

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 adrenoceptor (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.

The functions of G protein-coupled receptors (7MS or GPCRs) such as the β_2 adrenoceptor (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 *Hind*III + *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).

Abbreviations: β_2 AR, β_2 adrenoceptor; PKA, protein kinase A; GRK, G protein-coupled receptor kinase; G_s , stimulatory guanine nucleotide regulatory protein; G_i , inhibitory guanine nucleotide regulatory protein; ERK, extracellular signal-regulated kinase; PDE, phosphodiesterase; HEK, human embryonic kidney.

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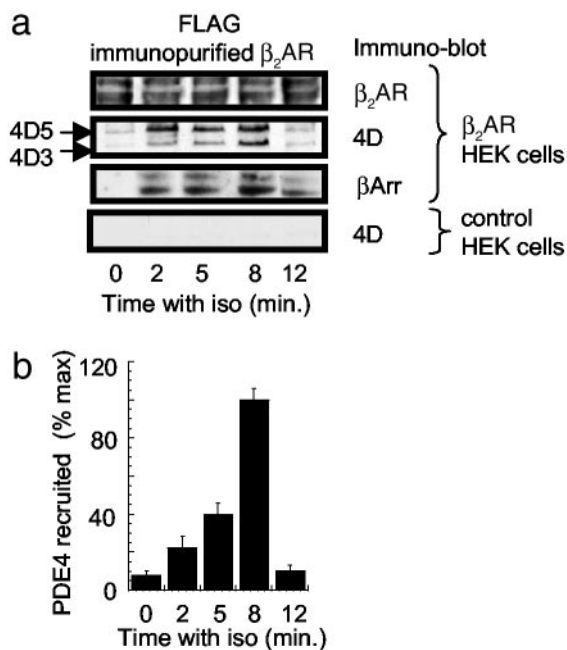


Fig. 1. Recruitment of PDE4D to the β_2 AR in HEK 293 cells. HEK 293 cells were challenged with isoprenaline (iso, 10 μ M) and harvested at the indicated times, and the β_2 AR, immunopurified on anti-FLAG antibody, was conjugated to beads. (a) HEK 293 cells transfected to express a FLAG-tagged β_2 AR. Blotting detects a doublet for the β_2 AR, PDE4D5 and PDE4D3 isoforms, and β -arrestin1/2 (β 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 β_2 AR-transfected HEK 293 cells (mean \pm SD for $n = 3$ experiments).

Cell Culture. HEK 293 cells lines stably overexpressing the FLAG-tagged β_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 β_2 AR. A rabbit polyclonal antiserum specific for the β_2 AR was obtained from Santa Cruz Biotechnology (Autogen Bioclear, Wittshire, U.K.). Immunoblotting was done as described (26–28) by using ≈ 20 - μ g protein samples. For immunopurification, cells were harvested in lysis buffer (25 mM HEPES/2.5 mM EDTA/50 mM NaCl/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 β_2 AR was done with 50 μ 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 β_2 AR.

In agreement with previous findings (10), isoprenaline stimulation of HEK 293 cells overexpressing the β_2 AR causes not only the transient recruitment of β -arrestin (Fig. 1) but also of both endogenous PDE4D3 and PDE4D5 long isoforms to the immunopurified β_2 AR (Fig. 1). β_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 β_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 β_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 β_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

