Adrenoceptors receptors (ARs) play a pivotal role in regulating cardiovascular response to catecholamines during stress. β2 ARs, prototypical G protein-coupled receptors (GPCRs), expressed in animal hearts, display dual coupling to both Gs and Gi proteins to control the adenylyl cyclase-cAMP dependent protein kinase A (PKA) pathway to regulate contraction responses. Here, we showed that the β2 AR coupling to Gi proteins was agonist dose-dependent and occurred only at high concentrations in mouse cardiac myocytes. Both the β2 AR-induced PKA activity, measured by fluorescence resonance energy transfer (FRET) imaging, and the increase in myocyte contraction rate displayed sensitivity to the Gi inhibitor pertussis toxin (PTX). Further studies revealed that activated β2 ARs underwent PKA phosphorylation at a broad range of agonist concentrations. Disruption of the PKA phosphorylation sites on the β2 AR blocked receptor/Gi coupling. However, a sufficient β2 AR/Gi coupling was also dependent on the G protein-coupled receptor kinase (GRK)-mediated phosphorylation of the receptors, which only occurred at high concentrations of agonist (≥100 nM). Disruption of the GRK phosphorylation sites on the β2 AR blocked receptor internalization and coupling to Gi proteins, probably by preventing the receptor’s transportation to access Gi proteins. Furthermore, neither PKA nor GRK site mutated receptors displayed sensitivity to the Gi specific inhibitor, Gi{\text{CT}}. Together, our studies revealed distinct roles of PKA and GRK phosphorylation of the β2 AR for agonist dose-dependent coupling to Gi proteins in cardiac myocytes, which may protect cells from overstimulation under high concentrations of catecholamines.

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Adrenoceptors receptors (ARs) play a pivotal role in regulating cardiovascular response to catecholamines during stress. Both β1- and β2 ARs are prototypical GPCRs expressed in animal hearts, which mediate the increases in cardiac contraction upon agonist stimulation through the Gi{\text{c}}-adenyl cyclase-cAMP-dependent PKA pathway (1). β2 ARs are also known to uniquely couple to inhibitory Gi proteins, which inhibits adenylyl cyclase to reduce cardiac contraction and initiates anti-apoptotic and cell growth signaling (2, 3). Although β2 AR coupling to Gi has been studied in reconstituted systems and in fibroblasts (4, 5), little is known about the regulation process and the circumstances under which this coupling occurs in cardiac cells, along with its physiological consequences.

Various studies indicate that phosphorylation of β2 AR plays a critical role in regulating differential G protein coupling. The β2 AR phosphorylation by PKA mediates the switch of coupling from Gs to Gi (6, 7), presumably operating in a feedback after receptor activation. Our previous studies have revealed that β2 AR/Gi coupling is also dependent on receptor internalization and recycling (8, 9). Meanwhile, Wang et al. have shown that direct inhibition of GRK2 prevents β2 AR/Gi coupling in mouse cardiac myocytes (8), supporting a role of the β2 AR phosphorylation by GRK in receptor/Gi coupling.

Studies have also revealed that β2 ARs are differentially phosphorylated by PKA and GRK in fibroblasts in an agonist dose-dependent manner (10–13). Although the PKA-mediated phosphorylation is sensitive to stimulation with subnanomolar concentrations, the GRK-mediated phosphorylation happens only when β2 ARs are stimulated with saturated concentrations (10–13). This differential regulation of receptor phosphorylation by different kinases and their effects on subsequent receptor trafficking indicate that the β2 AR/Gi coupling may be finely regulated in an agonist concentration-dependent manner.

Here, using a fluorescence resonance energy transfer (FRET) assay with a biosensor for PKA activity and a physiological cardiac contraction rate assay, we show for the first time that β2 AR/Gi coupling occurs only at a saturated concentration of isoproterenol (Iso) in cardiac cells. Stimulation induces the PKA-mediated phosphorylation of the β2 AR over a wide range of agonist concentrations, whereas the GRK-mediated phosphorylation occurs only at sufficiently high concentrations (≥100 nM) of Iso and is necessary for receptor internalization. We further demonstrate that mutations of the specific PKA or GRK phosphorylation sites on the β2 AR abolish receptor coupling to Gi protein. Our data suggest that the PKA-mediated phosphorylation is necessary for β2 AR/Gi coupling, but a sufficient receptor/Gi coupling is also dependent on the
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GRK-mediated phosphorylation and subsequent trafficking of the receptors.

