

Phosphodiesterases coordinate cAMP propagation induced by two stimulatory G protein-coupled receptors in hearts

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Inflammation is a significant player in the progression of heart failure and has detrimental effects on cardiac function. Prostaglandin (PGE)₂, a major proinflammatory prostanoid in the cardiovascular system, is a potent stimulus in inducing intracellular cAMP but minimally affects cardiac contractile function. Here, we show that the PGE₂ stimulation attenuates the adrenergic-induced cardiac contractile response in animal hearts. Stimulation with PGE₂ leads to stimulatory G protein (G_s)-dependent production of cAMP. However, the induced cAMP is spatially restricted because of its degradation by phosphodiesterase (PDE)4 and cannot access the intracellular sarcoplasmic reticulum (SR) for increasing calcium signaling and myocyte contraction. Moreover, pretreatment with PGE₂ significantly inhibits PKA activities at the SR induced by a β -adrenergic agonist, isoproterenol, and subsequently blocks isoproterenol-induced PKA phosphorylation of phospholamban and contractile responses in myocytes. Further analysis reveals that the PGE₂-induced cAMP/PKA is sufficient to phosphorylate and activate PDE4D isoforms, which, in turn, spatially inhibits the diffusion of adrenergic-induced cAMP from the plasma membrane to the SR. Inhibition of PDE4 rescues the adrenergic-induced increase in cAMP/PKA activities at the SR, PKA phosphorylation of phospholamban, and contractile responses in PGE₂-pretreated myocytes. Thus, this offers an example that one G_s-coupled receptor is able to inhibit the intracellular signaling transduction initiated by another G_s-coupled receptor via controlling the diffusion of cAMP, presenting a paradigm for G protein-coupled receptor (GPCR) signal transduction. It also provides a mechanism for the integration of signaling initiated by different neurohormonal stimuli, as well as long-term effects of chronically circulating proinflammatory factors in myocardium.

FRET | subcellular diffusion | cross-talk

Inflammation plays a significant role in the progression of heart failure (1, 2). The expression of prostaglandin (PGE)₂, a major proinflammatory prostanoid in cardiovascular system, is elevated in the hearts of patients and animals with myocardial infarction and heart failure (3, 4). Clinically, heart failure is characterized with gradual development of cardiac hypertrophy and myocyte death attributable to elevated β -adrenergic stimulation and cardiac dysfunction (5, 6). However, cardiac function under E series prostaglandin (EP) receptor signaling is not clear, and the impact on cardiac β -adrenergic signaling and function during the development of heart diseases remains to be addressed.

In human and animal hearts, PGE₂ and catecholamines activate divergent signaling cascades for distinct cellular and physiological function. EP₄ is the most abundantly expressed EP subtype in the myocardium (7). Biochemically, activation of EP₄ leads to an accumulation of cAMP through activation of stimulatory G (G_s) protein. However, the induced cAMP signal appears to be confined along the plasma membrane (PM) and has limited effect on myocyte contractile response (7). This confinement of cAMP is primarily regulated through cAMP

hydrolysis by PDE4 isoforms (8, 9). Consequently, PGE₂ stimulation fails to promote phosphorylation of proteins such as phospholamban (PLB), a critical player on the intracellular calcium storage compartment sarcoplasmic reticulum (SR) that releases calcium for contractile response. Catecholamines stimulate β adrenergic receptors (β ARs) to induce G_s protein activation and cAMP production. In contrast to PGE₂ stimulation, the adrenergic-induced cAMP signaling leads to robust PKA phosphorylation of substrates on the PM, the SR, and the myofibril, and promotes cardiomyocyte contractility (10, 11). Previous studies have also characterized the association of different PDE4D isoforms with β AR subtypes, which regulate subcellular distribution of the adrenergic signaling-induced cAMP activities (12, 13). Under agonist stimulation, these PDE4D isoforms dissociate from the activated β ARs to allow cAMP diffusion from the PM to the intracellular compartments (12, 13). Interestingly, early studies have indicated that PGE₂ treatment impairs the sympathetic regulation of cardiac contractile function in animal hearts (14). It is, thus, likely that the signaling induced by PGE₂ and β -adrenergic stimuli are integrated in the myocardium. We hypothesize that PGE₂ exerts inhibitory effects on β AR signaling cascades in the myocardium via a cross-talk by activating the β AR-associated PDE4D isoforms.