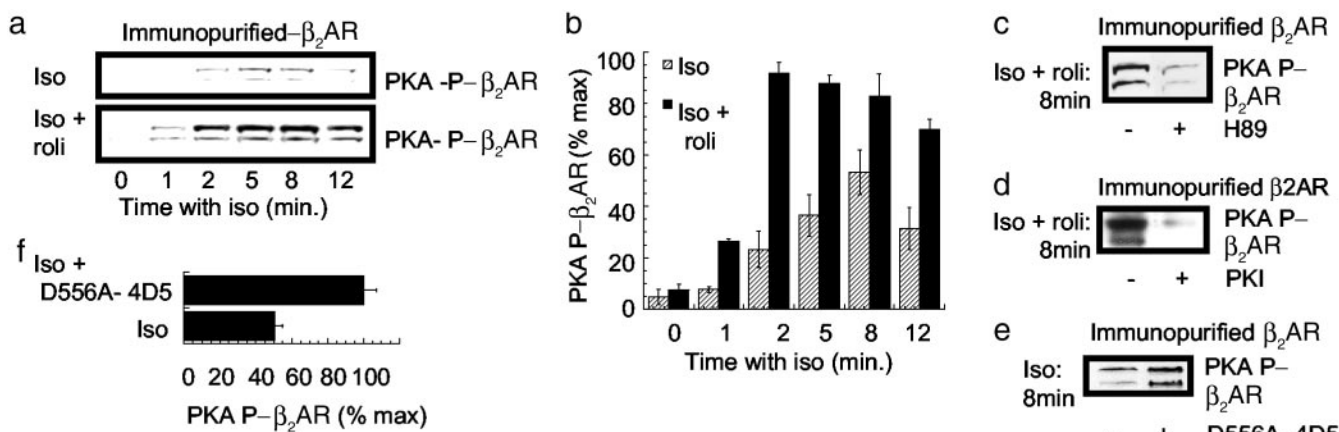


Fig. 2. Isoprenaline-induced phosphorylation of the β_2 AR in HEK 293 cells. In all these experiments at the indicated times, HEK 293 cells overexpressing the β_2 AR were harvested, and the PKA phosphorylation status of the anti-FLAG-immunopurified β_2 AR was assessed by using the PKA substrate antibody. In all instances lanes were loaded equally with immunopurified receptor. (a) Cells were challenged with either isoprenaline (Iso, 10 μ M) alone or together with rolipram (Iso + roli, 10 μ 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 μ M) plus rolipram (10 μ M) in either the absence or presence of the PKA inhibitor H89 (1 μ M). (d) Cells were treated for 8 min with isoprenaline (10 μ M) plus rolipram (10 μ 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 μ M) before analysis. (f) Quantification of the data shown in d for $n = 3$ experiments with means \pm SD.

