Phosphodiesterase-4 influences the PKA phosphorylation status and membrane translocation of G-protein receptor kinase 2 (GRK2) in HEK-293 β 2 cells and cardiac myocytes

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Membrane-recruitment of GRK2 (G-protein receptor kinase 2) provides a fundamental step in the desensitization process controlling GPCRs (G-protein-coupled receptors), such as the β_2 AR (β_2 -adrenergic receptor). In the present paper, we show that challenge of HEK-293 β 2 [human embryonic kidney cells stably overexpressing the FLAG-tagged β_2 AR–GFP (green fluorescent protein)] cells with the β -adrenoceptor agonist, isoprenaline, causes GRK2 to become phosphorylated by PKA (cAMP-dependent protein kinase). This action is facilitated when cAMP-specific PDE4 (phosphodiesterase-4) activity is selectively inactivated, either chemically with rolipram or by siRNA (small interfering RNA)-mediated knockdown of PDE4B and PDE4D. PDE4-selective inhibition by rolipram facilitates the isoprenaline-induced membrane translocation of GRK2, phosphorylation of the β_2 AR by GRK2, membrane translocation of β -arrestin and internalization of β_2 ARs. PDE4-selective inhibition also enhances the ability of isoprenaline to trigger the PKA phosphorylation

of GRK2 in cardiac myocytes. In the absence of isoprenaline, rolipram-induced inhibition of PDE4 activity in HEK-293 β 2 cells acts to stimulate PKA phosphorylation of GRK2, with consequential effects on GRK2 membrane recruitment and GRK2-mediated phosphorylation of the β_2 AR. We propose that a key role for PDE4 enzymes is: (i) to gate the action of PKA on GRK2, influencing the rate of GRK2 phosphorylation of the β_2 AR and consequential recruitment of β -arrestin subsequent to β -adrenoceptor agonist challenge, and (ii) to protect GRK2 from inappropriate membrane recruitment in unstimulated cells through its phosphorylation by PKA in response to fluctuations in basal levels of cAMP.

Key words: β_2 -adrenoceptor, cAMP-dependent protein kinase (PKA), G-protein receptor kinase 2 (GRK2), phosphodiesterase 4 (PDE4), rolipram.

INTRODUCTION

Extracellular signals provided by molecules such as hormones and neurotransmitters are transduced into cells by a large family of cell-surface receptors known as GPCRs (G-protein-coupled receptors) [1]. Agonist-occupied GPCRs undergo rapid desensitization, an adaptive response used by cells to constrain G-protein signalling [2]. Desensitization of GPCRs follows a universal mechanism that involves two families of proteins, the GRKs (G-protein receptor kinases) [2–5] and the arrestins [1].

Studies on the β_2 AR (β_2 -adrenergic receptor) have provided a key paradigm for understanding GPCR desensitization [1]. Following agonist stimulation, the β_2 AR binds to and activates the G-protein, G_i [1,6]. The resultant $G\beta\gamma$ subunits, free of $G\alpha$ subunits, recruit GRK2 to the membrane, allowing it to phosphorylate the β_2 AR [7–9]. This phosphorylation event triggers the recruitment of cytosolic β -arrestin to the membrane-bound β_2 AR [10]. Such binding of β -arrestin to the β_2 AR prevents productive coupling to $G\alpha_s$, and targets the β_2 AR for endocytosis [10]. Additionally, phosphorylation of the β_2 AR by PKA (cAMP-dependent protein kinase) allows it to switch its coupling to the G-protein, G_i , leading to activation of the ERK (extracellular-signal-regulated kinase) pathway [7].

PDEs (phosphodiesterases) provide the sole means of degrading cAMP in cells [11–13]. Currently, there is much interest in

members of the cAMP-specific PDE4 family [12,14,15]. PDE4-selective inhibitors have potential therapeutic benefits for treating asthma and chronic obstructive pulmonary disease, as well as acting as cognitive enhancers and antidepressants [15,16]. Targeted gene-knockout studies of *PDE4* genes indicate the key importance of these enzymes in inflammation and cognition [12], and a genetic analysis has linked the *PDE4D* gene to stroke in both Icelandic [17] and Swedish [18] populations.