Here, we show that the PGE₂-induced cAMP signal is confined within the PM domain and has minimal effects on PKA phosphorylation of PLB at the SR and contractile function in animal hearts. However, PGE₂ stimulation inhibits intracellular β AR signaling transduction by blocking propagation of cAMP from the PM to the SR, which blocks PKA phosphorylation of PLB and inhibits calcium cycling regulation in myocytes. Further dissection reveals that PGE₂ stimulation induces PKA phosphorylation of PDE4D isoforms, which prevents the β AR-induced cAMP diffusion to the SR. Consequently, the PGE₂ stimulation blocks the β AR-induced contractile responses in cardiomyocytes and in animal hearts. The elegant cross-talk between two G_s-coupled receptors in shaping subcellular distribution of second messenger cAMP presents a paradigm on G protein-coupled receptor (GPCR) signaling transduction for integration of multiple extracellular stimuli.

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Results

Prostaglandin Impairs β -Adrenergic Stimulation on Contractile Responses in Animal Hearts. Proinflammatory factor PGE₂ is a potent stimulus in inducing intracellular cAMP signaling but fails to promote cardiac contractile response (7). We envisioned that prostaglandin-induced PKA phosphorylation and activation of phosphodiesterase could impair signaling transduction induced by β -adrenergic stimulation in the myocardium and block adrenergic-induced contractile responses, offering a mechanism on how inflammation contributes to cardiac dysfunction. Using Langendorff perfusion of mouse hearts, we first tested different doses of PGE₂ on cardiac contractility and found that PGE₂ exerted a positive inotropic effect on the heart at minimal concentration of 1 μ M (Fig. 1A). In comparison, β -adrenergic stimulation with isoproterenol (ISO) induced a sensitive dose-dependent contractile response (Fig. 1B) with EC₅₀ at 1.99 nM and maximal response at the concentrations >10 nM. ISO was able to increase peak left ventricular developed pressure (LVDP) from 71.4 ± 13.0 to 115.3 ± 12.0 mmHg (61.8%; EC₅₀, -8.708), maximal dp/dt from $2,343.7 \pm 159.7$ to $5,984.4 \pm 622.8$ mmHg (150.6%; EC₅₀, -8.675), and minimum -dp/dt from $-1,787.8 \pm 325.2$ to $3,492.2 \pm 142.03$ mmHg/s (95.4%; EC₅₀, -8.729). In contrast, when the heart was pretreated with 0.1 μ M PGE₂, a concentration that was noneffective on cardiac function by itself, the ISO dose-response curve was shifted rightward, and the EC₅₀ of ISO was changed to ~199 nM, 100 times higher than the EC₅₀ of ISO alone. Furthermore, the maximal responses of LVDP (from 63.8 ± 3.9 to 81.5 ± 10.9 mmHg; 27.8%; EC₅₀, -6.891), dp/dt (from $2,229.5 \pm 54.3$ to $4,009.6 \pm 504.3$ mmHg/s; 77.1%; EC₅₀, -6.78) and -dp/dt (from $-1,840.8 \pm 179.0$ to $-3,127.7 \pm 750.9$ mmHg; 70.0%; EC₅₀, -6.577) were all substantially decreased. These data suggest that PGE₂ pretreatment blunts β -adrenergic responsiveness of cardiomyocytes.

To validate the contractile response, we examined phosphorylation level of a PKA substrate PLB. PLB is located at the SR

and plays a critical role in calcium cycling by regulating SERCA activity. Adrenergic stimulation, but not prostaglandin stimulation, significantly promotes both PKA and Ca²⁺/calmodulin-dependent protein kinase (CaMK)II phosphorylation of PLB in animal hearts. Pretreatment with 0.1 μ M PGE₂ abolished phosphorylation of PLB induced by ISO (Fig. 1C). These data suggest that although PGE₂ stimulation fails to induce cAMP and PKA activities at the SR, it inhibits the adrenergic-induced PKA activities at the SR for cardiac contractile response in animal hearts.