MATERIALS AND METHODS

Site-directed Mutagenesis and Recombinant Adenoviruses—pcDNA3.1-FLAG-tagged murine β₂AR was used as a template for mutagenesis. A protein kinase A mutant (PKAmut) β₂AR lacking protein kinase A (PKA) phosphorylation sites (serines 261, 262, 345, and 346) was generated by replacing the four serines with alanines. A GRK mutant (GRKmut) β₂AR—Ser(P)₂₆₁/₂₆₂ was detected with a monoclonal antibody from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). IRDye 680CW goat anti-rabbit secondary antibodies were used to detect Ser(P)₂₆₁/₂₆₂ and Ser(P)₃₅₅/₃₅₆ phosphorylation, respectively. Western blots were visualized with an Odyssey scanner (Li-cor Biosciences, Lincoln, NE) and quantified before normalization against the baseline levels.

titers of the viruses were assessed by comparison of receptor expression levels with both Western blot and ligand binding, as described previously (15). The GFP-GiCT virus used in contraction assays was a kind gift from Dr. Walter Koch (Thomas Jefferson University, Philadelphia, PA).

Cardiac Myocyte Contraction Assay—Measurement of spontaneous myocyte contraction was performed as described previously (16). Neonatal cardiac myocytes were isolated from mice lacking either β₁AR (β₁AR-knock-out; β₁AR-KO) or both β₁ and β₂AR genes (β₁β₂AR-KO). β₁β₂AR-KO myocytes were infected with murine β₂AR, PKAmut β₂AR, or GRKmut β₂AR at a multiplicity of infection of 100 and stimulated with 10 μM Iso. Cardiac myocytes were treated with G_i inhibitor pertussis toxin (PTX) at 0.3 μg/ml, 3 h before stimulation. GFP-GiCT virus was co-infected at a multiplicity of infection of 100 or, as indicated, along with the wild type or mutant β₂AR viruses. Contraction rate assays were performed the next day as described previously (16).

Receptor Phosphorylation—Neonatal cardiac myocytes were infected with FLAG-tagged wild type or mutant β₂ARs at a multiplicity of infection of 100. After 48 h of expression, cells were serum-starved for 2 h and stimulated with different doses of Iso for 5 min. For the time course experiments, cells were stimulated with 10 μM Iso for different times as indicated. Cells were placed into lysis buffer (10 mM Tris, pH 8.0, 1% Nonidet P-40, 150 mM NaCl, 2 mM EDTA, and protease and phosphatase inhibitor mixture (Thermo Scientific)) for 30 min at 4 °C. Samples were centrifuged, and the supernatant was resolved by SDS-PAGE mixture (Thermo Scientific)) for 30 min at 4 °C. Samples were centrifuged, and the supernatant was resolved by SDS-PAGE

FIGURE 1. Stimulation of the β₂AR induces agonist dose-dependent coupling to G_i in cardiac myocytes. A and B, β₁β₂AR-KO cardiac myocytes expressing the PKA biosensor AKAR2.2 were stimulated with 10 nM (A) or 10 μM (B) Iso. Myocytes were treated with G_i protein kinase A (PKA) or G_i protein kinase A (PKA) or G_i knockout (β₁AR-KO) or both β₁ and β₂AR genes (β₁β₂AR-KO). β₁β₂AR-KO myocytes were infected with murine β₂AR, PKAmut β₂AR, or GRKmut β₂AR at a multiplicity of infection of 100 and stimulated with 10 μM Iso. Cardiac myocytes were treated with G_i inhibitor pertussis toxin (PTX) at 0.3 μg/ml, 3 h before stimulation. GFP-GiCT virus was co-infected at a multiplicity of infection of 100 or, as indicated, along with the wild type or mutant β₂AR viruses. Contraction rate assays were performed the next day as described previously (16).
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Receptor Internalization and Recycling—After expressing the FLAG-β₂AR, the β₁β₂AR-KO cardiac myocytes were serum-starved for 2 h and stimulated with 10 nM or 10 μM Iso for different times. For receptor recycling, cells were stimulated with Iso for 10 min, rinsed, and refed with serum-free medium for different times. Cells were then fixed, permeabilized, and stained with anti-FLAG antibody, which was revealed with an Alexa-488 conjugated goat anti-mouse antibody (Invitrogen). Fluorescence images were taken with a CCD camera on a Zeiss microscope with Metamorph software. HEK293 cells were transfected with plasmids expressing the FLAG-β₂AR, PKA-mut β₂AR, and GRKmut β₂AR. Receptor internalization and recycling was quantified in HEK293 cells by using a fluorescence-linked immunosorbent assay (FLISA) to determine cell surface receptor levels as previously described (17). In brief, the FLAG-tagged receptors were labeled with anti-FLAG M1 antibody, which was revealed by the Alexa-488 goat-anti-mouse secondary antibody and detected by Spectramax M2 fluorometer.

