Phosphodiesterase 4 and Phosphatase 2A Differentially Regulate cAMP/Protein Kinase A Signaling for Cardiac Myocyte Contraction under Stimulation of β_1 Adrenergic Receptor[S]

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ABSTRACT

Activation of the β adrenergic receptor (βAR) induces a tightly controlled cAMP/protein kinase A (PKA) activity to ensure an agonist dose-dependent and saturable contraction response in animal heart. We have found that stimulation of β_1AR by isoproterenol induces maximal contraction responses at the dose of 1 μM in cardiac myocytes; however, cAMP accumulation continues to increase with higher agonist concentrations. Dose-dependent cAMP accumulation is tightly controlled by negative regulator phosphodiesterase 4 (PDE4) that hydrolyzes cAMP. At 1 nM isoproterenol, cAMP accumulation is minimal because of the hydrolysis of cAMP by PDE4, which leads to a small increase in PKA phosphorylation of phospholamban and troponin I (TnI), and contraction responses. Inhibition of PDE4 activity with rolipram enhances cAMP accumulation, yields maximal PKA phosphorylation of phospholamban and TnI, and

myocyte contraction responses. In contrast, at 10 μM isoproterenol, despite the negative effect of PDE4, cAMP accumulation is sufficient for maximal PKA phosphorylation of phospholamban and TnI. Inhibition of PDE4 with rolipram enhances cAMP accumulation, but not PKA phosphorylation and contraction responses. It is interesting that activities of both PKA and protein phosphatase 2A (PP2A) are enhanced under β_1AR activation with 10 μM isoproterenol, and PP2A is recruited to PKA/A kinase-anchoring protein complex. Inhibition of PP2A with okadaic acid further enhances the phosphorylation of phospholamban and TnI as well as contraction responses induced by 10 μM isoproterenol. Therefore, PP2A plays a key role in limiting PKA phosphorylation of phospholamban and TnI for myocyte contraction responses under β_1AR stimulation.

β adrenergic receptors (βARs) regulate cardiac contraction to enhance cardiac output in response to sympathetic nerve activity. It is well known that cardiac contraction is a saturable process, which is essential to prevent the heart from undergoing fibrillation or cardiac arrest. Among the adrenergic receptors expressed in myocardium, β_1AR serves as the primary receptor subtype in both human and murine hearts and is responsible for regulating cardiac contraction. Activated β_1ARs couple to G_s proteins to stimulate adenylyl cyclases, which synthesize second-messenger cAMP to activate PKA (Lefkowitz, 2007). PKA phosphorylates a wide range of substrates to enhance contraction, including L-type

calcium channels and phospholamban for regulating cytosolic calcium concentration and troponin I (TnI) for myofibril shortening (Xiang and Kobilka, 2003; Xiao et al., 2006). The Gs/cAMP/PKA system serves as a tightly controlled axis to conduct β_1AR signaling using negative regulators such as phosphodiesterases (PDEs) and protein phosphatases to fine-tune the output of signaling transduction.

PDEs hydrolyze cAMP to attenuate cAMP/PKA activity. Accumulating evidences have shown that intracellular cAMP induced by βAR signaling is regulated by different PDE enzymes in magnitude, time, and space, which are responsible for transiently increasing local PKA activity for a set of specific substrates (Kapiloff, 2002; Cooper, 2005; Mongillo and Zaccolo, 2006; Conti and Beavo, 2007; Houslay et al., 2007). PDEs 3 and 4 account for more than 90% of specific activity for cAMP hydrolysis in animal hearts (Richter et al., 2005). In particular, PDE4D isoforms have been shown to associate with βARs and regulate the receptor-induced cAMP accumulation in cardiac myocytes (Perry et al., 2002; Mon-

ABBREVIATIONS: βAR, β-adrenergic receptor; TnI, troponin I, PDE, phosphodiesterase; AKAP, A kinase anchoring proteins; IBMX, 3-isoproterenolbutyl-1-methylxanthine; PKA, protein kinase A; PKI, protein kinase A inhibitor; KO, knockout; PP2A, protein phosphatase 2A.

gillo et al., 2004; Xiang et al., 2005; Richter et al., 2008). However, recent studies suggest that cAMP accumulation is not in linear relation to the myocyte contraction responses induced by βAR signaling. When the function of PDE4 is disrupted by genetic deletion or pharmacological inhibition, βAR-induced cAMP can be greatly enhanced by more than 3-fold (Xiang et al., 2005). However, the higher cAMP accumulation fails to promote equivalent increases in contraction responses. We propose that further regulation downstream of cAMP accumulation plays a rate-limiting role in the contraction responses.

PKA is one of the major targets of cAMP involved in cardiac myocyte contraction. Upon binding cAMP, PKA is activated to phosphorylate a wide range of downstream proteins for myocyte contraction. In contrast, protein phosphatases dephosphorylate phosphorylated proteins. In cardiac tissues, protein phosphatase 2A (PP2A) has been identified as one of the major phosphatases associated with protein contraction machinery under βAR stimulation (Marks, 2001; Zhou et al., 2007). Phosphatases are often associated with scaffold protein A kinase anchoring proteins (AKAPs), which also anchor PKA holoenzymes. The locally bound PKA and phosphatase thus act together for tight regulation on phosphorylation of substrates. Thus, the tightly controlled PKA phosphorylation of substrates may be necessary to prevent myocytes from undergoing supermaximal contraction under βAR stimulation with high concentrations of agonist or when cAMP hydrolysis is perturbed. We hypothesize that increased PP2A activity plays a key role to prevent hyperphosphorylation of proteins for cardiac myocyte contraction responses under βAR stimulation.

Here, we explored agonist dose-dependent myocyte contraction induced by β_1AR signaling in neonatal and adult cardiac myocytes. We find that myocyte contraction stimulated by β_1AR signaling is a saturable process and is differentially controlled by PDE (on cAMP levels) and PP2A (on protein phosphorylation by PKA) downstream of receptor/G protein-uncoupling. At submaximal doses, PDE4 ensures a tight control of cAMP accumulation at minimal levels, which leads to a small increase of PKA activity and phosphorylation of contractile proteins for contraction. In contrast, at saturated doses, despite hydrolysis of cAMP by PDE4, activation and recruitment of PP2A to PKA complexes prevent hyperphosphorylation of contractile proteins under incremental cAMP/PKA activities, which ensures saturated contraction responses in cardiac myocytes.

Materials and Methods

Measurements of Cell Contraction. Adult mouse ventricular myocytes were isolated from hearts of 2- to 3-month-old male β_2AR-knockout (KO) FVB mice via a modified enzymatic technique (Zhou et al., 2000). Spontaneously beating neonatal cardiac myocytes were isolated from newborn pups from β_2AR-KO mice as described previously (Devic et al., 2001). We have characterized previously β_1ARs as the major βAR subtype responsible for adrenergic stimulation in cardiac myocytes. Thus, β_2AR-KO myocytes serve as an ideal model system to analyze activation of β_1AR signaling by isoproterenol, a βAR-specific agonist without the complication of signaling induced by endogenous β_2AR.

