# β-Arrestin-mediated PDE4 cAMP phosphodiesterase recruitment regulates β-adrenoceptor switching from G$_s$ to G$_i$

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Phosphorylation of the β$_2$ adrenoreceptor (β$_2$AR) by cAMP-activated protein kinase A (PKA) switches its predominant coupling from stimulatory guanine nucleotide regulatory protein (G$_s$) to inhibitory guanine nucleotide regulatory protein (G$_i$). β-Arrestins recruit the cAMP-degrading PDE4 phosphodiesterases to the β$_2$AR, thus controlling PKA activity at the membrane. Here we investigate a role for PDE4 recruitment in regulating G protein switching by the β$_2$AR. In human embryonic kidney 293 cells overexpressing a recombinant β$_2$AR, stimulation with isoprenaline recruits β-arrestins 1 and 2 as well as both PDE4D3 and PDE4D5 to the receptor and stimulates receptor phosphorylation by PKA. The PKA phosphorylation status of the β$_2$AR is enhanced markedly when cells are treated with the selective PDE4-inhibitor rolipram or when they are transfected with a catalytically inactive PDE4D mutant (PDE4D5-D556A) that competitively inhibits isoprenaline-stimulated recruitment of native PDE4 to the β$_2$AR. Rolipram and PDE4D5-D556A also enhance β$_2$AR-mediated activation of extracellular signal-regulated kinases ERK1/2. This is consistent with a switch in coupling of the receptor from G$_s$ to G$_i$, because the ERK1/2 activation is sensitive to both inhibitors of PKA (H89) and G$_i$ (pertussis toxin). In cardiac myocytes, the β$_2$AR also switches from G$_s$ to G$_i$ coupling. Treating primary cardiac myocytes with isoprenaline induces recruitment of PDE4D3 and PDE4D5 to membranes and activates ERK1/2. Rolipram robustly enhances this activation in a manner sensitive to both pertussis toxin and H89. Adenovirus-mediated expression of PDE4D5-D556A also potentiates ERK1/2 activation. Thus, receptor-stimulated β-arrestin-mediated recruitment of PDE4 plays a central role in the regulation of G protein switching by the β$_2$AR in a physiological system, the cardiac myocyte.

**T**he functions of G protein-coupled receptors (7MS or GPCRs) such as the β$_2$ adrenoreceptor (β$_2$AR) are highly regulated by their agonist-stimulated phosphorylation by both second messenger-stimulated kinases [protein kinase A (PKA) and protein kinase C (PKC)] and the specialized G protein-coupled receptor kinases (GRKs) (1). Phosphorylation of receptors by either PKA or PKC directly uncouples them from their cognate G proteins, thereby decreasing the amplitude of the evoked signal. Recently, studies have revealed that phosphorylation by PKA of some stimulatory guanine nucleotide regulatory protein (G$_s$)-coupled receptors (2–5) not only decreases their coupling to G$_s$ but switches their coupling to inhibitory guanine nucleotide regulatory protein (G$_i$) with two consequences: it further decreases the rate of cAMP generation, because G$_i$ activation inhibits adenylyl cyclase activity, and it couples the receptors to G$_i$-linked pathways such as activation of the extracellular signal-regulated kinases ERK1/2 and Akt.

Phosphorylation of 7MS receptors by GRKs promotes binding of arrestins to the phosphorylated receptors. β-Arrestin "desensitizes" the receptors by sterically interdicting signaling to the G proteins (6–8) and can also serve as an adaptor that links the

receptors to a variety of signaling pathways (e.g., mitogen-activated protein kinases and nonreceptor tyrosine kinases) and elements of the clathrin-dependent endocytic machinery (9).

Recently it was demonstrated (10) that the ability of the GRK/β-arrestin system to desensitize the β$_2$AR is not restricted to dampening the rate of cAMP generation, but it also increases the local rate of cAMP degradation. This is accomplished by an agonist-stimulated, β-arrestin-mediated recruitment of phosphodiesterase (PDE)4D cAMP-specific PDE isoforms to the receptor (10). Thus, expression in cells of a catalytically inactive PDE4D that competes with endogenous PDE4s for binding to β-arrestins increases the level of β$_2$AR-mediated activation of PKA at the plasma membrane. Because PDEs provide the sole route for degradation of cAMP in cells, these enzymes are poised to play a key role in controlling cAMP signaling (11–14). Of these enzymes, the PDE4 cAMP-specific PDE family has attracted much interest recently because PDE4-selective inhibitors, of which rolipram is the archetype, have therapeutic potential in a wide range of disorders (15–18). Four genes (PDE4A–PDE4D) encode >16 different PDE4 isoforms, each characterized by a unique N-terminal region and showing distinct regulatory properties and modes of intracellular targeting (12, 14, 19, 20).