The PDE4 family is encoded by four genes that generate a large number of isoforms expressed on a tissue and cell-type-specific basis [12,14]. Certain of these isoforms can interact specifically with other proteins, which include Src family tyrosine kinases [19], RACK1 (receptor for activated C-kinase 1) [20], myomegalin [21] and AKAPs (A-kinase-anchoring proteins) [22–24].

It has been shown that agonist-activated β_2 ARs can recruit PDE4 isoforms in complex with β -arrestins [25] and, in particular, PDE4D5 [26,27]. A pivotal role of β -arrestin-recruited PDE4 is in attenuating the ability of membrane-bound PKA to phosphorylate β_2 ARs [28,29], a process that switches β_2 AR coupling from G_s to G_i with consequential activation of ERK [7,30]. Indeed, the importance of PDE4D in regulating the PKA-mediated switching of β_2 AR coupling from G_s to G_i has additionally been elegantly demonstrated in cardiac myocytes from *PDE4D*-knockout animals [31].

Abbreviations used: β_2 AR, β_2 -adrenergic receptor; ERK, extracellular-regulated-protein kinase; GFP, green fluorescent protein; GPCR, G-protein-coupled receptor; GRK, G-protein receptor kinase; HEK-293 β 2, human embryonic kidney cells stably overexpressing the FLAG-tagged β_2 AR–GFP; PDE, phosphodiesterase; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; siRNA, small interfering RNA; TBS, Tris-buffered saline.

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In the present study, we have identified a novel role for PDE4 in regulating the initial stage of the β_2 AR-desensitization process. We show that PDE4 activity regulates GRK2 phosphorylation by PKA, which is known to result in an increased affinity of GRK2 for $G\beta\gamma$ subunits, thereby accelerating the recruitment of active GRK2 to the plasma membrane [32].

MATERIALS AND METHODS

Materials

PDE4D-specific antiserum [33], rabbit polyclonal antibodies against β -arrestin 1/2 [25], monoclonal antibodies used to detect native and phosphorylated forms of ERK1/2 [34] and monoclonal antibodies against phosphorylated (serine/threonine) PKA substrates [28] (Cell Signaling Technology) were all used as described previously. Antibodies against the β_2 AR, phospho- β_2 AR Ser³⁵⁵/Ser³⁵⁶, phospho- β_2 AR Ser³⁴⁵/Ser³⁴⁶ and GRK2 were obtained from Santa Cruz Biotechnology and were used according to the manufacturer's instructions. Anti-phospho-Ser⁶⁷⁰ GRK2-(44–202) was purchased from BioSource International. All other biochemicals were obtained from Sigma. Stock solutions of rolipram {4-[3-(cyclopentoxy)-4-methoxyphenyl]-2-pyrrolidinone}, H89 and cilostamide were prepared in DMSO.

Cell culture, transfection, immunoblotting and molecular reagents

HEK-293 β_2 cells [human embryonic kidney cells stably overexpressing the FLAG-tagged β_2 AR–GFP (green fluorescent protein)] were cultured as described previously [35]. Preparation and short-term culture of neonatal rat ventricular myocytes were as described previously [28]. Immunoprecipitation of the β_2 AR, GRK2 and phospho-PKA substrates was performed as described previously [28]. Membrane fractions (P2) for recruitment studies were prepared and analysed as in [25]. For determination of PKA-phosphorylated membrane GRK2, P2 pellet fractions (200 μ g of protein) were solubilized with lysis buffer [25 mM Hepes, pH 7.5, 2.5 mM EDTA, 50 mM NaCl, 50 mM NaF, 30 mM sodium pyrophosphate, 10% (v/v) glycerol and 1% (v/v) Triton X-100 containing CompleteTM EDTA-free protease inhibitor cocktail tablets (Roche)] and then PKA phosphorylated GRK2 immunopurified using a specific PKA phospho-substrate antibody, with detection by immunoblotting for GRK2 [28]. Proteins were separated by SDS/PAGE (4–12% Bis-Tris gels) and transferred on to nitrocellulose membranes for Western blotting. Protein concentrations were determined with BSA as standard [36]. Human GRK2-specific siRNA (small interfering RNA) was purchased from Santa Cruz Biotechnology. GRK2-siRNA (100 nM) was transfected into HEK-293 β_2 cells using LipofectamineTM 2000 (Invitrogen). A non-specific siRNA control (duplex XII) (Dharmacon) was obtained and used at the appropriate optimal concentration. Note that treating cells with this non-targeted (control) siRNA resulted in the same data as in untreated cells (results not shown). The selective knockdown of the entire PDE4B or PDE4D subfamilies in HEK-293 β_2 cells was performed as described previously by us in some detail [27]. Briefly, we designed double-stranded 21-mer RNA duplexes targeted at unique regions of sequence within each of these subfamilies. siRNA was obtained from Dharmacon and was developed so that efficient knockdown of the indicated species was achieved with no effect on expression of isoforms from the other PDE4 subfamilies found in these cells.