Adult myocytes were placed in a dish with HEPES buffer (Zhou et al., 2000) and electrically stimulated at 30 V/cm at 1 Hz at room temperature. Cell length was recorded with a charge-coupled device camera. Cell contraction shortening was analyzed by Metamorph software (Molecular Devices, Sunnyvale, CA) and normalized as the increase over the basal levels after being fitted to a sigmoidal curve. The maximal shortening was normalized to the baseline value or plotted as a percentage of the maximal response stimulated by 10 μM forskolin. Measurement of spontaneous neonatal cardiac myocyte contraction rate was carried out as described previously (Devic et al., 2001). The responses in myocyte contraction velocity after drug treatments was analyzed by Metamorph software (Spinale et al., 1997).

Drug Treatment. Myocytes were treated with the following inhibitors: rolipram (10 μM; Calbiochem, San Diego, CA) as a PDE4 inhibitor, cilostamide (10 μM, Calbiochem) as a PDE3 inhibitor, or 3-isoproterenolbutyl-1-methylxanthine (IBMX, 100 μM; Sigma, St. Louis, MO) as a nonselective PDE inhibitor (Alvarez et al., 1995; Sette and Conti, 1996). These agents were added to cells incubated at 37°C for 10 min before isoproterenol stimulation (10 μM, Sigma). In some assays, membrane-permeable myristoylated PKA inhibitor (PKI) amide 14 to 22 (2 $\times$ 10 μM, Calbiochem) or okadaic acid (1 μM; Alexis, San Diego, CA) was added to cells incubated at 37°C for 30 min before stimulation with isoproterenol or forskolin (10 μM, Sigma) (Wang et al., 2008).

Measurement of cAMP Accumulation. To measure intracellular cAMP levels, myocytes were cultured in six-well plates (2.5×10^5 cells/well). Cells were rinsed three times with 1$\times$ phosphate-buffered saline before feeding with serum-free Dulbecco's modified Eagle's medium (Mediatech, Herndon, VA) for 1 h. A time course of cAMP accumulation was carried out with both 1 nM and 10 μM isoproterenol. In both cases, cAMP accumulation peaked at 2 min after stimulation with isoproterenol (data not shown; Xiang et al., 2005). Cells were then stimulated with different doses of isoproterenol for 2 min. In some dishes, the PDE inhibitor rolipram or cilostamide was added 10 min before isoproterenol stimulation. cAMP accumulation was terminated by 0.1% trichloroacetic acid. The cAMP in the lysates was determined by radioimmunoassay similar to a method described previously (Xiang et al., 2005).

Immunoblotting. Neonatal or adult cardiac myocytes were stimulated with isoproterenol for 15 min at different concentrations (10 μM or 1 nM). In addition, myocytes were pretreated with the PDE inhibitor rolipram or cilostamide or with PKI before stimulation with isoproterenol. The lysates were separated by SDS-polyacrylamide gel electrophoresis for Western blot with antibodies to phospholamban (Affinity BioReagents, Golden, CO), phospho-Ser16-phospholamban (p-phospholamban; Badrilla, West Yorkshire, UK), TnI (Cell Signaling Technology, Danvers, MA), and phosphoSer23,24-TnI (Cell Signaling). 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, Lincoln, NE).

Coimmunoprecipitation. Neonatal cardiac myocytes were stimulated with 1 nM or 10 μM isoproterenol for 5 min before lysed in the coimmunoprecipitaton buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 2 mM EDTA, 50 mM NaF, 10% glycerol, 0.6% Nonidet P-40, and protease inhibitors). Lysates were cleared by centrifugation and subjected to immunoprecipitation with anti-PKA RII antibody (Santa Cruz Biotechnology, Santa Cruz, CA). The immunoprecipitates were resolved by SDS-polyacrylamide gel electrophoresis and blotted with antibodies against AKAP150 (Santa Cruz Biotechnology), PKA regulatory subunit (BD Biosciences Transduction Laboratories, Lexington, KY), PDE4 (Abcam, Cambridge, MA), and PP2A (BD Biosciences Transduction). Similar to immunoblots, 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).

Phosphatase and Protein Kinase A Assays. PP2A phosphatase activity was measured using the serine/threonine phosphatase assay system (Promega, Madison, WI). In brief, neonatal myocyte lysates were cleared by centrifugation at 16,000*g* for 10 min at 4°C.

Supernatant (20 μg) in the presence or absence of the serine-threonine phosphopeptide substrate (100 μM) was used for the assay based on the instructions of manufacturer. Relative PP2A activity was calculated and normalized against basal levels.

The same lysates were used to measure protein kinase A activity with PKA assay kits (Promega) in the same treatment conditions. This assay was carried out based on the instructions provided by the manufacturer. Likewise, the activities were calculated and normalized against basal levels.

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

Results

Differential Regulation of cAMP Accumulation and PKA Phosphorylation for Contraction under Dose-Dependent β_1AR Stimulation. To understand the mechanism on regulation of cAMP/PKA activity under β_1AR stimulation, cAMP accumulation induced by βAR-specific agonist isoproterenol was measured in β_1AR-KO neonatal cardiac myocytes. Stimulation of endogenous β_1ARs induced a dose-dependent maximal cAMP accumulation. We were surprised to find that cAMP accumulation was not saturated with concentrations of isoproterenol from 1 nM to 100 μM (Fig. 1A).

Recent studies have identified that PDE4D functionally associates with βAR signaling to regulate the contraction rate in neonatal cardiac myocytes (Xiang et al., 2005; Richter et al., 2008). We thus examined whether PDE4 enzymes control cAMP accumulation under dose-dependent isoproterenol stimulation of β_1ARs. Specific inhibition of PDE4 with rolipram enhanced the cAMP accumulation induced by 1 nM isoproterenol, resulting in a cAMP level similar to that induced by 10 μM isoproterenol alone (Fig. 1B). At 10 μM, the β_1AR-induced cAMP accumulation was further significantly enhanced by approximately 3-fold after pretreatment with rolipram (Fig. 1B). In comparison, specific inhibition of PDE3 enzymes with cilostamide did not affect the cAMP accumulation induced by either 1 nM or 10 μM isoproterenol (Fig. 1B).