Here we investigate how the mechanisms of PKA-mediated switching of β$_2$AR coupling from G$_s$ to G$_i$ and the β-arrestin-mediated recruitment of PDE4D interact to regulate the receptor-mediated activation of ERK1/2.

## Methods

**Reagents.** The D556A-PDE4D5 mutant in pcDNA3 was used as described (10). D556A-PDE4D5 was also transferred into the pAdTrack-cytomegalovirus (CMV) previrus vector by using *Hin*dIII + *Sal*I, which was inserted into the attenuated adenovirus genome (pAdEasy-1 plasmid) by cotransformation with *Pme*I-linearized pAdTrack-CMV-D556A-PDE4D5 construct into BJ5183 *Escherichia coli*. Recombinant plasmids were checked by digestion with *Pac*I, and positive clones were identified. Recombinant adenovirus was produced by transfecting human embryonic kidney (HEK) 293 cells with the recombinant D556A-PDE4D5-pAdEasy plasmids. The RSV-protein kinase inhibitor plasmid was a kind gift from Stan McKnight (Seattle) (21). The plasmid encoding the β$_2$AR with PKA serine target sites mutated was as described (5).

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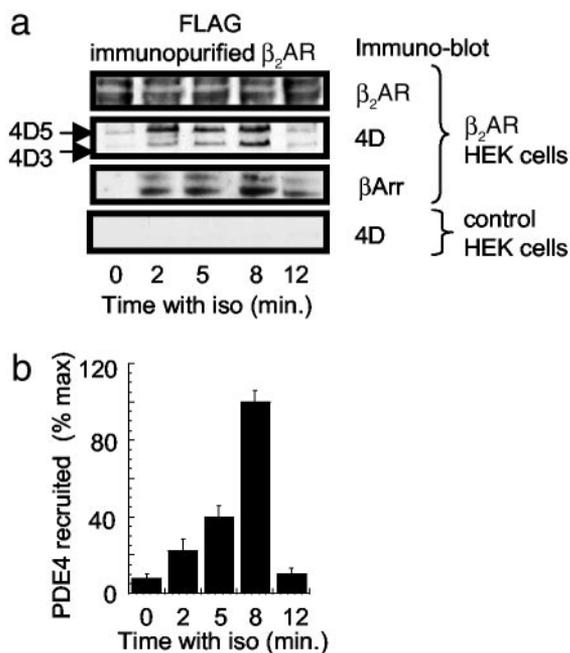
**Fig. 1.** Recruitment of PDE4D to the $\beta_2$AR in HEK 293 cells. HEK 293 cells were challenged with isoprenaline (iso, 10 $\mu$M) and harvested at the indicated times, and the $\beta_2$AR, immunopurified on anti-FLAG antibody, was conjugated to beads. (*a*) HEK 293 cells transfected to express a FLAG-tagged $\beta_2$AR. Blotting detects a doublet for the $\beta_2$AR, PDE4D5 and PDE4D3 isoforms, and $\beta$-arrestin1/2 ($\beta$Arr) isoforms. (*a Lower*) An identical FLAG immunopurification protocol was performed but on native HEK 293 cells, showing no PDE4D recruitment. (*b*) Total immunoreactive PDE4D recruited over time in the $\beta_2$AR-transfected HEK 293 cells (mean $\pm$ SD for *n* = 3 experiments).

**Cell Culture.** HEK 293 cells lines stably overexpressing the FLAG-tagged $\beta_2$AR-GFP were cultured as described (22). Preparation and short-term culture of neonatal rat ventricular myocytes were as described (23, 24), where 24 h after plating they were placed in low-serum medium.

**Immunological Reagents and Immunopurification.** The PDE4D4-specific antiserum used was as described (25). mAbs (New England Biolabs) were used to detect the native and phosphorylated forms of ERK1/2 as described (26–28). A phospho-serine PKA substrate antibody (Cell Signaling Technologies, Beverly, MA) was used to detect PKA phosphorylation of the $\beta_2$AR. A rabbit polyclonal antiserum specific for the $\beta_2$AR was obtained from Santa Cruz Biotechnology (Autogen Bioclear, Wittshire, U.K.). Immunoblotting was done as described (26–28) by using $\approx$20-$\mu$g protein samples. For immunopurification, cells were harvested in lysis buffer (25 mM Hepes/2.5 mM EDTA/50 mM NaC1/50 mM NaF/30 mM sodium pyrophosphate/10% glycerol/1% Triton X-100, pH 7.5, with added protease inhibitors) after specified treatments. Isolation of FLAG-tagged $\beta_2$AR was done with 50 $\mu$l of anti-FLAG M2 antibody conjugated to agarose (Sigma) by using 1 mg of cellular protein for 2 h at 4°C as described (27, 29). Samples were washed three times in lysis buffer before the proteins were solubilized in Laemmli (30) buffer. Proteins were separated by PAGE and transferred to nitrocellulose for Western blotting. Protein concentration was determined with BSA as standard (31).