PKA Activity Measurement through FRET Assay—β₁AR-KO or β₁β₂AR-KO cardiac myocytes were infected with virus expressing A-kinase activity reporter (AKAR2.2) as previously described (18). For β₁β₂AR-KO myocytes, cells were also infected with receptor virus for overnight expression. The procedure for PKA FRET activity was carried out as previously described (18).

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

RESULTS

Stimulation of the β₂AR Induces an Agonist Concentration-dependent Receptor/Gi Coupling in Cardiac Myocytes—By activation of the endogenous β₂AR with Iso in β₁AR-KO cardiac myocytes, we analyzed the receptor-induced PKA activity through a FRET assay using the fluorescent biosensor AKAR2.2. Activation of the β₂AR with 10 nM Iso induced a transient peak in PKA activity, which returned to base-line levels within 5 min (Fig. 1A). Pretreatment with PTX, which specifically inhibits Gᵢ proteins, did not significantly change the β₂AR-induced FRET response. However, a saturated concentration of 10 μM Iso resulted in a more sustained PKA FRET response, which was further enhanced with PTX pretreatment (Fig. 1B). This finding indicates that 10 μM Iso, but not 10 nM Iso, selectively induces β₂AR/Gᵢ₁ coupling for PKA activity in cardiac myocytes. We then used a cardiac contraction rate assay to examine the physiological consequences of this dose-dependent receptor/Gᵢ₁ protein coupling. We found that at 10 nM Iso, the β₁AR-induced increase in contraction rate was not PTX-sensitive (Fig. 1C), whereas PTX significantly enhanced the increase in contraction rate induced by 10 μM Iso (Fig. 1D). These data again suggest that the β₁AR couples to Gᵢ₁ proteins only after stimulation with high concentrations of agonist. Consistent with this notion, the PKA-mediated phosphorylation of phospholamban, a PKA substrate that is critical for the βAR-induced cardiac contractile response, was enhanced by PTX only after stimulation with 10 μM Iso (Fig. 1E). Previous reports have suggested that the PKA-mediated phosphorylation of the β₁AR enhances the receptor coupling to Gᵢ₁ protein (6, 7), and the GRK-dependent phosphorylation of the β₂AR occurs only after stimulation with high concentrations of agonist (13), making the phosphorylation of β₂AR a potential regulator of this dose-dependent Gᵢ₁ coupling in cardiac myocytes.
Agonist Stimulation Induces a Dose-dependent Distinct PKA and GRK Phosphorylation for β2AR Internalization and Signaling in Cardiac Myocytes—Analysis of the phosphorylation of β2AR in cardiac myocytes showed that the PKA-dependent phosphorylation at serines 261/262 occurred at subnanomolar concentrations of Iso and displayed a dose-dependent increase (Fig. 2, A and B). In contrast, the β2AR phosphorylation by GRK at serines 355/356 occurred only at 100 nM Iso or at higher doses (Fig. 2, A and C). After stimulation with saturated 10 μM Iso, the kinetics of the GRK- and PKA-mediated phosphorylation of the β2AR differed (Fig. 2D). The PKA-mediated phosphorylation showed a rapid increase, followed by a slow decrease, whereas the GRK-mediated phosphorylation was slower but more sustained over time (Fig. 2, E and F). It has been well documented that the GRK-mediated phosphorylation is involved in the endocytosis of β2AR in fibroblasts (19, 20), prompting us to explore the role of β2AR phosphorylation in receptor internalization in cardiac myocytes. The activated β2ARs showed little punctuate staining upon stimulation with 10 nM Iso in cardiac myocytes (Fig. 3A). However, stimulation with 10 μM Iso induced a time-dependent clustering of β2ARs in the cells, similar to those reported previously (8), which is a characteristic of receptor internalization (Fig. 3A). Moreover, the removal of Iso after 10 min of stimulation allowed the internalized receptor to return to the cell surface (Fig. 3A). These observations were further confirmed by quantification with a FLISA assay (Fig. 3B).