Prostaglandin and Adrenergic Stimulation Induces Distinct Subcellular cAMP-PKA Activities in Cardiomyocytes. We sought to directly measure PKA activities in different intracellular compartments with biosensors under the signaling cross-talk between EP and β AR in cardiomyocytes. To achieve this, we used the recently developed PKA biosensors that are targeted to the PM, the cytoplasm, and the SR (PM-AKAR3, cyto-AKAR3, and SR-AKAR3, respectively) (Fig. 2A and Fig. S1) (15, 16). In neonatal cardiomyocytes, direct stimulation of adenylyl cyclases with forskolin (FSK) induced strong PKA activities at the PM, less robust PKA activities in the cytoplasm, and much lower PKA activities at the SR (Fig. 2B-D). The pattern follows a diffusion of cAMP from the PM where cAMP is produced. In comparison, stimulation with ISO or PGE₂ induced lower increases in PKA activities at the PM than FSK (Fig. 2B-D). Interestingly, under ISO or PGE₂ stimulation, the PKA activities in the cytoplasm were much more robust than those at the PM, respectively (Fig. 2A-C). Furthermore, adrenergic stimulation induced significantly higher PKA activities at the SR than FSK stimulation, whereas PGE₂ failed to generate significant increase (Fig. 2D and E). The data indicate that the adrenergic-induced cAMP preferentially activates PKA at the intracellular compartments like the SR for myocyte contractile response, whereas PGE₂ stimulation completely fails to do so. In addition, dopamine induced modest PKA activities at the SR, whereas adenosine failed

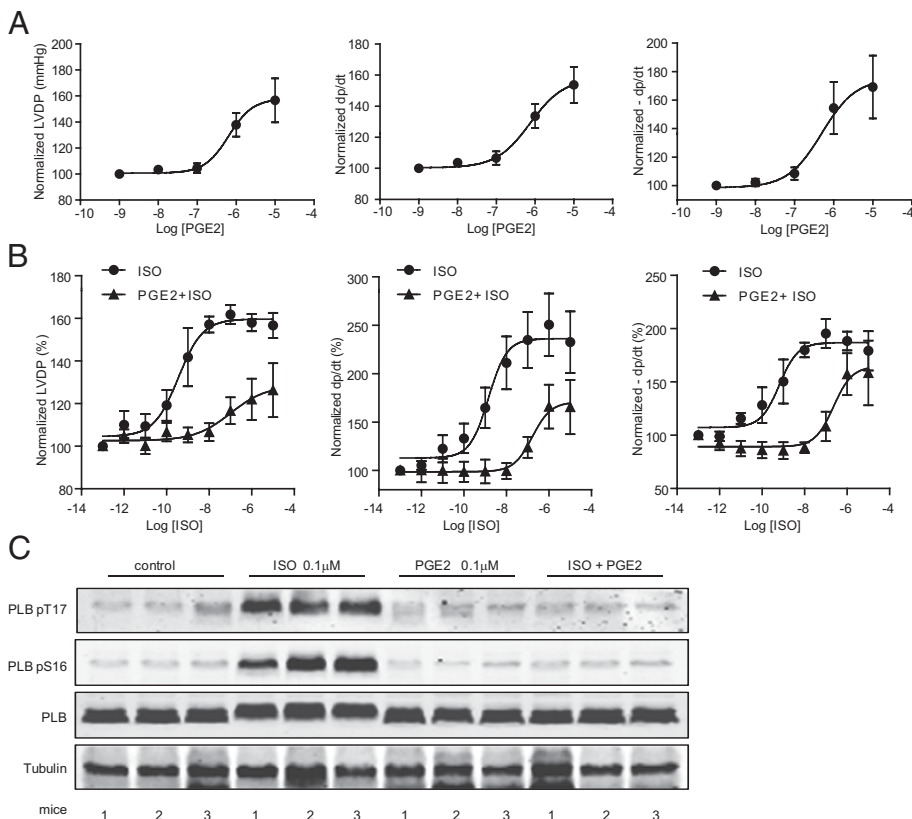


Fig. 1. PGE₂ inhibits adrenergic-induced phosphorylation of PLB and contractile response in animal hearts. Mouse hearts were cannulated for Langendorff perfusion with prostaglandin or ISO or with prostaglandin for 5 min before addition of ISO at concentrations indicated. The LVDP, maximal dp/dt, and minimal -dp/dt were analyzed and normalized against basal levels. (A) Cardiac functional responses to different concentrations of PGE₂. (B) Cardiac functional responses to different concentrations of ISO after PGE₂ (0.1 μ M) pretreatment. (C) Phosphorylation of PLB at the Ser16 site (PKA site) and Thr17 site (CaMKII site) after perfusion of the hearts with drugs indicated.