## Results

**Agonist-Recruited PDE4 Regulates the PKA Phosphorylation Status of the $\beta_2$AR.** In agreement with previous findings (10), isoprenaline stimulation of HEK 293 cells overexpressing the $\beta_2$AR causes not only the transient recruitment of $\beta$-arrestin (Fig. 1) but also of both endogenous PDE4D3 and PDE4D5 long isoforms to the immunopurified $\beta_2$AR (Fig. 1). $\beta_2$AR-mediated recruitment of PDE4 activity has been shown to regulate plasma membrane PKA activity (10), suggesting a role in regulating local cAMP levels after agonist-dependant recruitment. It is well established that PKA can cause the phosphorylation of the $\beta_2$AR (4), raising the possibility that specifically recruited PDE4 could influence this process through the regulation of membrane-associated PKA activity. Here we have followed this phosphorylation by immunopurifying the $\beta_2$AR and immunoblotting for its phosphorylation by PKA using a PKA substrate-specific antibody used in other studies to detect the PKA-specific phosphorylation of a variety of proteins (32–35). Challenge of HEK 293 cells with isoprenaline causes a time-dependent increase in PKA phosphorylation of the immunopurified $\beta_2$AR (Fig. 2 *a* and *b*). This effect is not only dramatically enhanced by the PDE4-selective inhibitor, rolipram (Fig. 2 *a* and *b*), but is also attenuated by
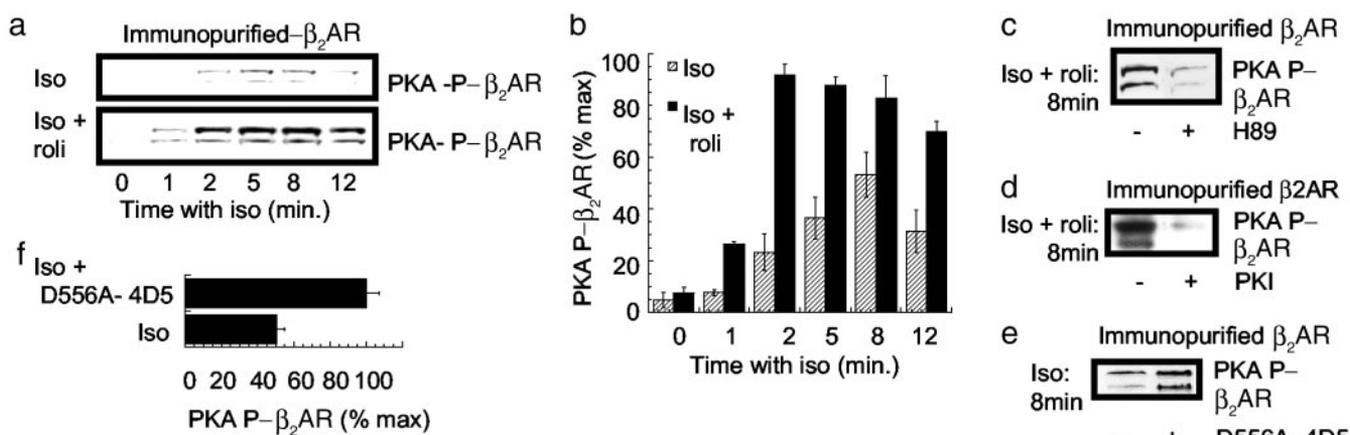