We then directly tested the role of phosphorylation in receptor internalization and signaling by using mutant β2ARs with the PKA or GRK phosphorylation sites replaced by alanines. The expressions of both PKAmut and GRKmut were quantified and equalized through Western blot and ligand binding (supplemental Fig. S1). Western blots of the mutant β2ARs also confirmed abolishment of the specific receptor phosphorylation events upon stimulation with 10 μM Iso (supplemental Fig. S2). Immunostaining of the wild type and PKAmut β2ARs
showed time-dependent internalization of the receptors after stimulation with 10 μM Iso (Fig. 4A). However, the GRKmut β2ARs failed to sufficiently internalize under Iso stimulation. Washout of Iso after 10 min of stimulation showed that the internalized receptors underwent recycling to the cell surface (Fig. 4B). Quantification through the FLISA assay confirmed that the wild type and mutant β2ARs were significantly internalized upon 10 min of stimulation with 10 μM Iso (Fig. 4C), supporting the necessary role of GRK phosphorylation in the internalization of the β2AR in cardiac myocytes. Together, the characterization of the mutant β2ARs revealed distinct actions of PKA and GRK on the receptor phosphorylation and trafficking in cardiac myocytes upon agonist stimulation, deeming the phosphorylation-deficient mutants suitable for closer examination on the effects on the receptor signaling in these cells.

By introducing either wild type or mutant β2AR into β1,β2AR-KO cardiac myocytes, we were able to examine the physiological effects of the phosphorylation of the β2AR upon receptor activation. Stimulation of the PKAmut β2AR induced a stronger contraction rate increase over that of the wild type (Fig. 5A). Stimulation of the GRKmut β2AR showed a contraction rate increase similar to that of the wild type, but this increase was much more sustained (Fig. 5A). Although the base-line contraction rates between the wild type and mutant β2ARs were not significantly different (Fig. 5B), the maximal
PKA and GRK Phosphorylation Differentially Regulate β2AR/Gi Coupling in Cardiac Myocytes—By analyzing PKA activity in cardiac myocytes induced by these mutant β2ARs, we were able to observe the kinetics of upstream signaling, which leads to contraction induced by the receptors. Stimulation of the wild type, PKAmut β2AR, or GRKmut β2AR with 10 μM Iso induced a stronger FRET increase in PKA activity, although the increase induced by the GRKmut β2AR was less than those of the wild type and PKAmut receptors (Fig. 6A). Inhibition of G proteins with PTX pre-treatment significantly enhanced the PKA FRET increase induced by activation of the wild type β2AR (Fig. 6B). However, the FRET response induced by the PKAmut β2AR was insensitive to PTX (Fig. 6C), indicating that this receptor was unable to couple to G protein. Interestingly, the FRET response induced by the GRKmut β2AR was also PTX-insensitive (Fig. 6D), indicating that the GRK-dependent phosphorylation was necessary for β2AR coupling to G protein.

Extending the PKA FRET results into the contraction rate assay reinforced the physiological effects of this phosphorylation-dependent G protein-coupling phenomenon. Although pretreatment with PTX significantly enhanced the wild type β2AR-induced increase in contraction rate upon stimulation with 10 μM Iso (Fig. 7, A and E), it did not significantly affect the PKAmut β2AR-induced increase in contraction rate (Fig. 7, B and E). Moreover, PTX did not enhance the GRKmut β2AR-induced increase in contraction rate; instead, it lowered the contraction rate increase (Fig. 7, C and E), which could be in part due to the enhanced base-line contraction rate by PTX treatment (Fig. 7D). These data suggest that both PKA and GRK phosphorylation are required for β2AR coupling to G protein, which thereafter affects myocyte contraction rate response.