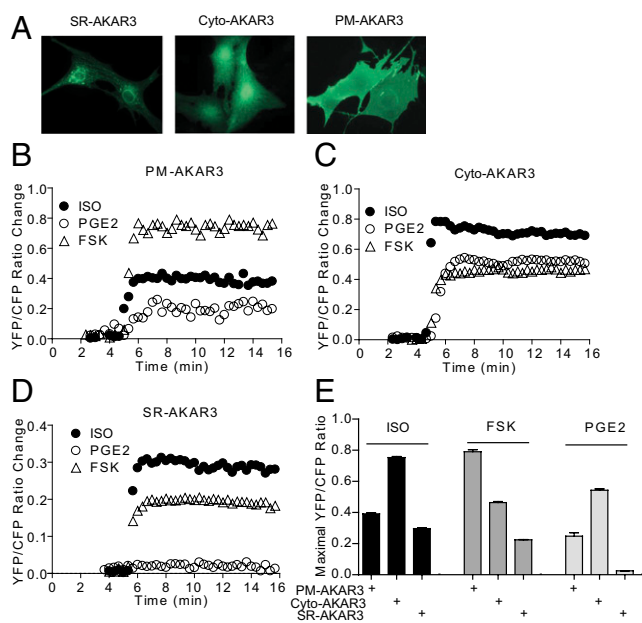


Fig. 2. PGE2 and ISO induce distinct subcellular cAMP signaling in cardiomyocytes. Neonatal cardiomyocytes were infected with PKA biosensors targeted to different subcellular organelles. The expression of biosensors was detected by immunofluorescence staining (A). (B–D) Neonatal cardiomyocytes expressing different PKA biosensors were stimulated with PGE2 (1 μ M), ISO (10 μ M), or FSK (10 μ M), and the changes in FRET ratio of the PKA biosensors were recorded. (E) The maximal increases in the FRET ratio of PKA biosensors were plotted.

to do so (Fig. S1 C and D). Together, different GPCRs have distinct effects in inducing local PKA activities at the SR in cardiomyocytes.

Prostaglandin Stimulation Confines Adrenergic Signaling in Cardiomyocytes. On GPCR stimulation, the small molecule cAMP is diffusible throughout the cell. However, the distribution of cAMP under specific GPCR stimuli is rather highly restricted because of hydrolysis of cAMP by a family of potent PDE enzymes. As a result, a specific set of PKA is activated for phosphorylation of substrates at the local vicinities. In rodent cardiomyocytes, PDE3 and PDE4 are two major PDE families expressed (17) to control cAMP distribution and diffusion in subcellular compartments. PDE3 and PDE4 can be selectively inhibited by cilostamide (CILO) and rolipram (ROL), respectively (Fig. S2A). PDE4, predominately located on the membrane (Fig. S2B), plays a major role in controlling cAMP activities induced by adrenergic stimulation in cardiomyocytes (12, 13). We hypothesized that the PGE2 inhibition on adrenergic signaling is mediated via activation of PDE4, which blocks cAMP diffusion and attenuates cardiac contractile response under adrenergic stimulation. Indeed, whereas PGE2 did not induce significant increase in PKA activities at the SR, pretreatment of myocytes with PGE2 blocked the ISO-induced PKA activities at the SR, but not the FSK-induced response (Fig. 3 A–C). Inhibition of PDE4 with ROL modestly enhanced the PGE2-induced PKA activities at the SR but significantly recovered the adrenergic-induced PKA activities at the SR in the presence of PGE2 pretreatment (Fig. 3 D and F). In contrast, inhibition of PDE3 with CILO did not significantly affect the PKA activities induced by PGE2, nor did it affect the PKA activities induced by ISO after PGE2 pretreatment (Fig. 3 E and F). As control, inhibition of PDE4 with ROL promoted PGE2-induced PKA activities at the SR and the PM, respectively (Fig. S3A and B) but did not affect the activities in the cytoplasm (Fig. S3B).