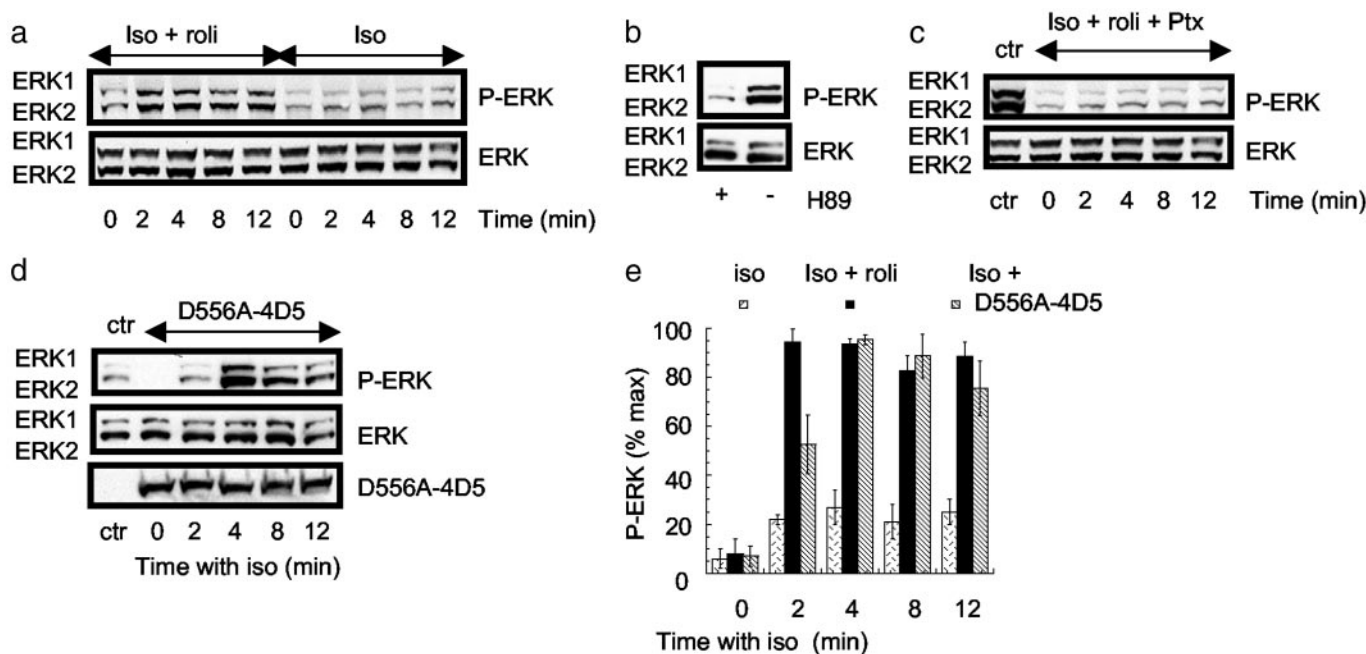


Fig. 3. β_2 AR-mediated activation of ERK in HEK 293 cells. These analyses were all done on HEK 293 cells overexpressing the β_2 AR. Each experiment was done three times. (a) Cells were treated with either isoprenaline (Iso, 10 μ M) alone or together with rolipram (Iso + roli, 10 μ M) before harvesting 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 μ M) together with rolipram (10 μ M) either in the absence or presence of H89 (1 μ 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 μ M) together with rolipram (10 μ 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 μ 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 μ M) alone or together with rolipram (10 μ M) as well as the effect of isoprenaline on cells that had been transfected to express the D556A-PDE4D5 construct. Shown are means \pm SD.

treatment with the PKA inhibitor H89 (Fig. 2c) and transfection of cells with the PKA protein kinase inhibitor (21) (Fig. 2d). In addition to this, challenge of HEK 293 cells for 8 min with isoprenaline (10 μ M) and rolipram (10 μ M) failed to cause PKA phosphorylation, as detected with the PKA substrate antibody, of a mutant form of the β_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 β_2 AR.

Because the PDE4-selective inhibitor rolipram inhibits both recruited and nonrecruited PDE4, we were unable to discriminate between the regulation of β_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 β -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 β -arrestin, preventing any β_2 -agonist-stimulated increase in receptor-associated PDE4 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 β -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 Zn²⁺-binding domain of PDE4D5 (Asp-556 \rightarrow Ala, D556A) ablated catalytic activity without altering its β -arrestin-binding properties. Thus, D556A-PDE4D5 competes with the endogenous and active PDE4 species for β -arrestin binding, preventing β_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 β_2 AR (Fig. 2e and f). Thus β -agonist-mediated recruitment of PDE4 regulates the PKA phosphorylation status of the β_2 AR.

Recruited PDE4 Regulates Switching of the Coupling of the β_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 β_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 β_2 AR-overexpressing HEK 293 cells, isoprenaline causes a small and transient increase in ERK1/2 activity (Fig. 3a), the time course of which parallels that observed for the PKA phosphorylation status of the immunopurified β_2 AR (Fig. 2a and b). This activation of ERK1/2 clearly depends on PKA activity, because it is enhanced greatly by the PDE4 inhibitor, rolipram (10 μ M) (Fig. 3a) and inhibited by the PKA inhibitor, H89 (Fig. 3b). Furthermore, the activation is G_i-dependent, because it is ablated by treatment with pertussis toxin (Fig. 3c), indicating that ERK1/2 are being stimulated by G_s to G_i switching by the β_2 AR. To assess to what extent PDE4 recruitment inhibits β_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. 3d and e) akin to that seen using rolipram (Fig. 3a and e), demonstrating that the recruited PDE4 plays a pivotal role in limiting the level of β_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