**Fig. 2.** Isoprenaline-induced phosphorylation of the $\beta_2$AR in HEK 293 cells. In all these experiments at the indicated times, HEK 293 cells overexpressing the $\beta_2$AR were harvested, and the PKA phosphorylation status of the anti-FLAG-immunopurified $\beta_2$AR was assessed by using the PKA substrate antibody. In all instances lanes were loaded equally with immunopurified receptor. (*a*) Cells were challenged with either isoprenaline (Iso, 10 $\mu$M) alone or together with rolipram (Iso + roli, 10 $\mu$M). Lanes were loaded equally with immunopurified receptor. (*b*) Quantification of the data in *a* for *n* = 3 experiments with means $\pm$ SD. (*c*) Cells were treated for 8 min with isoprenaline (10 $\mu$M) plus rolipram (10 $\mu$M) in either the absence or presence of the PKA inhibitor H89 (1 $\mu$M). (*d*) Cells were treated for 8 min with isoprenaline (10 $\mu$M) plus rolipram (10 $\mu$M) either without or with transfection of protein kinase inhibitor (PKI), the PKA inhibitor (21). (*e*) As indicated, cells were transfected to express D556A-PDE4D5 before being treated for 8 min with isoprenaline (10 $\mu$M) before analysis. (*f*) Quantification of the data shown in *d* for *n* = 3 experiments with means $\pm$ SD.
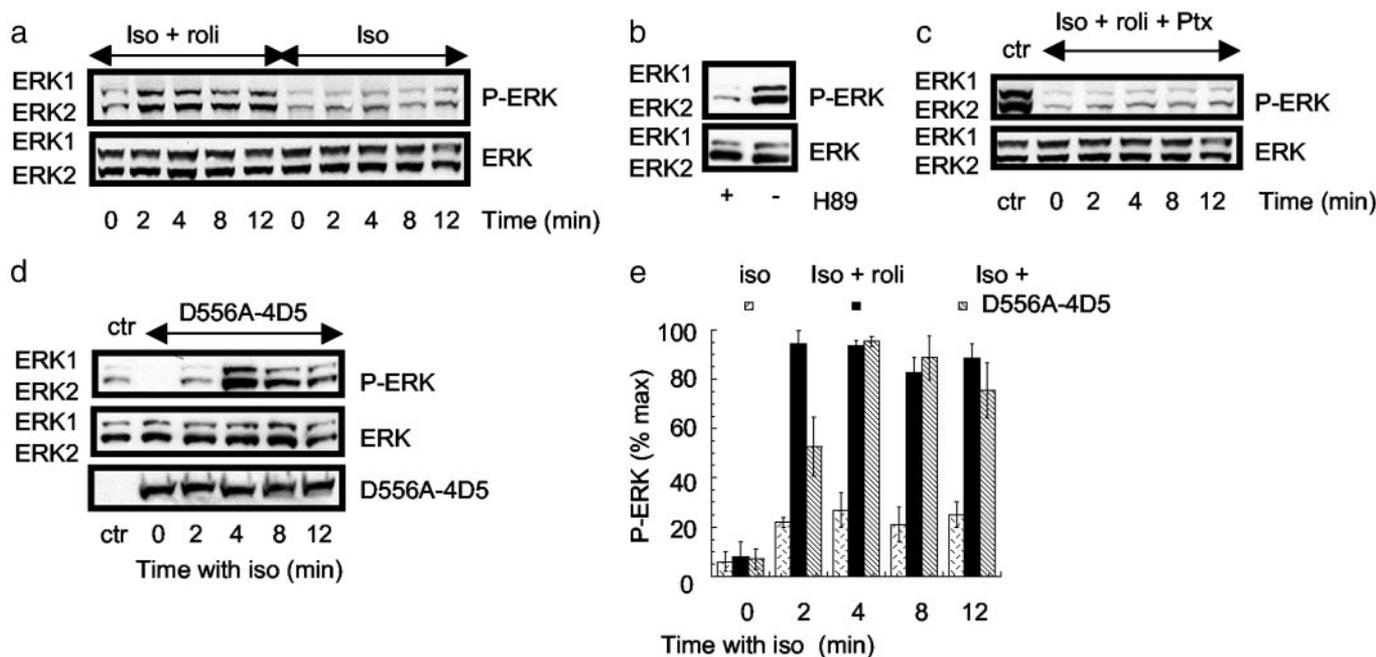
**Fig. 3.** $\beta_2$AR-mediated activation of ERK in HEK 293 cells. These analyses were all done on HEK 293 cells overexpressing the $\beta_2$AR. Each experiment was done three times. (*a*) Cells were treated with either isoprenaline (Iso, 10 $\mu$M) alone or together with rolipram (Iso + roli, 10 $\mu$M) before harvesting as indicated and immunoblotting for either total ERK or active P-ERK. The two immunoreactive species identified reflect the 42- and 44-kDa forms of ERK. (*b*) Cells were treated with isoprenaline (10 $\mu$M) together with rolipram (10 $\mu$M) either in the absence or presence of H89 (1 $\mu$M), harvested at 4 min, and immunoblotted for ERK and active P-ERK. (*c*) Cells were pretreated with pertussis toxin (25 ng/ml for 16 h) before challenge with isoprenaline (10 $\mu$M) together with rolipram (10 $\mu$M) and immunoblotted for ERK and P-ERK. A control (ctr) experiment was done in which cells that had not been subjected to pretreatment with pertussis toxin were challenged with isoprenaline and rolipram for 4 min before harvesting. (*d*) Cells were transfected with D556A-PDE4D5 before challenge with isoprenaline (10 $\mu$M) alone and then immunoblotted for ERK and P-ERK. A control experiment was done by using cells that had not been transfected with D556A-PDE4D5 but were challenged with isoprenaline for 4 min. (*e*) Quantification of the time course for ERK activation in cells treated with either isoprenaline (10 $\mu$M) alone or together with rolipram (10 $\mu$M) as well as the effect of isoprenaline on cells that had been transfected to express the D556A-PDE4D5 construct. Shown are means ± SD.

