dt with wash](image)

E

![Blot showing protein expression of pS16-PLB, pT17-PLB, PLB with Con, Iso, Iso + Rol, Iso + Wash + Rol](image)
under stimulation with 1 μM isoproterenol (Fig. 4C), which can enhance enzymatic activity for cAMP degradation (21). Among PDE4D isoforms, we identified that only the PDE4D8 isoform displayed a significant association with β₁AR 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 β₁AR-KO myocytes enhanced contraction rate response induced by 1 μM isoproterenol (Fig. 4F). 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 μM isoproterenol in β₁AR-KO myocytes (Fig. 4, G and H). Consequently, overexpression of catalytically inactive PDE4D8-D498A enhanced the myocyte contraction rate increases induced by 1 μM isoproterenol, whereas wild-type PDE4D8 did not (Fig. 4G). Further analysis revealed that scaffold protein SAP97 is critical to connect PDE4D8 to β₁AR. Knockdown of SAP97 with shRNA disrupted the association between β₁AR and PDE4D8 (Fig. 5A) and enhanced both the magnitude and duration of cAMP FRET response in wild-type cardiac myocytes induced by 1 μM isoproterenol (Fig. 5B). These data indicate a critical role of PDE4D8 association with β₁AR for tuning cAMP amplitude and duration after isoproterenol stimulation.

To assess the duration of the β₁AR-induced cAMP signal, we added rolipram at different durations after the first transient cAMP FRET response induced by 1 μM isoproterenol in β₁AR-KO myocytes. Strikingly, the rolipram-induced second cAMP FRET response was evident after 8 h of stimulation with a decay t₁/₂ 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 t₁/₂ of 3.4 h (Fig. 6, B and D). Meanwhile, we also examined whether we could detect similar response in wild-type myocytes. 1 μM 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. 7A). After 4 or 8 h of stimulation with 1 μM 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.](image)

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 μM isoproterenol (ISO) for 20 min, followed by addition of the PDE4 inhibitor rolipram (Rol, 10 μM). C, hearts were perfused with 1 μM 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 μM). 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 2. PDE4 masks the cAMP signal induced by β₁AR in primary cardiac myocytes and prefrontal cortical neurons under stimulation with isoproterenol.](image)

Cardiac myocytes or prefrontal cortical neurons from β₁AR-KO mice expressing the cAMP biosensor ICUE were stimulated with 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 μM 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 μM 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' diodeoxyadenosine triphosphate (2',5' DDA, 100 μM) (F) before addition of the PDE4 inhibitor rolipram (10 μM). The changes in FRET ratio were recorded.

![FIGURE 3. Knockdown of SAP97 with shRNA disrupted the association between β₁AR and PDE4D8.](image)
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. 7E). 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 β1AR-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 β1AR in cardiac myocytes, we introduced a mutation to the β1AR C-terminal PDZ motif that selectively disrupted the receptor binding to SAP97 and PDE4D8 but did not affect the receptor association with Gs (Fig. 8A). When introduced into β1AR-KO cardiac myocytes, the mutant β1AR induced much stronger and sustained cAMP FRET responses than the wild-type receptor (Fig. 8B) and promoted sustained increases in contraction rate during 8-hour stimulation (Fig. 8C). In contrast, wild-type β1AR induced transient increases in contraction rate that returned to baseline level. Accordingly, stimulation of wild-type β1AR induced transient PKA phosphorylation of PLB in cardiac myocytes, whereas the mutant β1AR 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 β1ARs continuously stimulated AC to produce cAMP in primary cardiac myocytes and prefrontal cortical neurons (Fig. 2, B
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FIGURE 4. β₁AR forms a macrocomplex with both Gₛ 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-β₁AR antibody. The precipitates were examined in Western blot analyses with the indicated antibodies. IP, immunoprecipitation. B, β₁-AR-KO cardiac myocytes expressing HA-β₁AR antibody were stimulated with 1 nM isoproterenol (ISO). Cell lysates were subjected to immunoprecipitation with anti-HA antibody. The precipitates 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, β₁-AR-ko cardiac myocytes expressing HA-β₁AR antibody were stimulated with the PDE4D isoforms. Cell lysates were subjected to immunoprecipitation with anti-HA antibody. The proteins that were pulled down were detected in Western blot analyses.

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 t½ 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 β1AR 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-β1AR. Cell lysates were subjected to immunoprecipitation (IP) with the indicated antibody. The β1AR and PDE4D8 that were pulled down were detected in Western blot analyses (IB). B, β1AR-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 β1AR promotes a long-acting equilibrium between cAMP synthesis and degradation.** β1AR-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, 1 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.
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, β2AR 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 β2AR, β1AR 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 β1AR recycling to the cell surface by PKA-mediated phosphorylation of the receptor at serine 312 (25–27). Together, the processes enable β1ARs 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...
clearly not with binding of antagonist. The mechanism underlying the observed long-lasting signaling of β1AR under stimulation with 1 nM isoproterenol is unclear at this point.

Interestingly, this persistent β1AR 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). When the PDE activity is inhibited by pharmacological

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inhibitor(s) or by disrupting the association with β₁AR, the receptor is able to promote a persistent cAMP signal in cardiac myocytes and prefrontal cortical neurons. Together, our data reveal a persistent β₁AR 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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