Confocal analyses

HEK-293 β_2 cells were plated out on to poly(L-lysine)-treated coverslips (18 mm $\times$ 18 mm) at approx. 40% confluence. After treatment with the indicated ligands, cells were fixed for 10 min

in 4% (w/v) paraformaldehyde followed by three washes with TBS (Tris-buffered saline; 0.136 M NaCl and 0.02 M Tris/HCl, pH 7.6). Cells were permeabilized and blocked as described previously [22]. The primary antibody used specifically to detect phosphorylation of the β_2 AR on Ser³⁵⁵/Ser³⁵⁶ by GRK2 was diluted to the required concentration in diluting buffer (blocking buffer diluted 1:1 with TBS) and added to the cells for 2 h at 21 °C. After washing, cells were incubated with 200 μ l of 1:400 diluted Alexa Fluor[®] 594 fluorophore-conjugated IgG (Molecular Probes) for 1 h. Cells were then washed extensively with TBS, mounted on microscope slides and visualized using the Zeiss Pascal laser-scanning confocal microscope.

RESULTS

The isoprenaline-stimulated PKA phosphorylation of GRK2 is amplified by inhibition of PDE4

Challenge of HEK-293 β_2 cells with the β -adrenoceptor agonist, isoprenaline, caused the time-dependent, PKA phosphorylation of GRK2 (Figure 1). We gauged this in two distinct ways. First, GRK2 was immunoprecipitated from HEK-293 β_2 cells using a GRK2-specific antibody. The resultant immunoprecipitate was probed for both GRK2 (Figure 1a, lower panel) and, using an anti-(PKA substrate) antibody, PKA-phosphorylated GRK2 (Figure 1a, upper panel). This revealed a single immunoreactive species, migrating at 79 kDa, as would be expected for GRK2. The anti-(PKA substrate) antibody revealed a time-dependent appearance of PKA-phosphorylated GRK2 in response to isoprenaline (Figures 1a and 1b). Similarly, when the anti-(PKA substrate) antibody was used to immunoprecipitate PKA-phosphorylated proteins from HEK-293 β_2 cells, and then the anti-GRK2 antibody was used to probe the immunopurified samples, a single 79 kDa species was detected following 5 min of isoprenaline treatment (Figures 1c and 1d).

PDEs provide the sole means of degrading cAMP in cells [11]. In HEK-293 β_2 cells, approx. 60% of the cAMP PDE activity is contributed by PDE4 [25,27]. The functional significance of PDE4 action can be assessed by chemical ablation using the PDE4-selective inhibitor, rolipram [12,14]. In the present study, we observed that the presence of the PDE4-selective inhibitor rolipram accelerated the ability of isoprenaline to cause the PKA phosphorylation of GRK2 (Figures 1a–1d).

No such immunoreactive species was evident when the cells were treated with isoprenaline and rolipram in the presence of either of the PKA inhibitors, H89 (1 μ M) (Figures 1a and 1c) or KT5720 (results not shown).

Antisera specific to GRK3, GRK4, GRK5 and GRK6 failed to detect these species in HEK-293 β_2 cells (results not shown), suggesting that GRK2 is the major species in these cells.