treatment with the PKA inhibitor H89 (Fig. 2*c*) and transfection of cells with the PKA protein kinase inhibitor (21) (Fig. 2*d*). In addition to this, challenge of HEK 293 cells for 8 min with isoprenaline (10 $\mu$M) and rolipram (10 $\mu$M) failed to cause PKA phosphorylation, as detected with the PKA substrate antibody, of a mutant form of the $\beta_2$AR, in which the sites for PKA phosphorylation had been mutated to alanine (data not shown; ref. 5). These data are consistent with agonist-dependent PKA phosphorylation of the $\beta_2$AR.

Because the PDE4-selective inhibitor rolipram inhibits both recruited and nonrecruited PDE4, we were unable to discriminate between the regulation of $\beta_2$AR phosphorylation by PKA exerted specifically by recruited PDE4 and that exerted by all cellular PDE4s. To address this point we exploited a dominant negative approach used previously to show that $\beta$-agonist-recruited PDE4 selectively regulates plasma membrane PKA (10). This involved generating a catalytically inactive form of PDE4D5 able to displace endogenous active PDE4D species from $\beta$-arrestin, preventing any $\beta_2$-agonist-stimulated increase in receptor-associated PDE activity. The rationale for generating such a species was to make as subtle a change as possible to the enzyme to ablate catalytic activity while retaining its ability to bind $\beta$-arrestin. To do this we took advantage of the known 3D structure of the PDE4 catalytic domain (36). The insertion of a single point mutation in the $Zn^{2+}$-binding domain of PDE4D5 (Asp-556 $\rightarrow$ Ala, D556A) ablated catalytic activity without altering its $\beta$-arrestin-binding properties. Thus, D556A-PDE4D5 competes with the endogenous and active PDE4 species for $\beta$-arrestin binding, preventing $\beta_2$-agonist-stimulated increases in receptor-associated PDE4 activity (10). When D556A-PDE4D5 was expressed in cells, it dramatically enhanced

the ability of isoprenaline to cause PKA-mediated phosphorylation of the $\beta_2$AR (Fig. 2 *e* and *f*). Thus $\beta$-agonist-mediated recruitment of PDE4 regulates the PKA phosphorylation status of the $\beta_2$AR.

**Recruited PDE4 Regulates Switching of the Coupling of the $\beta_2$AR Between the $G_s$ and $G_i$ Signaling Pathways in HEK 293 Cells.** In untransfected HEK 293 cells, PKA-mediated switching of the $\beta_2$AR from $G_s$ to $G_i$ coupling is evident when isoprenaline stimulation leads to activation of ERK1/2 through a pathway that is sensitive to both the PKA inhibitor H89 and $G_i$ inhibitor pertussis toxin (2). In the $\beta_2$AR-overexpressing HEK 293 cells, isoprenaline causes a small and transient increase in ERK1/2 activity (Fig. 3*a*), the time course of which parallels that observed for the PKA phosphorylation status of the immunopurified $\beta_2$AR (Fig. 2 *a* and *b*). This activation of ERK1/2 clearly depends on PKA activity, because it is enhanced greatly by the PDE4 inhibitor, rolipram (10 $\mu$M) (Fig. 3*a*) and inhibited by the PKA inhibitor, H89 (Fig. 3*b*). Furthermore, the activation is $G_i$-dependent, because it is ablated by treatment with pertussis toxin (Fig. 3*c*), indicating that ERK1/2 are being stimulated by $G_s$ to $G_i$ switching by the $\beta_2$AR. To assess to what extent PDE4 recruitment inhibits $\beta_2$AR switching, we used the dominant negative approach by transfecting the HEK 293 cell line with D556A-PDE4D5. Challenge of these cells with isoprenaline causes a marked potentiation of ERK1/2 phosphorylation (Fig. 3 *d* and *e*) akin to that seen using rolipram (Fig. 3 *a* and *e*), demonstrating that the recruited PDE4 plays a pivotal role in limiting the level of $\beta_2$AR switching in these cells.