Despite the activity of rolipram to potentiate cAMP levels when stimulated with 10 μM isoproterenol, rolipram fails to further enhance the maximal contraction rates at this concentration (Xiang et al., 2005). Therefore, we hypothesized that PKA-mediated phosphorylation of proteins involved in cardiac myocyte contraction, a process downstream of cAMP accumulation limited the effects on contraction rate. To de-
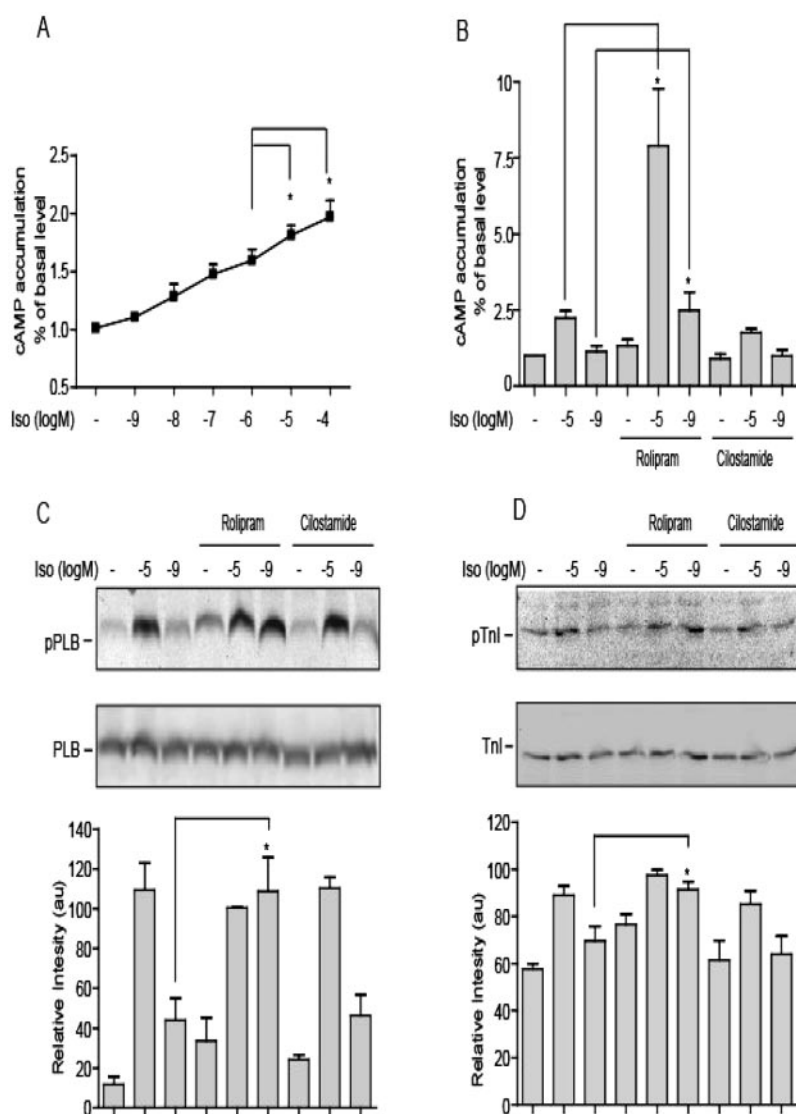

Fig. 1. Activation of β_1ARs induces a dose-dependent cAMP accumulation and PKA phosphorylation of phospholamban in β_2AR-KO neonatal cardiac myocytes. A, activation of endogenous β_1ARs by isoproterenol induced an agonist dose-dependent cAMP accumulation in β_2AR-KO myocytes. B, inhibition of PDE4 with rolipram but not PDE3 with cilostamide enhanced the cAMP accumulation induced by β_1AR activation with 10 nM or 10 μM isoproterenol. C, inhibition of PDE4 with rolipram but not PDE3 with cilostamide selectively enhanced the phosphorylation of phospholamban induced by β_1AR activation with 1 nM isoproterenol. D, inhibition of PDE4 with rolipram but not PDE3 with cilostamide selectively enhanced the phosphorylation of TnI induced by β_1AR activation with 1 nM isoproterenol. ∗, $P < 0.05$ by Student's t test.

termine this, we examined the phosphorylation of two important proteins in contraction, phospholamban and TnI. Stimulation with 10 μM isoproterenol induced a significant increase in phosphorylation of phospholamban at its PKA phosphorylation site, serine residue 16 (Fig. 1, C and D). In contrast, stimulation of β_1ARs with 1 nM isoproterenol, a threshold concentration required to induce significant myocyte contraction responses (Fig. 2, A and B), induced a small increase in phosphorylation of phospholamban at the same residue (Fig. 1C). However, rolipram selectively enhanced the phosphorylation of phospholamban induced by 1 nM but not 10 μM isoproterenol (Fig. 1C). With rolipram, the phospholamban phosphorylation induced by 1 nM isoproterenol was equivalent to that induced by 10 μM isoproterenol, suggesting a complete phosphorylation of serine 16 on phospholamban under these conditions (Fig. 1C). In accordance with our cAMP data in Fig. 1C, inhibition of PDE3 with cilostamide did not significantly affect the β_1AR-induced phosphorylation of phospholamban by either 1 nM or 10 μM isoproterenol (Fig. 1C). Likewise, stimulation with 10 μM isoproterenol induced a significant increase in the phosphorylation of TnI in myocytes. In contrast, 1 nM caused only a modest increase in TnI phosphorylation (Fig. 1D). Rolipram, but not cilostamide, selectively enhanced the phosphorylation of TnI induced by 1 nM isoproterenol. Neither rolipram nor cilostamide affected the phosphorylation of TnI induced by 10 μM isoproterenol (Fig. 1D). Together, these data indicate that isoproterenol-stimulated PKA phosphorylation of phospholamban and TnI is dose-dependent and is regulated by PDE4 activity.

PDE4 Selectively Controls Contraction Responses to β_1AR Activation at Submaximal Agonist Concentrations. We then examined myocyte contraction rates upon activation of β_1ARs with different doses of isoproterenol in neonatal cardiac myocytes. Activation of β_1ARs induced a dose-dependent increase on myocyte contraction rate, which peaked at 1 μM (Fig. 2, A and B). At 1 nM, isoproterenol induced a small but significant increase on myocyte contraction rate over baseline level (Fig. 2, A and B). After 1 nM stimulation, the addition of 10 μM isoproterenol further enhanced the β_1AR-induced contraction rate to a level equivalent to that when stimulated directly by 10 μM (data not shown).