We also measured cAMP activities directly using a cAMP biosensor targeted at the SR (SR-ICUE3). Pretreatment of myocytes

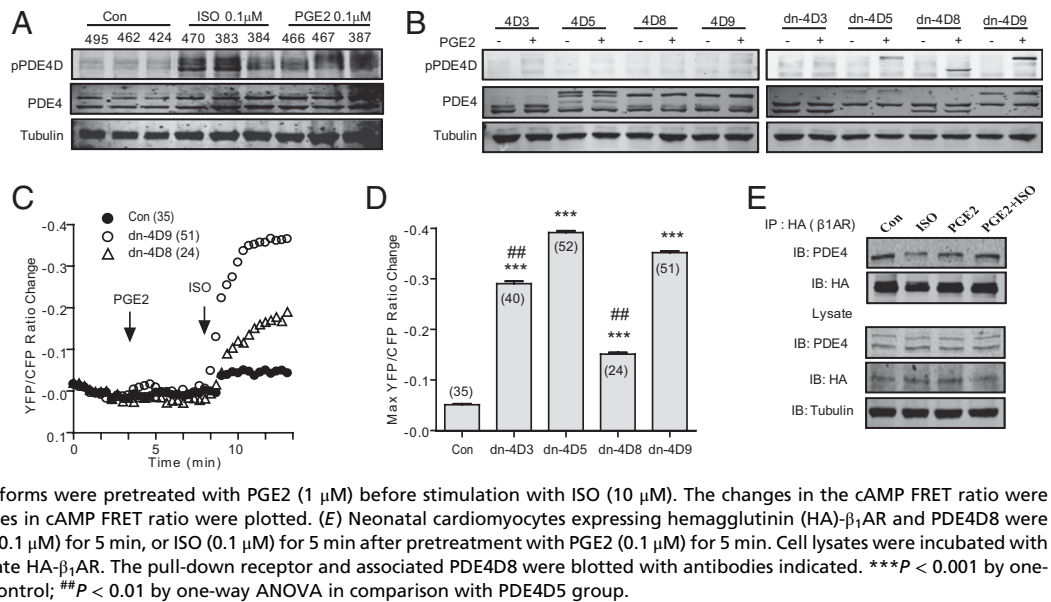
with PGE2 completely blocked the adrenergic stimulation-induced cAMP accumulation at the SR but not the FSK-induced response (Fig. 3G). Moreover, inhibition of PDE4 with ROL, but not PDE3 with CILO, recovered the adrenergic-stimulated cAMP at the SR (Fig. 3 H–J). These data indicate that PGE2 activates PDE4 enzymes to prevent diffusion of the adrenergic stimulation-induced cAMP to the intracellular SR.

We then determined which β AR and EP subtypes are involved in the cross-talk. EP3 and EP4 are major subtypes expressed in rodent cardiomyocytes (7). Pretreatment with EP3 selective antagonist L-798106 or EP4 antagonist L-161982 did not affect the basal PKA activities at the SR in cardiomyocytes, nor did it affect the PGE2-induced PKA activities at the SR (Fig. S4A). However, EP4 antagonist L-161982 alone or together with EP3 antagonist L-798106 completely blocked the inhibitory effects of PGE2 on ISO-induced PKA activities at the SR (Fig. S4A). Meanwhile, in β_2 AR-KO myocytes, pretreatment with PGE2 did not affect ISO-induced PKA activities at the SR (Fig. S4B); in contrast, in β_1 AR-KO myocytes, pretreatment with PGE2 inhibited ISO-induced PKA activities, which was rescued by inhibition of PDE4 with ROL or by inhibition of PDE3 with CILO (Fig. S4B). Moreover, EP4 did not associate with either β_1 AR or β_2 AR when expressed in HEK293 cells, suggesting that the cross-talk occurs via downstream intracellular mechanisms (Fig. S5).