**Dominant Negative PDE4D5 Amplifies Isoprenaline-Mediated ERK Activation in Cardiac Myocytes.** Next, we set out to explore the involvement of PDE4 recruitment in a primary cell-culture
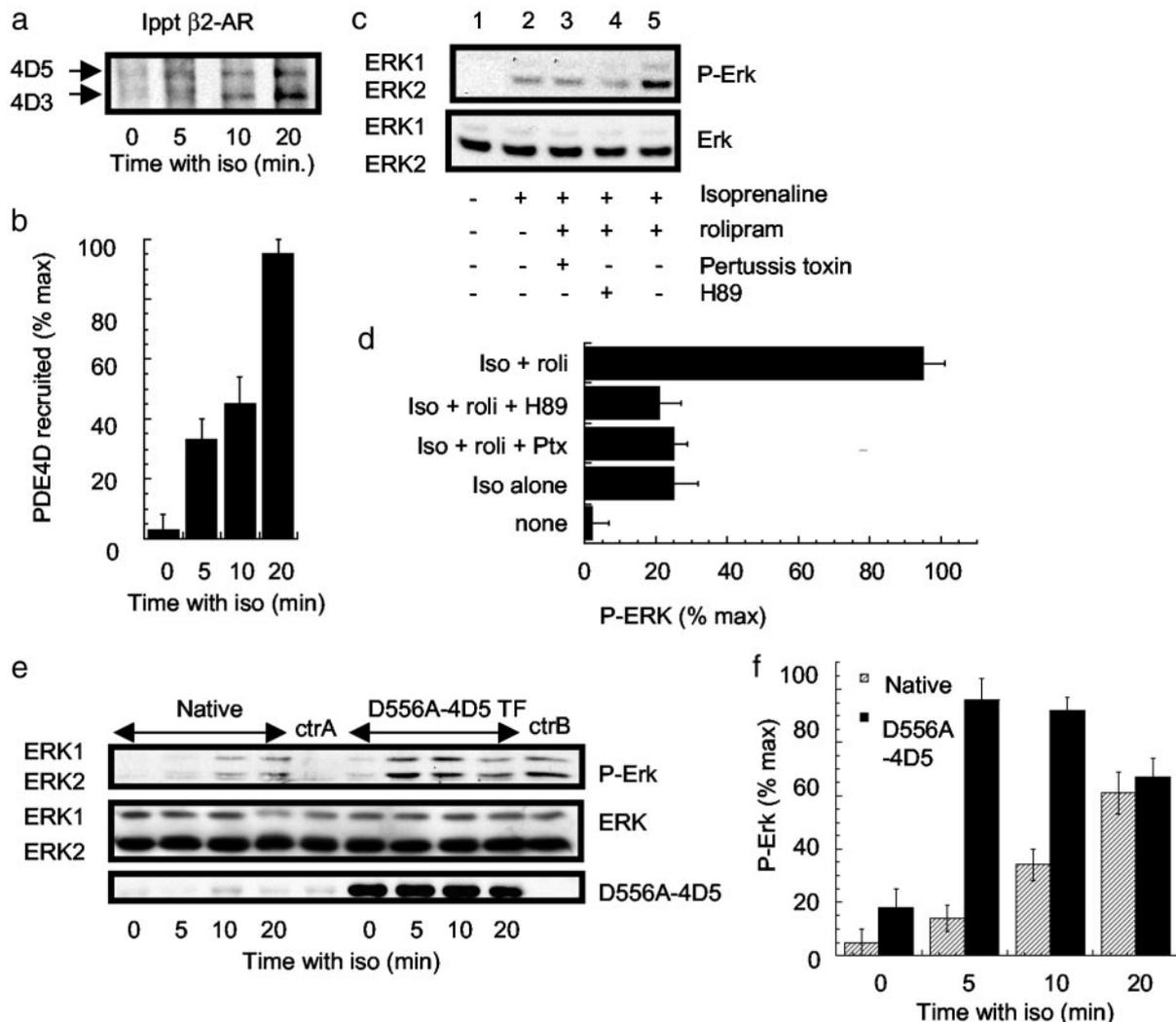
**Fig. 4.** $\beta_2$AR-mediated activation of ERK in primary cardiac myocytes. (*a*) Myocytes were challenged as indicated with isoprenaline (iso, 10 $\mu$M) and harvested, and the immunopurified $\beta_2$AR was immunoblotted for PDE4D. PDE4D5 (105 kDa, *Upper*) and PDE4D3 (95 kDa, *Lower*) isoforms were identified. (*b*) Quantification of the data shown in *a* (means $\pm$ SD). (*c*) Myocytes were challenged with the indicated ligands for 10 min before Western blotting for ERK and P-ERK. In some instances, as indicated, cells were pretreated for 16 h with 25 ng/ml pertussis toxin, 10 $\mu$M isoprenaline, 10 $\mu$M rolipram, or 1 $\mu$M H89. (*d*) Quantification of the data shown in *c* for three experiments with means $\pm$ SD. (*e*) Myocytes were challenged for the indicated times with isoprenaline (10 $\mu$M) before Western blotting for ERK and P-ERK. DN-4D5 indicates cells transfected to express D556A-PDE4D5 by adenovirus-mediated gene transfer as evaluated by immunoblotting. The level of recombinant inactive PDE4D5 was 50- to 60-fold greater than that of endogenous PDE4D3 and PDE4D5 species, which made up the greater fraction ($\approx$60%) of the total PDE4 activity in these cells. Control experiments (ctrA) were performed with cells exposed to adenovirus allowing GFP expression alone. Here ctrA indicates cells exposed to control virus and treated for 10 min with isoprenaline (10 $\mu$M), and ctrB indicates such cells treated for 10 min with both isoprenaline (10 $\mu$M) and rolipram (10 $\mu$M). (*f*) Quantification of the time-course data shown in *e* for three experiments with means $\pm$ SD. Each experiment was performed three times.