Inhibition of PDE4 with rolipram selectively enhanced myocyte contraction rates induced by 1 and 10 nM, but not by 100 nM, 1 μM, or 10 μM isoproterenol (Fig. 2B). With rolipram, contraction rates under different doses of isoproterenol stimulation were maximized (Fig. 2B). Therefore, inhibition of all PDE activities with IBMX, but not inhibition of PDE3 with cilostamide, enhanced the myocyte contraction rate increase induced by β_1AR signaling at 1 nM isoproterenol (Fig. 2C). The enhanced responses after IBMX treatment were similar to those after rolipram treatment, supporting previous observations that PDE4 is the major PDE controlling the dose-dependent saturation of contraction responses.
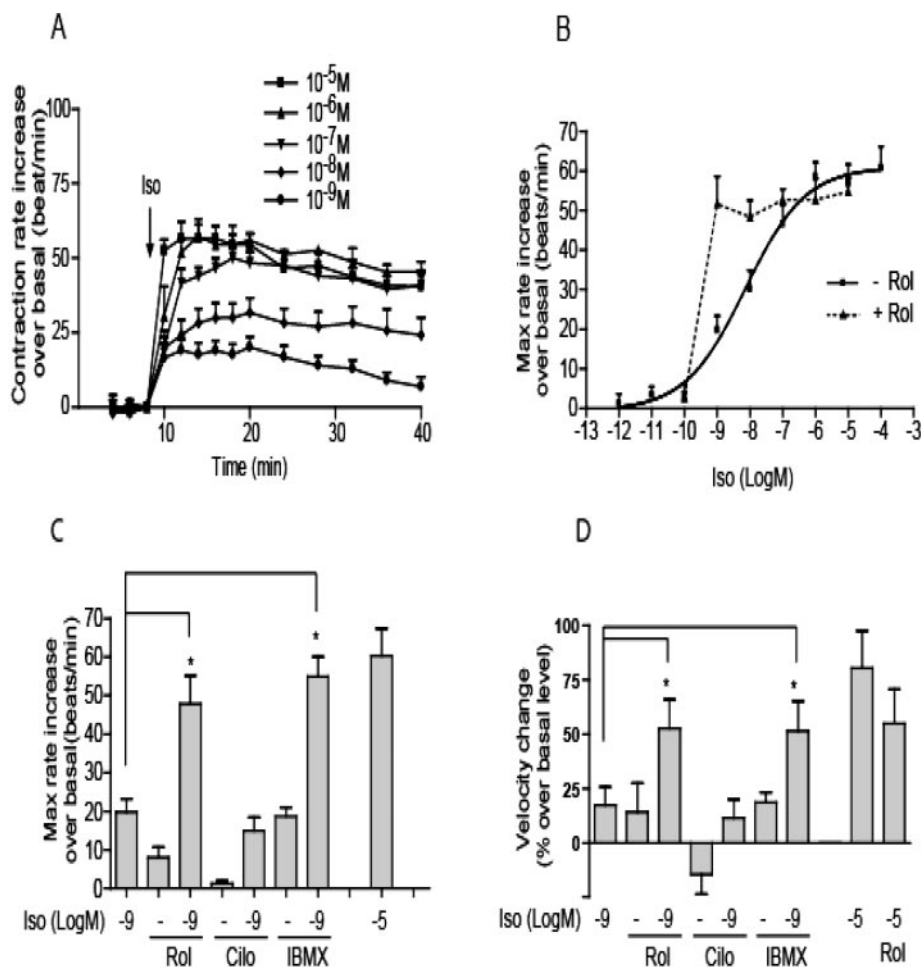

Fig. 2. Inhibition of PDE4 enhances myocyte contraction induced by activation of β_1ARs at submaximal doses of isoproterenol in β_2AR-KO neonatal cardiac myocytes. A, activation of endogenous β_1ARs by isoproterenol induced a dose-dependent contraction rate increase in β_2AR-KO myocytes. B, inhibition of PDE4 with rolipram selectively enhances the maximal contraction rate increases induced by β_1AR activation with submaximal concentrations but not with saturated concentrations of isoproterenol. C, inhibition of PDE4 and inhibition of all PDEs with IBMX but not inhibition of PDE3 significantly enhanced the maximal contraction rate responses induced by β_1AR activation with 1 nM isoproterenol. D, inhibition of PDE4 and inhibition of all PDEs with IBMX but not inhibition of PDE3 significantly enhanced the maximal contraction velocity responses induced by β_1AR activation with 1 nM isoproterenol. In contrast, inhibition of PDE4 had minimal effect on the contraction rate (C) and velocity (D) responses induced by β_1AR activation with 10 μM isoproterenol. The contraction response curves represent the mean $\pm$ S.E. of beating dishes from at least three different myocyte preparations. $*$, $P < 0.05$ by Student's t test.

In addition, the myocyte contraction velocity, an indicator of myocyte contractility, also displayed significant increases after stimulation of β_1ARs with isoproterenol (Fig. 2D). Although 1 nM isoproterenol stimulated a 17% increase in contraction velocity over baseline level, 10 μM induced an 80% increase in contraction velocity (Fig. 2D). Rolipram significantly enhanced the β_1AR-induced contraction velocity increase induced by 1 nM but not by 10 μM isoproterenol. This increase was equivalent to that induced by 10 μM isoproterenol alone (Fig. 2D). In addition, IBMX, but not cilostamide, significantly enhanced the contraction velocity increases induced by 1 nM isoproterenol (Fig. 2D). Together, these data suggest that inhibition of PDE4 with rolipram enhances myocyte contraction rate and velocity to the maximal levels even when stimulating β_1ARs at a submaximal concentration of 1 nM isoproterenol.

PP2A Is Activated and Recruited to PKA to Prevent Supermaximal Contraction Responses under Maximal Stimulation of β_1ARs. The discrepancy between the increasing cAMP accumulation and the saturated PKA phosphorylation of phospholamban and TnI under high doses of