In these studies, HEK-293 β_2 cells were challenged with isoprenaline after a 10 min pre-incubation with rolipram. However, PKA phosphorylation of GRK2 was clearly evident in rolipram-pre-treated cells before the addition of isoprenaline (zero time; Figures 1a–1c). Further examination showed that addition of rolipram alone elicited the time-dependent PKA phosphorylation of GRK2 and that H89 ablated this effect (Figures 1e and 1f). However, despite the fact that 40% of total cAMP PDE activity in HEK-293 β_2 cells is contributed by PDE3, the PDE3-selective inhibitor, cilostamide, failed to trigger the PKA phosphorylation of GRK2 (Figure 1e).

We have developed siRNA reagents and transfection conditions that allow for the selective ablation (> 95%) of expression and activity of PDE4B, represented by PDE4B2, and PDE4D, represented by PDE4D3 and PDE4D5, in HEK-293 β_2 cells [27]. Under such conditions, we observed that ablation of either PDE4B or

Figure 1 GRK2 is phosphorylated by PKA in HEK-293 cells

(a) HEK-293 β 2 cells were challenged with either isoprenaline (10 μ M) alone or isoprenaline following a 10 min pre-treatment with rolipram (10 μ M) in the presence and absence of the PKA inhibitor H89 (1 μ M). Cells were harvested in 3T3 lysis buffer and GRK2 was immunopurified from 300 μ g of cellular protein before being blotted with an anti-(phospho-PKA substrate) antibody. Confirmation of equal GRK2 loading is shown using an anti-GRK2 antibody. (b) Quantification of three such independent experiments (means $\pm$ S.D.) of which that shown in (a) is a typical example. (c) The presence of PKA-phosphorylated GRK2 was determined by immunoprecipitation using the anti-(phospho-PKA substrate) antibody and subsequent blotting with a GRK2-specific antibody. (d) Quantification for three such independent experiments (means $\pm$ S.D.) as shown in (c). (e) HEK-293 β 2 cells were challenged with either rolipram (10 μ M) alone, rolipram and H89 (1 μ M) or cilostamide (1 μ M) alone for the indicated times. (f) Quantification of three independent experiments as shown in (e) for rolipram alone. (g) Identification of PKA-phosphorylated GRK2 in HEK-293 β 2 cells transfected with either scrambled siRNA or siRNA specific for PDE4B or siRNA specific for PDE4D under resting conditions (c), challenged for 5 min with isoprenaline or for 5 min with isoprenaline and H89. (h) Quantification of three independent experiments (means $\pm$ S.D.) as shown in (g). Blots here and all other Figures are typical of separate experiments performed three times. Ab, antibody; IH, isoprenaline and H89; Iso/I, isoprenaline; Roli, rolipram; si4B, siRNA specific for PDE4B; si4D, siRNA specific for PDE4D; sub, substrate.

PDE4D subfamilies resulted in an enhanced ability of isoprenaline to cause the PKA phosphorylation of GRK2 (Figures 1g and 1h). Such data provide independent support to data from chemical ablation of PDE4 activity achieved using rolipram (Figure 1a). However, while siRNA-mediated knockdown of either PDE4B or PDE4D alone failed to increase the PKA phosphorylation status

of GRK2 in resting (unstimulated) cells (Figures 1g and 1h), knockdown of both PDE4B and PDE4D together did achieve such an increase (Figures 1g and 1h). Clearly, such combined knockdown mimics the effect of rolipram, which inhibits both of these PDE4 families. However, that inhibition of the exclusively membrane-bound PDE3 had no effect suggest that these PDEs

exert compartmentalized actions in HEK-293 β 2 cells. Indeed, in many cell types, it has been shown that selective inhibition of PDE3 and PDE4 results in very different functional effects [13,37].

PDE4 inhibition and membrane translocation of GRK2

Challenge of HEK-293 β 2 cells with isoprenaline elicited the time-dependent membrane translocation of the cytosolic protein kinase, GRK2 (Figures 2a and 2b). However, in the added presence of rolipram, this recruitment not only was accelerated, but also was clearly transient in nature (Figures 2a and 2b). Such transience was abolished by additionally treating cells with the MEK (mitogen-activated protein kinase/ERK kinase) inhibitor, UO126 (Figures 2a and 2b).