system in which $\beta_2$AR switching is known to occur (37). It has been reported (37) that the $\beta_2$AR stimulates ERK1/2 activity in cardiac myocytes by a $G_i$- and PKA-dependent mechanism. Here we show that their treatment with isoprenaline also causes a time-dependent recruitment of both endogenous PDE4D3 and PDE4D5 to the immunoprecipitated $\beta_2$AR (Fig. 4 *a* and *b*) similar to that seen in HEK 293 cells (Fig. 1 *a* and *b*). Concomitantly, isoprenaline challenge also increases ERK1/2 phosphorylation (Fig. 4*c*). This effect increases dramatically after the addition of rolipram through a process attenuated in cells treated with either pertussis toxin or H89 (Fig. 4 *c* and *d*), which indicates that $\beta_2$AR switching to $G_i$ leads to ERK activation in cardiac myocytes and is controlled by PDE4 activity in these cells. Furthermore, when the catalytically inactive D556A-PDE4D5 is expressed in cardiac myocytes by using adenoviral-mediated gene transfer, the ability of isoprenaline to activate ERK1/2 is

enhanced (Fig. 4 *e* and *f*) to a level similar to that seen after rolipram treatment, whereas no enhancement is seen in cells treated with the nonrecombinant adenovirus (Fig. 4*e*). Taken together, these data indicate that, in cardiac myocytes, switching by the $\beta_2$AR from coupling to $G_s$ to $G_i$, measured by ERK1/2 activation, is regulated by the recruitment of PDE4 activity to the $\beta_2$AR.

## Discussion

The $\beta_2$AR has long served as a prototype for understanding the function and regulation of 7MS receptors (1). The findings presented here demonstrate the complex series of controls that serve not only to regulate the intensity of receptor signaling down a specific pathway but how this signaling can be redirected between different pathways. Traditionally, the role of the $\beta_2$AR has been seen as eliciting activation of adenylyl cyclase via

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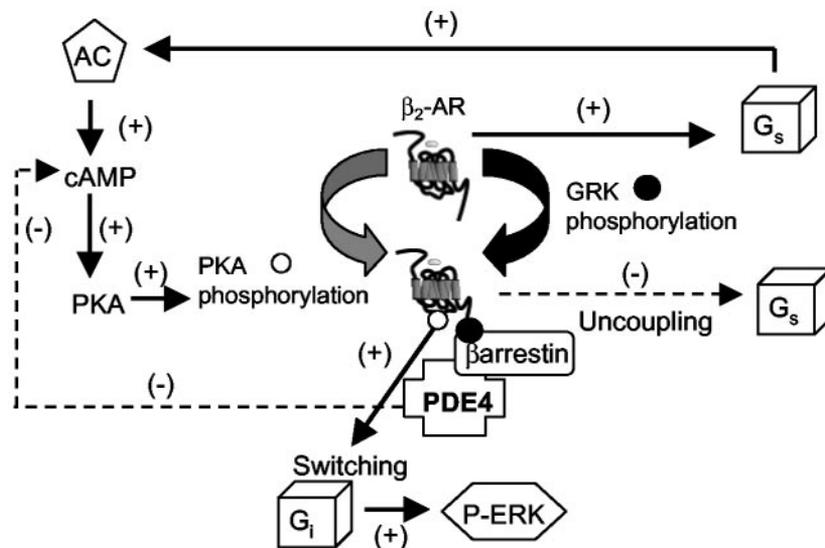
**Fig. 5.** A schematic representation of the role of arrestin-recruited PDE4 in regulating the ''switching'' of the $\beta_2$AR from $G_s$ to $G_i$ stimulation. Agonist occupancy of the $\beta_2$AR initially leads to coupling to $G_s$, which causes activation of adenylyl cyclase, elevated cAMP levels, and activation of PKA, which is able to phosphorylate the $\beta_2$AR. Concomitantly, agonist occupancy also leads to GRK-mediated phosphorylation of the $\beta_2$AR, which allows for the recruitment of $\beta$-arrestin together with bound PDE4. PKA phosphorylation of the $\beta_2$AR confers switching from $G_s$ to $G_i$, with consequent activation of ERK1/2. However, $\beta$-arrestin-recruited PDE4 provides a negative feedback loop, the role of which is to attenuate local cAMP levels and thus the ability of membrane PKA to phosphorylate the $\beta_2$AR. This action of $\beta$-arrestin-recruited PDE4 is uncovered by dominant negative PDE4, which replaces the active endogenous recruited PDE4 to ablate the negative feedback loop and thus accentuate switching to $G_i$.