isoproterenol stimulation suggests that either PKA activity is completely saturated or negative regulation exists downstream of cAMP/PKA activity to attenuate the PKA phosphorylation levels of the substrates. We speculated that PP2A may play a role in controlling the PKA-mediated phosphorylation of the contractile proteins. First, we examined the role of PKA activity in β_1AR-induced phosphorylation of phospholamban at serine 16. Inhibition of PKA activity with PKI significantly reduced the phosphorylation of phospholamban induced by 10 μM isoproterenol (Fig. 3A). PKI also completely abolished the effect of rolipram on the β_1AR-induced phosphorylation of phospholamban with 1 nM isoproterenol (Fig. 3A). These data confirmed that PKA activity is required for increasing phosphorylation of phospholamban at serine 16 under βAR stimulation. Second, we examined the role of PP2A activity in the β_1AR-induced phosphorylation of the substrates. Inhibition of PP2A with okadaic acid further enhanced the phosphorylation of phospholamban and TnI under stimulation with the saturated 10 μM isoproterenol (Fig. 3, B and C). Okadaic acid also enhanced the phosphorylation of phospholamban under stimulation of 10 μM
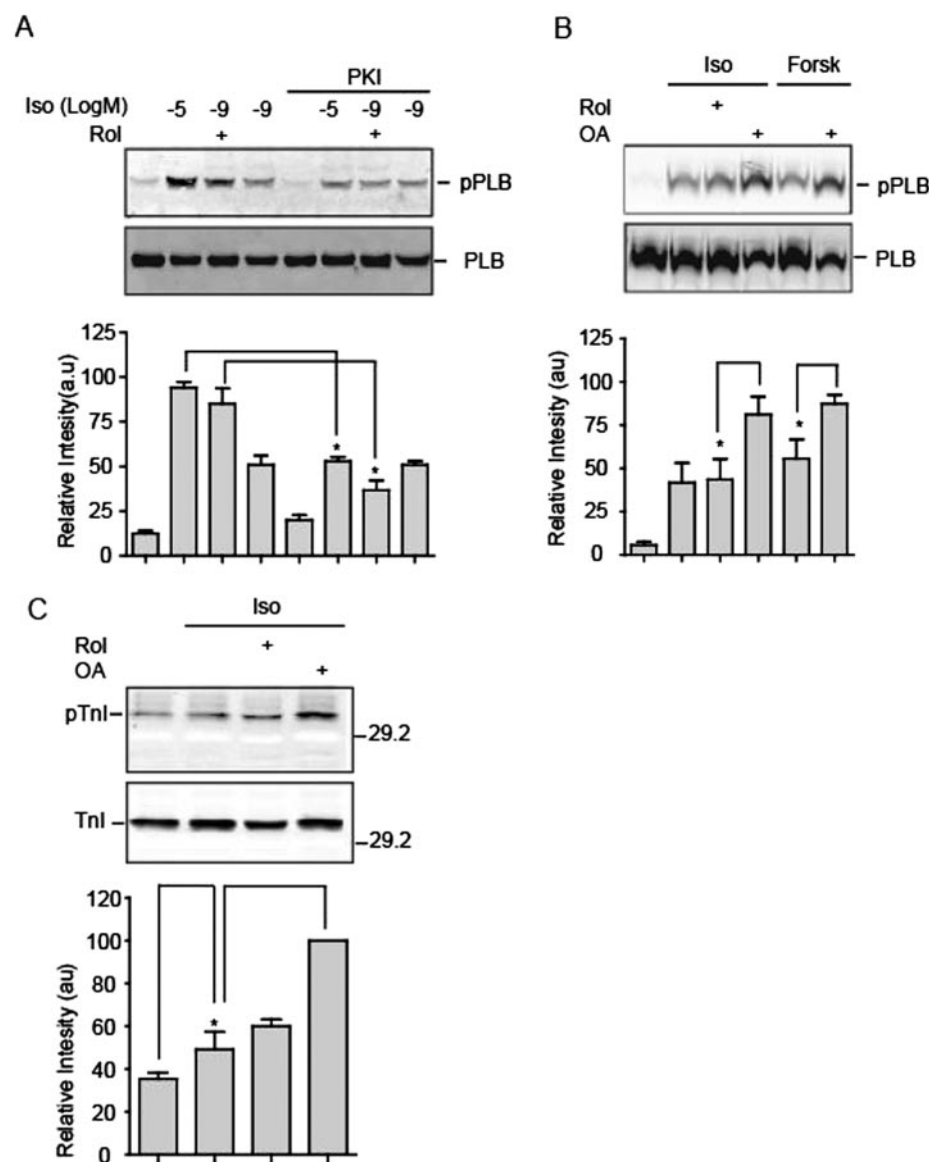

Fig. 3. PKA and PP2A regulate PKA phosphorylation of phospholamban and TnI induced by activation of β_1ARs in β_2AR-KO cardiac myocytes. A, inhibition of PKA with PKI attenuated the PKA phosphorylation of phospholamban induced by β_1AR activation with either 10 μM isoproterenol or 1 nM isoproterenol after rolipram treatment. B, inhibition of PP2A with okadaic acid enhanced the PKA phosphorylation of phospholamban induced by β_1AR activation with either 10 μM isoproterenol or 10 μM forskolin. C, inhibition of PP2A with okadaic acid enhanced the PKA phosphorylation of TnI induced by β_1AR activation with 10 μM isoproterenol. *, $P < 0.05$ by Student's t test.

forskolin (Fig. 3B). Neither rolipram nor okadaic acid changed the receptor densities on the cell surface of cardiac myocytes, and okadaic acid did not significantly alter the cAMP accumulation induced by β_1AR activation with 10 μM isoproterenol (Supplemental Fig. S1).

To further understand the mechanism underlying the saturated PKA phosphorylation of contractile proteins and contraction responses under incremental β_1AR signaling, we examined both PKA and PP2A activities upon receptor activation by either 1 nM or 10 μM isoproterenol. PKA activity was enhanced after stimulation at 10 μM isoproterenol, which was inhibited

by the PKA-specific inhibitor PKI (Fig. 4A). Moreover, PKI reduced both contraction rate and velocity increases induced by β_1AR signaling with either 10 μM isoproterenol or 1 nM isoproterenol after pretreatment with rolipram (Fig. 4, B and C). It is interesting that PP2A activity was also significantly increased upon receptor activation at 10 μM isoproterenol, and the increase was attenuated by PKI (Fig. 4D). Inhibition of PP2A with okadaic acid enhanced contraction rate induced by 10 μM isoproterenol or forskolin (Fig. 4E). Okadaic acid alone enhanced the myocyte contractile velocity, suggesting that neonatal myocyte contraction rate and velocity are differentially
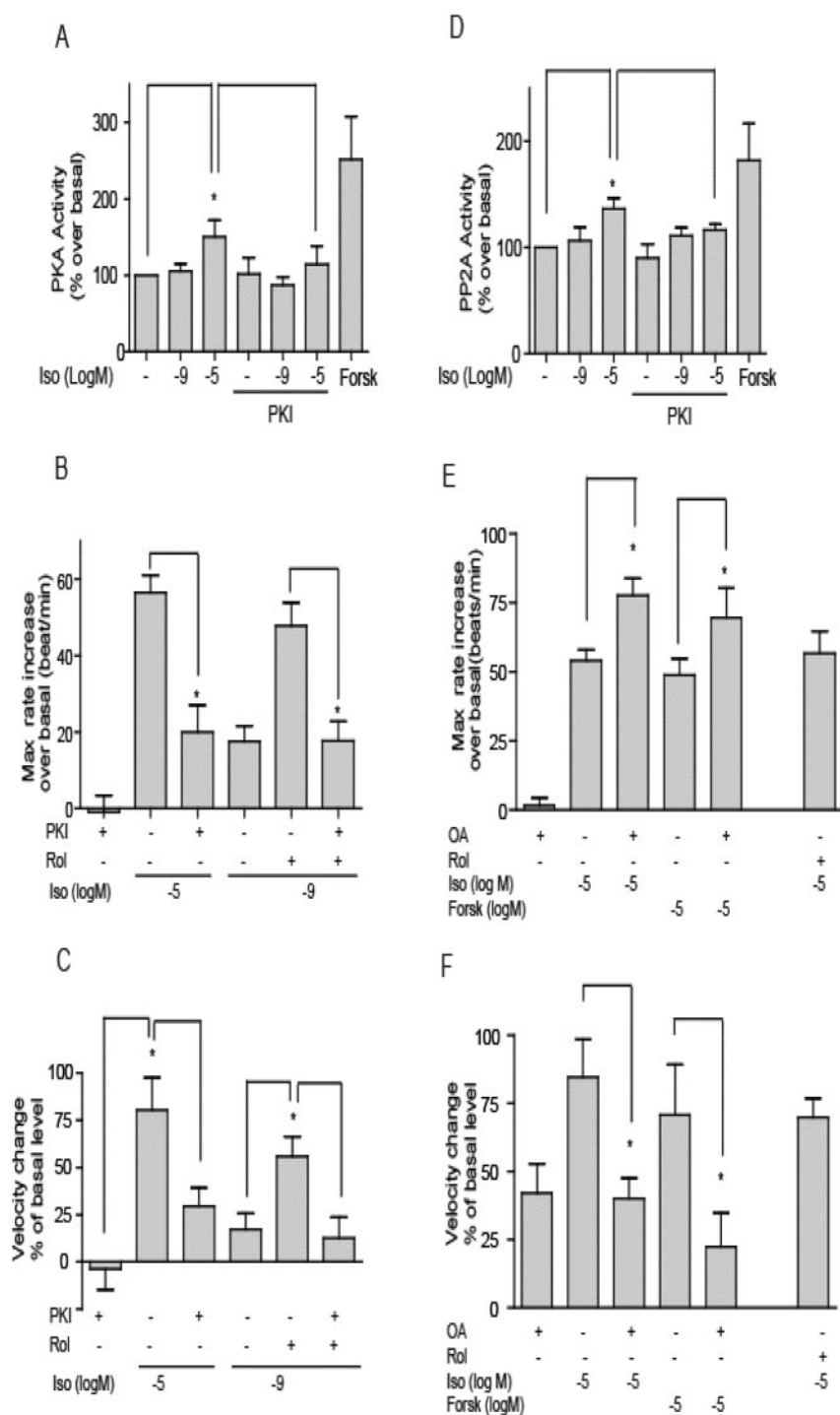