We further employed a specific inhibitor of the Gi protein consisting of the C-terminal portion of Gαi2 (GCT) (21) to probe receptor/Gi coupling by either wild type or mutant β2AR.
Together, we showed that inhibition of Gi with GiCT significantly enhanced the maximal contraction rate induced by the wild type β2AR and enhanced the maximal contraction rate induced by the mutant PKAmut/β2AR. Similar to that of PTX treatment (Fig. 7C), inhibition of Gi with GiCT did not affect the base-line contraction rates (Fig. 8A). We then introduced GiCT along with wild type or mutant β2AR into β1β2AR-KO cardiac myocytes. Overexpression of GiCT significantly enhanced the wild type β2AR-induced increase in contraction rate (Fig. 8, B and F), which was similar to that obtained with PTX treatment. However, GiCT did not affect the PKAmut β2AR-induced increase in contraction rate (Fig. 8, C and F). Overexpression of GiCT did not affect the base-line contraction rates (Fig. 8E). Together, we showed that inhibition of Gi with GiCT significantly enhanced the maximal contraction rate induced by the wild type but not by the PKAmut and GRKmut β2ARs.

DISCUSSION

By measuring PKA activity and cardiac contraction rate increase, we show for the first time that β2AR activation of Gi proteins occurs selectively at high concentrations but not at low concentrations of agonist. Moreover, we show that the receptor coupling to Gi proteins is differentially regulated by the PKA- and GRK-mediated phosphorylation of the activated β2ARs. At both low and high concentrations of agonist, the activated β2ARs undergo the PKA-mediated phosphorylation. In comparison, only high concentrations of agonist induce the GRK-mediated phosphorylation of the β2ARs for subsequent internalization, which is also necessary for sufficient receptor coupling to Gi proteins (8, 9). Our studies link together various components of the β2AR, including receptor phosphorylation, receptor trafficking, and differential receptor/G protein coupling in cardiac cells, which may allow the activation of the β2AR signaling pathway to function as either a stimulatory or protective mechanism for cardiac cells under different levels of stress.

It has been well documented that the PKA-mediated phosphorylation of β2AR enhances the receptor coupling affinity to Gi protein in a reconstituted system, and mutation of the PKA phosphorylation sites blocks receptor coupling to Gi in HEK293 fibroblasts (6, 7). In this study, we have further resolved the necessity of the PKA-dependent phosphorylation of β2AR in coupling to Gi proteins in cardiac myocytes. Activation of the murine β2AR by both low and high concentration of isoproterenol leads to phosphorylation of the receptor by PKA. At high concentrations of isoproterenol, the phosphorylation levels of the β2AR by PKA are further enhanced in comparison with those at low concentrations. This is probably due to dissociation of phosphodiesterase 4D isoforms from the activated receptors (data not shown), which leads to higher local PKA activity for receptor phosphorylation. However, under stimulation with low concentrations of isoproterenol, the activated β2ARs do not couple to Gi, even if the PKA-mediated phosphorylation occurs (Figs. 1 and 2). However, disruption of the PKA phosphorylation sites on the β2AR with mutagenesis blocks the receptor coupling to Gi (Figs. 6–8). Together, these data indicate that PKA phosphorylation is necessary but not sufficient for the activated β2AR coupling to Gi in cardiac myocytes. In contrast, the β2ARs undergo GRK-dependent phosphorylation only under stimulation with high concentrations of Iso (Fig. 2). Disruption of the GRK sites prolongs the Iso-induced phosphorylation of the activated β2AR, leading to receptor internalization. These data suggest that PKA-mediated phosphorylation of the β2AR is necessary for sufficient receptor coupling to Gi proteins.
myocyte contraction rate response (Fig. 5A), which is consistent with the notion that the phosphorylation of β₂AR by GRK is required for rapid receptor desensitization (22). We do not observe obvious change in the overall PKA activity induced by the GRKmut (Fig. 6A), probably because the change is small and occurs in the local environment of the receptor and thus is not sensed by PKA probes presented throughout the cytosol. However, similar to those of the PKAmut, the PKA FRET and contraction rate responses induced by the GRKmut β₂AR do not show sensitivity to the inhibition of Gᵢ protein (Figs. 6–8), indicating that the GRK-mediated phosphorylation is required for sufficient coupling to Gᵢ. One possibility is that the GRK phosphorylated receptors undergo internalization to promote the access of the receptor to Gᵢ protein. Another possibility is that the GRK-mediated phosphorylation of the β₂AR directly enhances the binding affinity of the receptor to Gᵢ protein. However, evidence so far argues that the GRK phosphorylation-dependent receptor trafficking is more likely the key for sufficient receptor/Gᵢ coupling. To support this notion, either phosphorylation is necessary for receptor trafficking that appears to control the accessibility of β₂AR to Gᵢ protein in cardiac myocytes.