Again, it was evident that rolipram pre-treatment sufficed to stimulate GRK2 translocation (Figure 2a). This occurred in a time-dependent manner and was ablated by the addition of H89 (Figure 2c). Thus challenge of HEK-293 β 2 cells with rolipram alone can cause both the PKA phosphorylation of GRK2 (Figures 1e and 1f) and its time-dependent membrane translocation (Figures 2c and 2d). That the PKA-phosphorylated form of GRK2 can be membrane-recruited, following isoprenaline treatment, is shown by immunoprecipitation with the anti-(PKA substrate) antibody from solubilized membranes and probing with the anti-GRK2 antibody (Figure 2d). This recruitment of phosphorylated GRK is increased in the presence of rolipram and is abolished by treatment with H89 (Figure 2d).

PDE4 inhibition and phosphorylation of the β_2 AR by GRK2

Isoprenaline challenge of HEK-293 β 2 cells led to the time-dependent phosphorylation of the β_2 AR by GRK2 (Figures 3a and 3e). This action, which we detected by probing β_2 AR immunoprecipitates with a specific phospho-antiserum, was ablated upon siRNA-mediated knockdown of GRK2 (Figure 3b).

Pre-treatment of HEK-293 β 2 cells with rolipram accelerates the ability of isoprenaline to trigger phosphorylation of the β_2 AR by GRK2 (Figures 3a and 3e). Indeed, challenge with rolipram alone causes the time-dependent GRK2 phosphorylation of the β_2 AR (Figures 3d and 3f), corresponding with the observed recruitment of GRK2 (Figure 2c). The ability of rolipram to promote the GRK2 phosphorylation of the β_2 AR was also ablated upon GRK2 knockdown (Figure 3c). Concomitant with GRK2 recruitment to the membrane (Figures 1g and 1h), GRK-mediated phosphorylation of the β_2 AR increased following siRNA-mediated knockdown of both PDE4B and PDE4D. As in cells treated with rolipram alone, siRNA-mediated ablation of PDE4B and PDE4D from cells also increased the GRK phosphorylation status of the β_2 AR in resting (unstimulated) cells (Figure 3g).

PDE4 inhibition and membrane recruitment of β -arrestin

It has been shown previously that isoprenaline triggers the time-dependent transient and concomitant membrane recruitment of both β -arrestin and PDE4D in HEK-293 β 2 cells [25]. Although we confirm this in the present study, we additionally show that this transient effect occurs more rapidly in isoprenaline-challenged cells that had been pre-treated with rolipram (10 μ M, 10 min) (Figure 4). Indeed, we noted that challenge of HEK-293 β 2 cells with rolipram alone sufficed to cause membrane recruitment of both β -arrestin, PDE4D3 and PDE4D5 (Figure 4e). Comparing these time courses, it is apparent that the concomitant membrane recruitment of β -arrestin and PDE4D3/5 occurred subsequently to the recruitment of GRK2 (Figure 2c) and GRK phosphorylation of the β_2 AR (Figures 3d and 3f). All such

Figure 2 Membrane translocation of GRK2 in HEK-293 cells

(a) Cells were challenged with isoprenaline (10 μ M) for the indicated times in either the presence or absence of UO126 (10 μ M), and either with or without rolipram (10 μ M) pre-treatment (10 min). Membrane (P2 fraction) protein (50 μ g) was blotted for GRK2 in each instance. (b) Quantification of three experiments as in (a) (means $\pm$ S.D.) with 100% as the maximal effect seen with isoprenaline and rolipram. (c) Cells were treated for the indicated times with rolipram (10 μ M) in the absence and presence of H89 (1 μ M) and 50 μ g of membrane protein was blotted for GRK2. (d) Membrane (P2) fractions were analysed from cells treated with either isoprenaline (10 μ M) alone or isoprenaline after pre-treatment with rolipram (10 μ M; 10 min) or isoprenaline after pre-treatment with rolipram and H89 (10 μ M) together for 10 min. The membrane fractions (200 μ g of protein) were solubilized in lysis buffer, and PKA-phosphorylated proteins were immunoprecipitated (IP) using an anti-(phospho-PKA substrate) antibody, subjected to SDS/PAGE (4–12% Bis-Tris gels) and immunoblotted for GRK2. Iso, isoprenaline; Roli, rolipram; UO, UO126.

rolipram-induced translocations were ablated by H89 (Figures 2c and 4e).