Fig. 4. PKA and PP2A regulate myocyte contraction responses induced by activation of β_1ARs in β_2AR-KO neonatal cardiac myocytes. A, PKA activity was significantly increased under β_1AR signaling induced by 10 μM but not 1 nM isoproterenol. B and C, inhibition of PKA with PKI significantly reduced the maximal contraction rate and velocity responses induced by β_1AR activation with either 10 μM isoproterenol or 1 nM isoproterenol after treatment with rolipram. D, PP2A activity was significantly increased under β_1AR signaling induced by 10 μM but not 1 nM isoproterenol. E, inhibition of PP2A with okadaic acid enhanced the maximal contraction rate responses induced by β_1AR activation with 10 μM isoproterenol or forskolin. F, inhibition of PP2A with okadaic acid enhanced the myocyte contraction velocity and blunted the maximal contraction velocity responses induced by β_1AR activation with 10 μM isoproterenol or forskolin. *, $P < 0.05$ by Student's t test.

regulated by protein phosphorylation at baseline levels. Pretreatment with okadaic acid also blunted the contractile velocity upon isoproterenol or forskolin stimulation (Fig. 4F).

An increasing number of studies indicate that both cAMP and PKA activities are highly localized in cardiac myocytes. In our study, the effect of PP2A on the phosphorylation levels of phospholamban and TnI by PKA suggests that PP2A and PKA are closely localized together with the substrates. Indeed, we observed a basal level of PP2A associated with PKA/AKAP complexes in cardiac myocytes at resting state (Fig. 5A). The association was enhanced upon receptor activation with 10 μM isoproterenol (Fig. 5, B and C). In contrast, the PDE4 association with PKA/AKAP complex did not change upon stimulation with either 1 nM or 10 μM isoproterenol (Fig. 5, B and D). Together, our data suggest that PP2A serves as a negative feedback to tightly control PKA-mediated phosphorylation of downstream phospholamban and TnI when β_1ARs are activated with 10 μM isoproterenol.

PDE4 and PP2A Differentially Control Adult Myocyte Shortening. To examine whether the effect of PP2A and PDE4 on βAR signaling is maintained in adult myocytes, we examined the effects of PP2A and PDE4 on βAR-induced adult myocyte shortening. Activation of β_1ARs induced a rapid increase in myocyte shortening (Fig. 6A). The maximal shortening responses displayed an isoproterenol dose-dependent manner (Fig. 6B). With rolipram, myocyte shortening induced by 1 nM but not 10 μM isoproterenol was selectively enhanced; the maximal shortening was equivalent to that induced by 10 μM isoproterenol alone (Fig. 6C). We were surprised to find that inhibition of PDE3 with cilostamide

disrupted the rhythmic contraction under pacing condition. A close examination showed that myocytes displayed arrhythmic beating with higher frequencies (Supplemental Fig. S2A), suggesting an essential role of PDE3 for basal cAMP/PKA activity critical for maintaining paced contraction. Likewise, inhibition of PKA with PKI caused arrhythmic contraction under pacing conditions by reducing both myocyte contraction frequency and shortening (Supplemental Fig. S2B). In contrast, inhibition of PP2A with okadaic acid enhanced the myocyte contraction shortening induced by a 10 μM concentration of both isoproterenol and forskolin (Fig. 6D).

To rule out that β_1AR signaling is altered by gene deficiency in β_2AR-KO myocytes, we examined myocyte shortening in wild-type adult cardiac myocytes. Activation of βARs with both 1 nM and 10 μM isoproterenol induced maximal shortening responses similar to those observed in β_2AR-KO myocytes (Fig. 6E). Inhibition of PDE4 with rolipram induced a minimal increase in myocyte shortening (Fig. 6E). With rolipram, myocyte shortening induced by 1 nM but not 10 μM isoproterenol was selectively enhanced; the maximal shortening was equivalent to that induced by 10 μM isoproterenol alone (Fig. 6E). In addition, okadaic acid enhanced myocyte contraction shortening induced by 10 μM concentration of both isoproterenol and forskolin (Fig. 6F). Together, these data confirm that PDE4 and PP2A differentially control an agonist dose-dependent, tightly regulated, and saturable myocyte contraction induced by β_1AR signaling in both β_2AR-KO and wild-type adult cardiac myocytes.