To explore whether the EP inhibition of adrenergic signaling is a general mechanism, we measured the cAMP activities in the nucleus in mouse embryonic fibroblasts under different stimulations. ISO and FSK induced robust cAMP activities in the nucleus in mouse embryonic fibroblasts (MEFs), whereas PGE2 failed to do so (Fig. S6 A–C). Pretreatment with PGE2 significantly reduced the ISO-induced cAMP signal in the nucleus (Fig. S6C). Inhibition of PDE4 with ROL, but not inhibition of PDE3 with CILO, abolished the inhibitory effect of PGE2 on the adrenergic-induced cAMP signal in the nucleus of MEFs (Fig. S6 D–F). Meanwhile, dopamine, urocortin, and relaxin all activate Gs-coupled receptors, but none of them induced cAMP activities in the nucleus in MEFs (Fig. S7 A–E). Pretreatment with dopamine almost completely inhibited the ISO-induced cAMP activities in the nucleus (Fig. S7 B and F). Urocortin partially inhibited the cAMP activities, whereas relaxin failed to do so (Fig. S7 D and E). In contrast, none of these agonists inhibited the cAMP activities in the nucleus induced by FSK (Fig. S7F). Together, these data indicate that the EP inhibition of adrenergic signaling is a general mechanism selectively shared by some Gs-coupled GPCRs.

Prostaglandin Activates β AR-Associated PDE4D to Restrict cAMP Signaling Under Adrenergic Stimulation. The members of PDE4 family can be activated via PKA phosphorylation (18). We examined the PKA phosphorylation of PDE4 isoforms in myocardium after Langendorff perfusion with drugs. Perfusion of mouse hearts with either PGE2 or ISO significantly increases in PKA phosphorylation of PDE4 isoforms in myocardium (Fig. 4A). Among the PDE4 family, we focused on PDE4D subfamily because its members are associated with adrenergic signaling in cardiomyocytes (12, 13). When wild-type PDE4D isoforms were overexpressed in H9C2 cardiac myoblasts, PGE2 failed to increase PKA phosphorylation of the isoforms (Fig. 4B), in agreement with the fact that overexpressed PDE4Ds suppress adrenergic-induced cAMP signal (19). However, PGE2 increased PKA phosphorylation of the dominant-negative forms of PDE4D5 and PDE4D9, and to a lesser extent, PDE4D8 and PDE4D3 (Fig. 4B). Accordingly, overexpression of dominant-negative PDE4D5 and PDE4D9 in neonatal cardiomyocytes fully rescued the adrenergic-induced cAMP signal at the SR after pretreatment with PGE2, and dominant-negative PDE4D3 and PDE4D8 showed partial rescue effects (Fig. 4 C and D). Stimulation with ISO promotes dissociation of PDE4D isoforms from the activated β_1 AR or β_2 AR, respectively (Fig. 4E and Fig. S8A) (12, 13). Whereas stimulation with PGE2 did not affect the β AR/PDE4D complexes, it completely blocked the dissociation of the β AR/

Fig. 4. PGE2 induces PKA phosphorylation of phosphodiesterase 4D isoforms to attenuate cAMP signaling. (A) Animal hearts undergoing Langendorff perfusion with PGE2 (0.1 μ M) or ISO (0.1 μ M) were harvested. The tissues lysates were blotted with antibody against the PKA phosphorylated PDE4D. (B) H9C2 cardiac myoblasts overexpressing different wild-type or dominant-negative (dn) PDE4D isoforms were stimulated with PGE2 before being lysated. The cell lysates were blotted with antibody against the PKA phosphorylated PDE4D. (C) Neonatal cardiomyocytes expressing the SR-targeted cAMP biosensors together with different dominant-negative PDE4D isoforms were pretreated with PGE2 (1 μ M) before stimulation with ISO (10 μ M). The changes in the cAMP FRET ratio were recorded. (D) The maximal increases in cAMP FRET ratio were plotted. (E) Neonatal cardiomyocytes expressing hemagglutinin (HA)- β_1 AR and PDE4D8 were treated with PGE2 (0.1 μ M) or ISO (0.1 μ M) for 5 min, or ISO (0.1 μ M) for 5 min after pretreatment with PGE2 (0.1 μ M) for 5 min. Cell lysates were incubated with anti-HA beads to immunoprecipitate HA- β_1 AR. The pull-down receptor and associated PDE4D8 were blotted with antibodies indicated. *** P < 0.001 by one-way ANOVA in comparison with control; ## P < 0.01 by one-way ANOVA in comparison with PDE4D5 group.



by two Gs-coupled receptors presents a paradigm in GPCR signaling.