Phosphorylation of GRK2 by ERK

Challenge of HEK-293 β 2 cells with isoprenaline caused a time-dependent increase in the level of ERK-phosphorylated GRK2 at the membrane (Figures 5a and 5b). Isoprenaline-induced recruitment of ERK-phosphorylated GRK2 not only was accelerated in the presence of rolipram, but also was shown to be

Figure 3 Phosphorylation of the β_2 AR in HEK-293 cells by GRK2

(a) Cells were challenged with isoprenaline ($10 \mu\text{M}$) for the indicated times either with or without rolipram ($10 \mu\text{M}$) pre-treatment (10 min). Lysate protein ($50 \mu\text{g}$) was blotted for either phosphorylation of the β_2 AR by GRK2 on Ser³⁵⁵/Ser³⁵⁶ or for total β_2 AR in each instance. (b) Cells were challenged with isoprenaline alone, control cells were pre-treated with scrambled siRNA, while test cells were evaluated after specific siRNA-mediated GRK2 knockdown. Lysates ($50 \mu\text{g}$) were probed as in (a). (c) Cells, treated or not with rolipram ($10 \mu\text{M}$) for 10 min, were probed as in (a), control cells were pre-treated with scrambled siRNA (scr), while test cells were evaluated after specific siRNA-mediated GRK2 knockdown (siR-GRK2). The extent of GRK2 knockdown was complete, as indicated in (b). (d) Cells were challenged with rolipram ($10 \mu\text{M}$) alone for the indicated times and analysed as in (a). (e) Quantification of three experiments as in (a) (means $\pm$ S.D.) with 100% as the maximal effect seen with isoprenaline and rolipram. (f) Quantification of three experiments as in (d) (means $\pm$ S.D.) with 100% relating to the maximal effect as seen in (e) with isoprenaline and rolipram. (g) Control cells and cells pre-treated with specific siRNA to PDE4B (siB) and PDE4D (siD), were challenged with isoprenaline. Knockdown of PDE4B and PDE4D, for each sample, is shown in the upper two panels with immunoblots (third panel) for the β_2 AR showing that loading of the gel was consistent. GRK phosphorylation of the β_2 AR is shown in the lower panel by probing with a specific antiserum. Iso, isoprenaline; Roli, rolipram.

Figure 4 Membrane recruitment of PDE4D and β -arrestin

(a) Cells were challenged with isoprenaline ($10 \mu\text{M}$) for the indicated times either with or without, as indicated, 10 min rolipram ($10 \mu\text{M}$) pre-treatment. In each instance, $50 \mu\text{g}$ of membrane (P2 fraction) protein was blotted to assess the level of β -arrestin. The major band identified here is β -arrestin 2. (b) Quantification of three experiments as in (a) (means $\pm$ S.D.) with 100% as the maximal effect seen with isoprenaline and rolipram. (c) Cells treated as in (a) and immunoblotted for PDE4D, which identifies PDE4D3 and PDE4D5 isoforms only in these cells. (d) Quantification of three experiments as in (c) (means $\pm$ S.D.) with 100% as the maximal effect seen with isoprenaline and rolipram. (e) Cells were treated with rolipram ($10 \mu\text{M}$) for the times indicated in the presence or absence of H89, as indicated. P2 membrane fraction ($50 \mu\text{g}$) was blotted for β -arrestin and PDE4D. Iso, isoprenaline; R/roli, rolipram.

profoundly transient in nature (Figures 5a and 5b). In fact, the transience of this effect bears comparison with the time-dependent membrane recruitment of GRK2 (Figures 2a and 2b). However, in contrast with such an action, there was no indication at all that 10 min pre-treatment of HEK-293 β_2 cells with rolipram caused phosphorylation of GRK2 by ERK to occur (Figure 5a). In addition, HEK-293 β_2 cells challenged with isoprenaline in the presence of rolipram were found to have enhanced activation of ERK, but no ERK activation was observed in the presence of rolipram alone (Figure 5c; detailed negative time courses of 0–15 min with $10 \mu\text{M}$ rolipram alone are not shown).