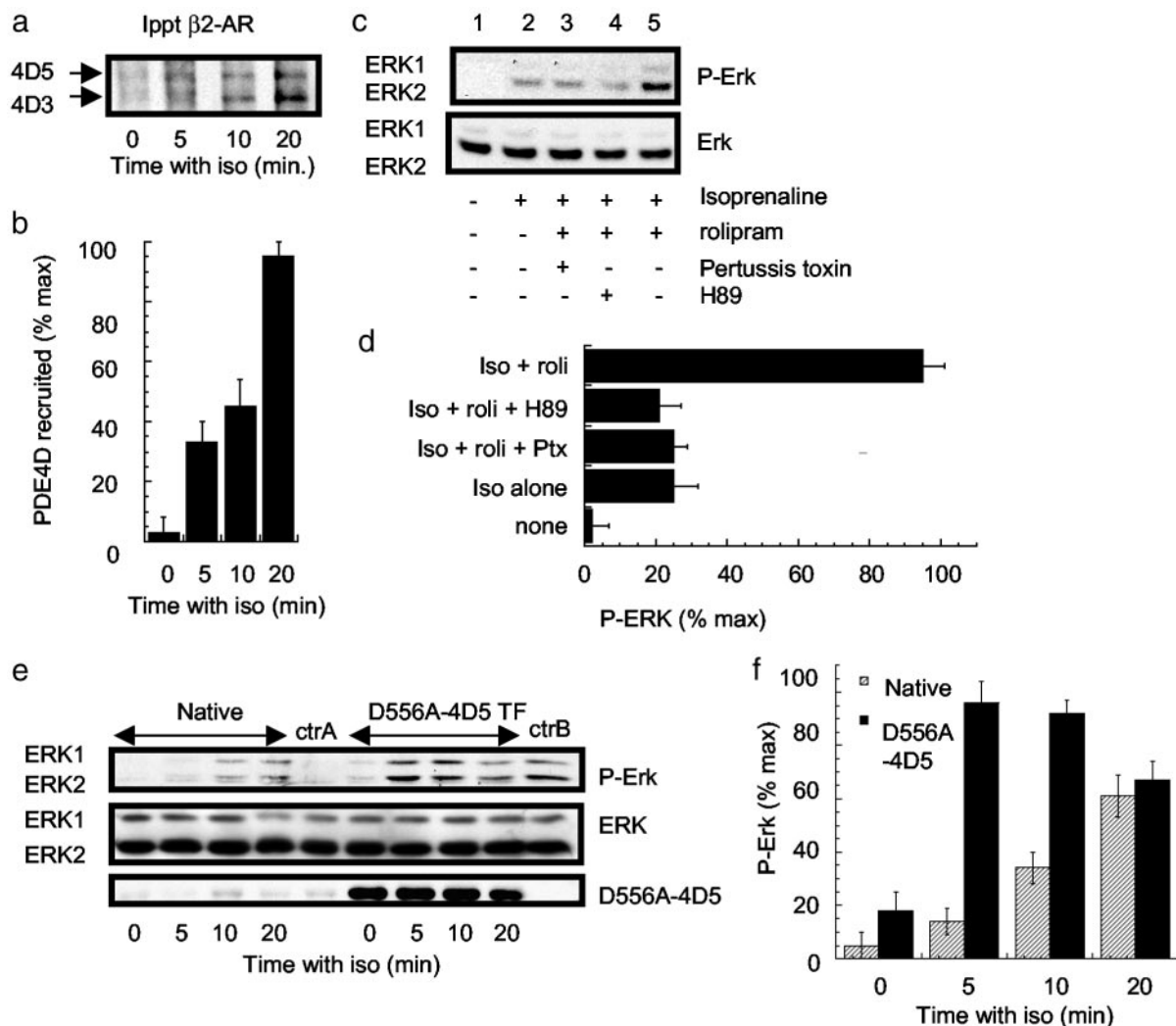


Fig. 4. β_2 AR-mediated activation of ERK in primary cardiac myocytes. (a) Myocytes were challenged as indicated with isoprenaline (iso, 10 μ M) and harvested, and the immunoprecipitated β_2 AR was immunoblotted for PDE4D. PDE4D5 (105 kDa, Upper) and PDE4D3 (95 kDa, Lower) isoforms were identified. (b) 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 μ M isoprenaline, 10 μ M rolipram, or 1 μ 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 μ 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 μ M), and ctrB indicates such cells treated for 10 min with both isoprenaline (10 μ M) and rolipram (10 μ 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 β_2 AR switching is known to occur (37). It has been reported (37) that the β_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 β_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. 4c). 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 β_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. 4e). Taken together, these data indicate that, in cardiac myocytes, switching by the β_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 β_2 AR.