EPs and β ARs represent two distinct families of GPCRs that are coexpressed in many cells such as cardiomyocytes, neurons, and astrocytes. Despite the activation of both receptors transduces extracellular stimuli to the cAMP and PKA signaling pathway, these stimuli can lead to divergent and sometimes even opposing cellular processes in cells. As proinflammatory factors, the prostaglandin-induced cAMP and PKA activities play important roles in diversified inflammatory responses in fibroblasts in the hearts (20) and in microglia cells in the brain (21) but have limited roles in promoting muscle contraction (7) and possibly neuron excitation (21). In contrast, the β AR-induced cAMP has a broad reach into different intracellular compartments. Consequently, activation of PKA leads to a wide range of responses, including muscle contraction and relaxation and neuron excitation. Traditionally, it is thought that these two signaling machineries are physically segregated via enrichment in different lipid raft microdomains on the PM (22), through which the cAMP is confined at distinct subcellular locations by PDE4 enzymes (8, 9). Thus, the stimulated signaling exerts different cellular responses via targeting selective sets of substrates.

However, it is also well known that EPs and β ARs act reciprocally to blunt the function of one another in vivo. In the myocardium, early studies show that PGEs have a negative effect on inotropy under sympathetic regulation (14). In microglia cells, EP signaling promotes activation of glia cells, whereas β AR activation inhibits the inflammatory response. These studies suggest that these two signaling systems converge and interact intracellularly. Here, we show that PGE2 stimulation of EP4 induces cAMP, which activates PKA to phosphorylate PDE4 isoforms (Fig. 4). This phosphorylation enhances the activities of PDE4 isoforms (18) that not only play a role in attenuating the EP signaling (9) but also blocks the diffusion of cAMP induced by β AR to the intracellular SR and nucleus. The observation is also consistent with the functional association between these PDE4 isoforms and β AR subtypes (12, 13); however, PGE2 stimulation also blocks the dissociation of PDE4 isoforms from both β AR subtypes (Fig. 4E and Fig. S8A). As a result, PGE2 treatment attenuates the adrenergic-induced contractile response in myocytes, as well as in animal hearts. The elegant and elaborate cross-talk between these two Gs-coupled receptors in shaping subcellular distribution of second messenger, which is shared by some but not all Gs-coupled receptors. This study provides a mechanism for understanding coordination and integration of extracellular stimuli for diversified

cellular functions under stress. It also offers an aspect in understanding long-term effect of chronically circulating proinflammatory factors in myocardium.

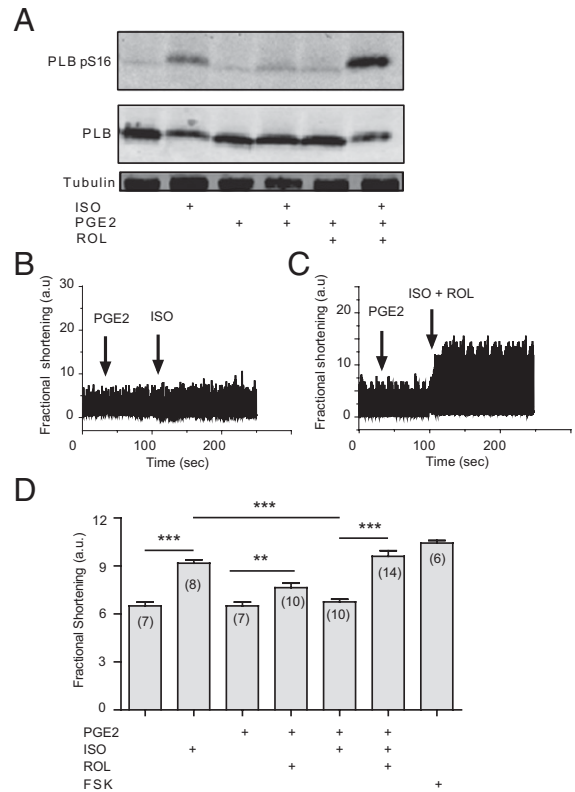


Fig. 5. Inhibition of PDE4 abolishes the inhibitory effect of PGE2 on the adrenergic signaling for myocyte contractile response. Adult cardiomyocyte were stimulated with different PGE2 (1 μ M), ISO (10 μ M), FSK (10 μ M), or ROL (1 μ M) as indicated. The phosphorylation of PDE4D and PLB at the PKA site were blotted with anti-phospho PLB antibody (A), the increases in ionotropic contractile response were recorded (B and C), and the maximal increases were plotted (D). ** P < 0.05 and *** P < 0.001 by one-way ANOVA.