Cardiac contraction in response to adrenergic stimulation is a tightly controlled process to ensure steady increases in cardiac output without overstimulation. Our observation of an agonist concentration-dependent switch of the activated β₂AR coupling from Gₛ to Gᵢ may be critical for such fine regulation. At low concentrations of catecholamines, the activated receptors will couple only to Gₛ for a stimulatory effect on cAMP/PKA activities for increasing cardiac contraction. However, in the presence of high concentrations of catecholamines, the activated β₂ARs will switch from Gₛ to Gᵢ to reduce cAMP/PKA activities in cardiac tissues while preventing the overstimulation of cardiac contraction. The mechanism for how high concentrations of agonists can induce receptor phosphorylation by different kinases for subsequent switch of G protein coupling is currently not known. Structural studies reveal that higher concentrations of agonists induce conformational changes distinct

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**FIGURE 8.** GᵢCT inhibits β₂AR/Gᵢ coupling for myocyte contraction rate response upon agonist stimulation. A, the maximal responses in contraction rate induced by the β₂AR were enhanced by expression of GᵢCT along with the wild type β₂AR in β₂AR-KO cardiac myocytes. B–D, the β₂AR-KO cardiac myocytes expressing the β₂AR, PKAmut β₂AR, or GRKmut β₂AR were stimulated with 10 μM ISO in the presence or absence of GᵢCT. The response in myocyte contraction rate was measured. E and F, the basal levels of contraction rates and the maximal increases in contraction rates after stimulation in the presence or absence of GᵢCT were plotted. ***, p < 0.001; ****, p < 0.0001 when compared with the wild type receptor by two-way ANOVA. *p < 0.05 when compared with the group without GᵢCT in Student’s t test.
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from those induced by low concentrations (17), which may enhance the receptor binding affinity to GRK. Alternatively, low concentrations may selectively activate the Gs protein-pre-coupled pool of receptors with high affinity binding, whereas higher concentrations of agonists activate both pre-coupled and non-coupled pools of receptors. The latter pool of receptors may be involved in coupling to Gi proteins. The mechanism thus remains to be addressed. This protective mechanism by receptor/Gi coupling may be essential for preventing a high frequency of contractions, such as fibrillation during stress. In addition, β2AR/Gi coupling can play a protective role against the apoptotic effects induced by adrenergic/Gs stimulation (24–27).

More than a simple extension of the biochemical characterization of the β2AR, this study provides insightful information on the physiological effects of receptor phosphorylation by PKA and GRK. More importantly, it opens up questions about the nature of phosphorylation and its role in receptor desensitization, a concept that has been established in the last couple of decades. Undoubtedly, phosphorylation seems to play a role in diminishing the effects of receptor activation in cardiac myocytes, since abolishing those phosphorylation sites enhanced the contraction rate over the wild type upon stimulation (Fig. 4). However, we find that receptor phosphorylation appears to be critical in regulating contraction through coupling to different G proteins to protect cardiac cells from overwhelming agonist stimulation. Our study provides a clear step toward understanding the nature of receptor regulation by PKA- and GRK-mediated phosphorylation in a physiological context.

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