Discussion

The β_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 β_2 AR has been seen as eliciting activation of adenylyl cyclase via

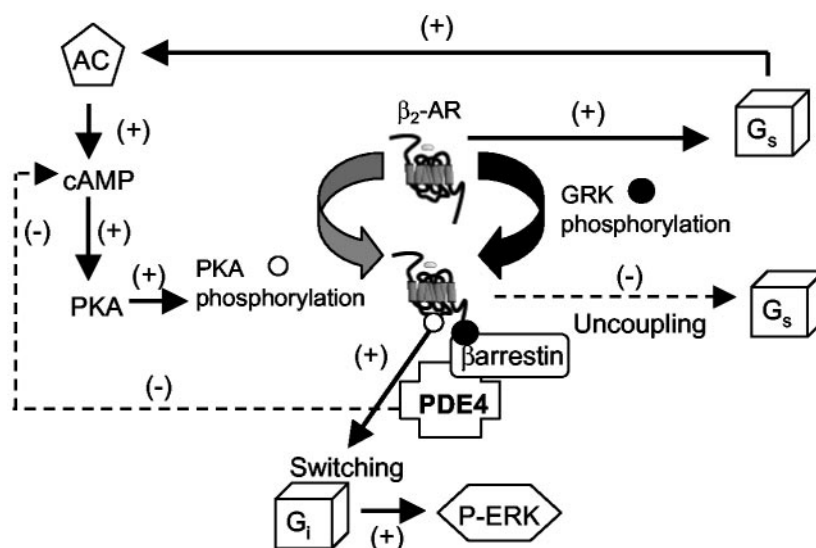


Fig. 5. A schematic representation of the role of arrestin-recruited PDE4 in regulating the “switching” of the β_2 AR from G_s to G_i stimulation. Agonist occupancy of the β_2 AR initially leads to coupling to G_s , which causes activation of adenylyl cyclase, elevated cAMP levels, and activation of PKA, which is able to phosphorylate the β_2 AR. Concomitantly, agonist occupancy also leads to GRK-mediated phosphorylation of the β_2 AR, which allows for the recruitment of β -arrestin together with bound PDE4. PKA phosphorylation of the β_2 AR confers switching from G_s to G_i , with consequent activation of ERK1/2. However, β -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 β_2 AR. This action of β -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 β -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 β_2 AR. This modification seemingly is unconnected to the primary recruitment of β -arrestin, which occurs through GRK-mediated phosphorylation. Instead, it serves to reprogram the G protein-coupling specificity of the β_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 β -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 β_2 AR and hence its ability to couple to G_i and effect ERK activation. Indeed, it has been shown that the β_2 AR can interact with PKA anchor proteins able to specifically recruit PKA and thereby facilitate PKA phosphorylation of the β_2 AR (41, 42). It thus would seem that β -arrestin-recruited PDE4 controls the activity of such a privileged pool of PKA, which determines the PKA phosphorylation status of the β_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 β_2 AR signaling (4). Because PDE4 isoforms show specific expression patterns (14, 18, 20), susceptibility of

the β_2 AR to control by PDE4 activity will depend very much on cell type. Indeed, the complexity of β_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 β -arrestin (with β -arrestin 2 preferred by the β_2 AR), PDE4, PKA subunits, and PKA anchor proteins, very different patterns of β_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 β_2 AR signaling. Occupancy of the β_2 AR causes recruitment of the arrestin scaffold protein with bound PDE4. β -Arrestin-recruited PDE4 regulates the functioning of a (plasma) membrane-associated PKA activity that serves to phosphorylate the β_2 AR. This recruited PDE4 controls the PKA-mediated switching of the β_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 β -arrestin-mediated recruitment of signal-modifying proteins such as PDE4 to the β_2 AR can regulate receptor activity.

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