Materials and Methods

Adenovirus Construction. Adenoviruses expressing individual wild-type and dominant-negative forms of PDE4D-mcherry that lack catalytic activity because of point mutation (e.g., D556A in PDE4D5 isoform) were described previously (13). The regular cytoplasmic, PM- and SR-specific PKA activity biosensors cyto-AKAR3, PM-AKAR3, and SR-AKAR3 were published previously (16, 23). The SR-specific cAMP biosensor SR-ICUE3 was generated from ICUE3 with the same strategy used for SR-AKAR3 (16), and the nuclear-specific cAMP biosensor NLS-ICUE3 was generated with the same strategy used for NLS-AKAR3 (16, 23). These reporters were subcloned into the shuttle vector of the AdEasy system for virus production according to the instructions of the manufacturer (MP Biomedical).

Langendorff Perfusion Heart Preparation. Animal experiments were performed following the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*, and all procedures were approved by the Institutional Animal Care and Use Committee at the University of Illinois at Urbana and Temple University. The isolated heart perfusion technique was described previously (24). See *SI Materials and Methods* for more details.

Cardiomyocyte Culture and Adenoviral Infection. Ventricular neonatal and adult cardiomyocytes were isolated from wild-type FVB animals and cultured as described previously (25). For expressing the PKA or cAMP activity biosensors, neonatal myocytes were infected with adenoviruses at the multiplicity of infection (MOI) of 100 in serum-free Dulbecco modified Eagle/F12 media. After 24 h, myocytes were fixed with 4% paraformaldehyde and blocking with 5% goat serum. The fluorescent images were captured by Leica SP2 Vis Laser confocal microscope. Other experiments were carried out after expression for 24h.

Fluorescent Resonance Energy Transfer Measurement. Myocytes expressing PKA or cAMP biosensors were rinsed and maintained in PBS for fluorescent resonance energy transfer (FRET) recording as described previously (25). Cells were imaged on a Zeiss Axiovert 200M microscope with a 40 \times /1.3NA oil-immersion objective lens and cooled CCD camera. Dual emission ratio

imaging was acquired with a 420DF20 excitation filter, a 450DRLP dichroic mirror, and two emission filters (475DF40 for cyan and 535DF25 for yellow). The acquisition was set with 200-ms exposure in both channels and 20-s elapses. Images in both channels were subjected to background subtraction, and ratios of yellow-to-cyan color were calculated at different time points.

Neonatal Myocyte Contraction Rate Assay and Adult Myocyte-Shortening Assay. Measurement of spontaneous contraction rate and adult myocyte shortening was carried out as described previously (25). Responses in myocyte beating rate and contractile shortening after drug treatments were analyzed by Metamorph software. Myocytes were stimulated with FSK (Sigma), ISO (Sigma), or PGE2 (Sigma) at indicated doses and times. Cells were also treated with ROL (Calbiochem) or CILO (Calbiochem) at indicated doses and times.

Western Blotting. Drug-treated myocytes were lysed in lysis buffer containing 25 mM Hepes (pH 7.5), 2.5 mM EDTA, 50 mM NaCl, 30 mM sodium pyrophosphate, 10% (vol/vol) glycerol, and 1% (vol/vol) Triton X-100 and protease inhibitor mixture tablets (Pierce) after washing twice with ice-cold PBS. Cell lysates were separated by SDS/PAGE for Western blotting with antibodies against phosphorylated serine 16 and threonine 17 of PLB and PLB (Badrilla), PDE4 and phosphorylated PDE4D S190 (Fabgenix), γ -tubulin, GFP, and phosphorylated PKA substrate (SCBT). Primary antibodies were visualized with IRDye 680CW goat-anti mouse or with IRDye 800CW goat-anti rabbit secondary antibodies using an Odyssey scanner (Li-cor biosciences). Quantification of phosphoproteins was performed using densitometry software Quantity One. Arbitrary phosphorylation units were calculated, and results were plotted against controls.

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

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