Discussion

Agonist Dose-Dependent Contraction Responses in Cardiac Myocytes. Stimulation of β_1ARs induces dose-dependent increases of contraction rate and contractility, which are maximized at 1 μM isoproterenol. We were surprised to find that cAMP accumulation is not saturated at the same dose. Nevertheless, the contraction responses are dependent on the elevated PKA activities because inhibition of PKA with PKI dramatically reduces the β_1AR-induced contraction responses. Further analysis revealed that the β_1AR/cAMP/PKA pathway can be differentially regulated by PDE4 (on cAMP level) and PP2A (on protein phosphorylation level by PKA). This dual regulation is essential to prevent hyperphosphorylation of phospholamban and TnI by PKA and supermaximal contraction responses upon β_1AR stimulation with increasing concentrations of isoproterenol. At low doses of agonist, inhibition of PDE4 leads to higher cAMP accumulation and subsequently higher levels of PKA phosphorylation of phospholamban and TnI. However, at high doses of agonist, inhibition of PDE4 fails to promote higher levels of PKA phosphorylation of phospholamban and TnI, despite the fact that it significantly enhances the cellular cAMP accumulation. This saturation of PKA phosphorylation of phospholamban is unlikely because PKA has completely phosphorylated all phospholamban proteins, as inhibition of PP2A significantly enhances the phosphorylation level of phospholamban by PKA. Therefore, although PDE4 is essential in confining cAMP accumulation at modest levels, PP2A plays a key role to prevent hyperphosphorylation of phospholamban and TnI by PKA in case cAMP accumulation is exuberated in cardiac myocytes. Together, PDE4 and PP2A function as dual levels
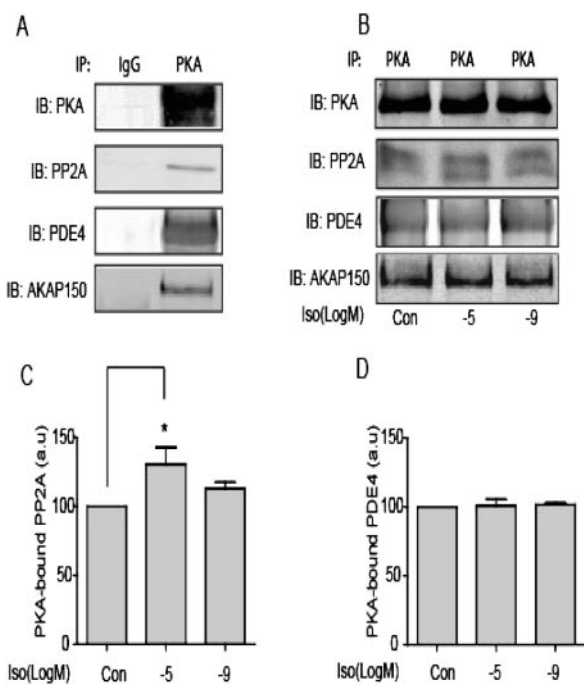

Fig. 5. Association of PP2A and PKA is enhanced under β_1AR signaling in β_2AR-KO neonatal cardiac myocytes. A, PP2A and PDE4 associate with PKA/AKAP79 complex in neonatal cardiac myocytes, which were immunoprecipitated with anti-PKA antibody before Western blot. B, the association of PP2A to PKA/AKAP150 complex was significantly increased under β_1AR signaling induced by 10 μM but not 1 nM isoproterenol. However, the association of PDE4 with the PKA/AKAP150 complex was not enhanced by β_1AR activation. The quantitative data of the PKA-associated PP2A and PDE4 in Western blots are plotted in C and D. *, $P < 0.05$ by Student's t test.

of protective measurements to prevent supermaximal contraction responses.

PDE4 Selectively Controls Dose-Dependent PKA Phosphorylation of Phospholamban and TnI Induced by β_1AR Signaling at Submaximal Concentrations. The

accumulated cAMP under βAR stimulation is hydrolyzed by PDEs. In cardiac myocytes and human embryonic kidney 293 cells, without inhibition of PDE activity, cAMP accumulation under βAR stimulation exhibits a transient increase that peaks at 2 min followed by a rapid decrease (Xiang et al.,
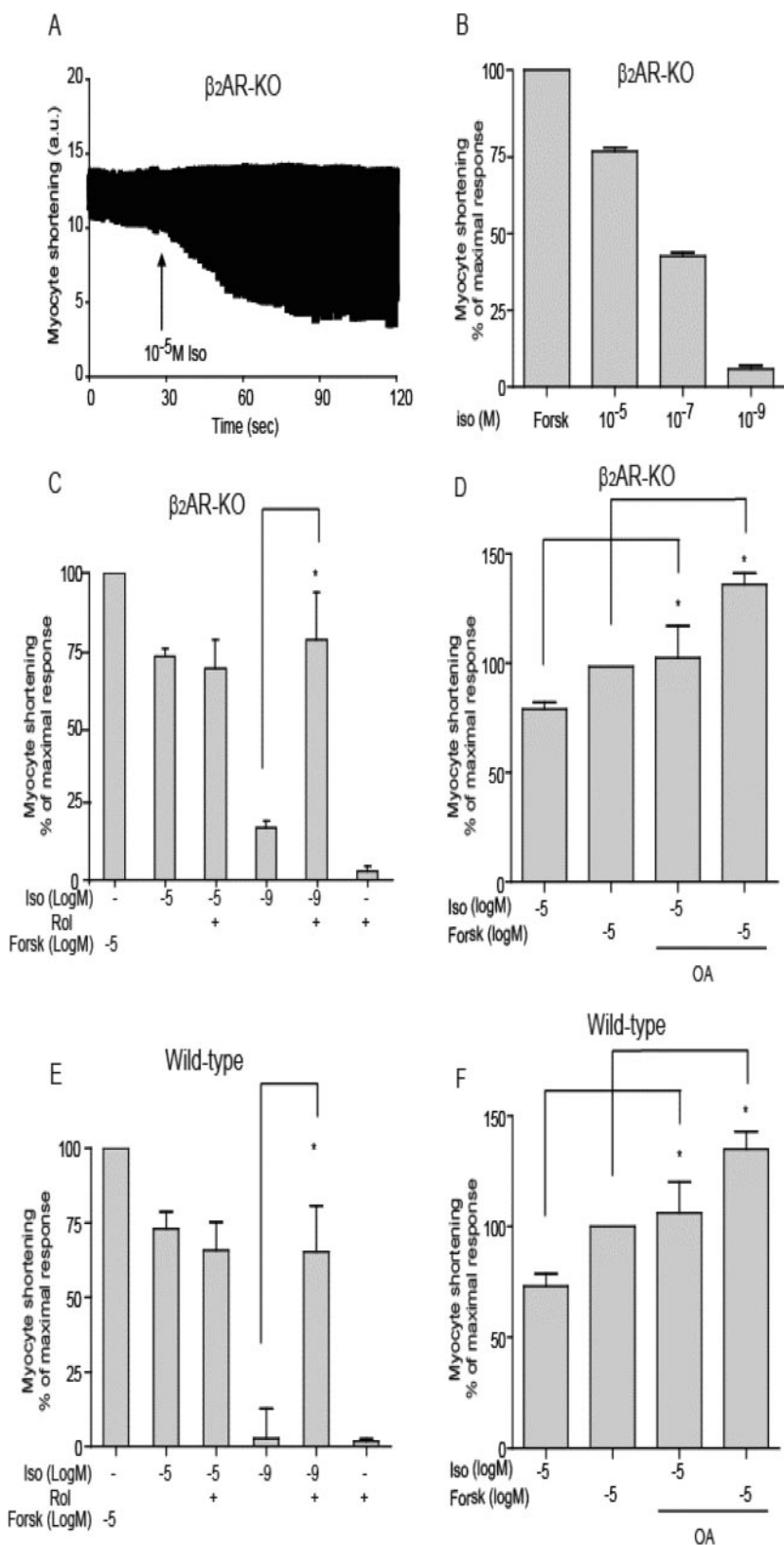