coupling to $G_s$. Such coupling, in itself, is regulated rapidly through GRK-mediated receptor phosphorylation, with the ensuing recruitment of cytosolic $\beta$-arrestin preventing receptor coupling to $G_s$. Long-term control then occurs through receptor internalization and degradation pathways.

However, it has been recognized for some time that PKA itself, which is activated by the ensuing rise in cAMP levels, can serve to phosphorylate the $\beta_2$AR. This modification seemingly is unconnected to the primary recruitment of $\beta$-arrestin, which occurs through GRK-mediated phosphorylation. Instead, it serves to reprogram the G protein-coupling specificity of the $\beta_2$AR such that it couples less well to $G_s$ (desensitization) and can now couple to $G_i$ (2–5). Such switching mechanisms have also been described for the vasoactive intestinal peptide (38) and prostacyclin receptors (39, 40). Arrestin serves as an agonist-recruitable signaling scaffold protein (9). We have demonstrated (10) that PDE4 cAMP-specific PDEs form a key part of this complex such that the $\beta$-arrestin-mediated recruitment of PDE4 to the receptor can regulate membrane PKA activity. The present work adds a functional dimension to this. Thus the recruited PDE4 itself can determine the PKA phosphorylation status of the $\beta_2$AR and hence its ability to couple to $G_i$ and effect ERK activation. Indeed, it has been shown that the $\beta_2$AR can interact with PKA anchor proteins able to specifically recruit PKA and thereby facilitate PKA phosphorylation of the $\beta_2$AR (41, 42). It thus would seem that $\beta$-arrestin-recruited PDE4 controls the activity of such a privileged pool of PKA, which determines the PKA phosphorylation status of the $\beta_2$AR itself.

These data serve to enhance our understanding of the targeting of PDE4 isoforms to specific intracellular sites (14, 19, 20) and of the sophistication of control processes that allow for the reprogramming of $\beta_2$AR signaling (4). Because PDE4 isoforms show specific expression patterns (14, 18, 20), susceptibility of

the $\beta_2$AR to control by PDE4 activity will depend very much on cell type. Indeed, the complexity of $\beta_2$AR signaling pathways is extended further by the occurrence of isoforms of various regulatory proteins involved. Thus very different targeting and regulation is seen for the various RI and RII subunits of PKA, PKA anchor proteins (43), and also for Raf isoforms, which show either positive or negative coupling to changes in cAMP levels and an ERK phosphatase, the action of which is inhibited by PKA (44). Thus, depending on the cellular expression of isoforms of $\beta$-arrestin (with $\beta$-arrestin 2 preferred by the $\beta_2$AR), PDE4, PKA subunits, and PKA anchor proteins, very different patterns of $\beta_2$AR phosphorylation by PKA and thus switching to $G_i$ pathways and ERK may ensue.

Nonetheless, in both HEK 293 cells and the more physiological primary cardiac myocytes, we have here identified a paradigm for the control of $\beta_2$AR signaling. Occupancy of the $\beta_2$AR causes recruitment of the arrestin scaffold protein with bound PDE4. $\beta$-Arrestin-recruited PDE4 regulates the functioning of a (plasma) membrane-associated PKA activity that serves to phosphorylate the $\beta_2$AR. This recruited PDE4 controls the PKA-mediated switching of the $\beta_2$AR from coupling to $G_s$ and the activation of adenylyl cyclase to coupling to $G_i$ and hence signaling through the ERK and, presumably, other pathways (Fig. 5). These findings demonstrate an additional aspect of the complex ways in which the $\beta$-arrestin-mediated recruitment of signal-modifying proteins such as PDE4 to the $\beta_2$AR can regulate receptor activity.

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