Fig. 6. PDE4 and PP2A differentially regulate β_1AR-induced contraction shortening in β_2AR-KO and wild-type adult cardiac myocytes. A, stimulation of β_1ARs with 10 μM isoproterenol induced a rapid shortening increase in β_2AR-KO myocytes. B, stimulation of β_1ARs induces an agonist dose-dependent maximal contraction shortening in β_2AR-KO myocytes. C, inhibition of PDE4 with rolipram enhanced the maximal myocyte shortening induced by β_1AR activation with 1 nM isoproterenol but did not affect the maximal shortening induced by β_1AR activation with 10 μM isoproterenol in β_2AR-KO myocytes. D, inhibition of PP2A with okadaic acid enhanced the maximal myocyte shortening induced by β_1AR activation with 10 μM isoproterenol or forskolin in β_2AR-KO myocytes. In the wild-type myocytes (E), inhibition of PDE4 with rolipram enhanced the maximal myocyte shortening induced by βAR activation with 1 nM isoproterenol but did not affect the maximal shortening induced by βAR activation with 10 μM isoproterenol. F, inhibition of PP2A with okadaic acid enhanced the maximal myocyte shortening induced by βAR activation with 10 μM isoproterenol or forskolin in the wild-type myocytes. *, $P < 0.05$ by Student's t test.

2005; Violin et al., 2008; data not shown). We were surprised to find that cAMP accumulation induced by βAR stimulation does not seem to be saturated under increasing concentrations of agonist, which is most likely regulated by the negative effect of PDE activities. Both PDE3 and PDE4 are highly expressed in cardiac myocytes and account for the majority of PDE activities for cAMP degradation (Mongillo et al., 2004). PDE4 enzymes are enriched in both M and Z lines in proximity to βAR (Mongillo et al., 2004). Indeed, PDE4D8 isoforms directly bind to β_1ARs at steady state and dissociate from the receptor upon agonist stimulation. This is in contrast to the agonist-induced and arrestin-dependent recruitment of PDE4D3 and PDE4D5 to β_2ARs (Perry et al., 2002; Baillie et al., 2003; Richter et al., 2008). The dissociation between PDE4D8 and β_1AR is likely in part because of the relative low affinity between the receptor and arrestin (Shiina et al., 2000, 2001). Moreover, PDE4 can be phosphorylated by PKA to enhance the enzymatic activities for cAMP degradation (Conti et al., 2003; McConnachie et al., 2006; Willoughby et al., 2006). Consistent with these studies, our data show that PDE4, but not PDE3 controls cAMP accumulation induced by β_1AR signaling in murine cardiac myocytes.

In addition, our data confirm that PDE3 has no significant role in subsequent PKA phosphorylation of phospholamban and TnI in cardiac myocytes under βAR stimulation. However, inhibition of PDE3 activity in adult cardiac myocytes abolishes the rhythmic contraction under pacing condition. PDE3 has been shown to localize at intracellular compartments and plays an essential role in controlling calcium release in both sarcoplasmic reticulum and mitochondria (Mongillo et al., 2004; Kerfant et al., 2007). The loss of paced contraction after inhibition of PDE3 is probably due to calcium releasing from sarcoplasmic reticulum and mitochondria by elevated PKA phosphorylation of calcium channels. In the same vein, the paced contraction is disrupted after inhibition of PKA with PKI. Together, our data indicate the distinct roles of PDE3 and PDE4 in maintaining basal and stimulated contraction, respectively, in adult cardiac myocytes.

Activation and Recruitment of PP2A Tightly Control the Maximized PKA Phosphorylation of Phospholamban and TnI in Cardiac Myocytes. The apparent discrepancy between cAMP signaling and myocyte contraction responses under βAR stimulation is explained by the levels of PKA phosphorylation of phospholamban and TnI (Fig. 1 and 2). There is ample evidence showing that cAMP accumulation under extracellular hormone stimulation is highly localized in cardiac myocytes (Zaccolo and Pozzan, 2002; Mongillo et al., 2004; Warrier et al., 2005; Nikolaev et al., 2006; Rich et al., 2007). It has also been reported that PKA activity forms a gradient depending on cAMP diffusion in neonatal rat cardiac myocytes (Saucerman et al., 2006). Therefore, the access of cAMP/PKA signaling to downstream substrates for myocyte contraction must be restricted in certain functional "domains". However, inhibition of PP2A with okadaic acid can further enhance the phosphorylation of phospholamban and TnI under 10 μM isoproterenol stimulation in cardiac myocytes (Fig. 3). Thus, the maximized PKA-mediated phosphorylation under β_1AR signaling is unlikely because of saturation of PKA activity or limitation of phospholamban availability. In contrast, the maximal PKA phosphorylation on contractile proteins is probably maintained through equilibrium between phosphorylation by PKA and dephosphoryla-

tion by PP2A within the functional "domains". This notion is further supported by evidence that PP2A is activated in a PKA-dependent manner under high doses of isoproterenol stimulation, and the enzymes are recruited to PKA/AKAP complexes to counterbalance the incremental cAMP/PKA activities. This novel mechanism ensures the tightly controlled and saturated phosphorylation of contractile proteins in local contractile "domains". Thus, our data provide the first direct biochemical evidence that PKA phosphorylation of phospholamban stimulated by β_1AR signaling is differentially controlled by PDE4 and PP2A in an agonist dose-dependent manner. Both PDE4 and PP2A target completely different substrates, and each will have different limiting factors. Our study does not rule out the possibilities that PKA phosphorylation of other targeted proteins under inhibition of PP2A could be discrete from the ones targeted by inhibition of PDE4. However, we have observed that both PP2A and PDE4 associate with PKA/AKAP complexes, suggesting that they do share common downstream targets such as phospholamban.

Together, our data indicate that the adrenergic stimulation-induced myocyte contraction is negatively regulated at multiple levels in a dose-dependent manner, which serves as protective measurements to prevent heart from over-beating. At low concentration of agonist, PDE4 controls cAMP accumulation and subsequent PKA activation and phosphorylation of contractile proteins. However, when high concentrations of agonists are present or the PDE4-mediated cAMP degradation is perturbed, the excessive cAMP activities can no longer lead to higher phosphorylation of contractile proteins partly because PP2A plays a critical role in maintaining the maximal phosphorylation level. The high level of cAMP may "spill over" from the normal contractile "domains" and access other cellular proteins for cell damage and for gene modification (Schmitt and Stork, 2002).

In summary, we demonstrate that the activation of β_1AR signaling induces agonist dose-dependent and saturable contraction responses in both neonatal and adult cardiac myocytes, which are significantly blunted by the inhibition of PKA activity. In cardiac myocytes, PKA-mediated phosphorylation but not cAMP level is correlated to agonist-induced contraction responses upon β adrenergic stimulation. At submaximal concentrations of agonist, PDE4 selectively enhances PKA phosphorylation of phospholamban and contraction responses. In contrast, at high concentrations of agonist, PP2A is activated and recruited to PKA to counterbalance the high cAMP/PKA activities to ensure a saturated PKA phosphorylation of phospholamban and contraction. These findings offer insights in understanding the signaling mechanisms of β_1AR in cardiac myocytes at normal and stimulating conditions.

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