PDE4 inhibition and the trafficking and phosphorylation status of the β_2 AR in HEK-293 β_2 cells

The GRK-phosphorylated form of the β_2 AR (Ser³⁵⁵/Ser³⁵⁶) can be detected using a specific antibody, as has been extensively characterized previously by others [38]. We have exploited this powerful new reagent in order to define the temporal and spatial accumulation of the GRK2-phosphorylated form of the β_2 AR *in situ* in HEK-293 β_2 cells using confocal analyses (Figure 6). In these studies, the green fluorescent channel detects the GFP-tagged β_2 AR, and the red channel detects the antibody used to detect the GRK2-phosphorylated form of the β_2 AR. Thus the

Figure 5 ERK-phosphorylated GRK2 in HEK-293 cells

(a) Cells were challenged with isoprenaline (10 μ M) for the indicated times either with or without, as indicated, 10 min rolipram (10 μ M) pre-treatment. In each instance, 50 μ g of membrane (P2 fraction) protein was blotted to assess the level of ERK-phosphorylated GRK2 present. (b) Quantification of three experiments as in (a) (means $\pm$ S.D.) with 100% as the maximal effect seen with isoprenaline and rolipram. (c) Cells were challenged with isoprenaline (10 μ M) for the indicated times either with or without, as indicated, 10 min rolipram (10 μ M) pre-treatment. In each instance, 50 μ g of lysate protein was blotted to assess the level of pERK. I/Iso, isoprenaline; R/Roli, rolipram.

merged images for these two channels will identify the GRK2-phosphorylated form of the β_2 AR as a yellow signal (Figure 6). Using this approach, we see that challenge of HEK-293 β_2 cells with isoprenaline alone caused the rapid GRK2 phosphorylation of the β_2 AR in a time-dependent manner (Figure 6). Consistent with biochemical data (Figure 3), GRK2 phosphorylation of the β_2 AR commenced some 3–5 min after isoprenaline challenge and continued over the 10 min time course studied (Figure 6). Also evident is that internalization of the β_2 AR began after 5 min of isoprenaline stimulation and that a major fraction of the β_2 AR population was internalized some 10 min after isoprenaline challenge (Figure 6). In the presence of rolipram, GRK2 phosphorylation and internalization of the β_2 AR in response to isoprenaline were observed to occur more rapidly (Figure 6). Additionally, the presence of rolipram accelerated the rate of internalization of the β_2 AR in response to isoprenaline (Figure 6).

However, treatment of HEK-293 β_2 cells with rolipram alone led to the time-dependent appearance of GRK-phosphorylated β_2 AR in HEK-293 β_2 cells (Figure 6), which is consistent with our biochemical studies (Figure 3d).

Rolipram triggers PKA phosphorylation of GRK2 in cardiac myocytes

Challenge of cardiac myocytes with isoprenaline caused the time-dependent PKA phosphorylation of GRK (Figure 7a) and also caused membrane recruitment of GRK2 (Figure 7b). These responses were amplified by pre-treatment with rolipram (Figure 7). In addition pre-treatment of cardiac myocytes with rolipram (10 μ M) alone was sufficient to cause both PKA phosphorylation of GRK and membrane recruitment of GRK2 (Figure 7). These actions of rolipram were ablated by the addition of H89 (Figure 7b).

DISCUSSION

Previous studies have shown that recruitment of GRK2 to the membrane and resulting phosphorylation of the associated GPCRs can be regulated by the activity of kinases [3,4,39]. PKA [32], as well as PKC (protein kinase C) [40], has been shown to phosphorylate GRK2, thereby increasing the ability of GRK2 to interact with G $\beta\gamma$ subunits [7,8,41,42]. This enhances membrane recruitment of GRK2 and, in consequence, the ability of GRK2 to phosphorylate the GPCR [4,9]. In the present study, we identified a novel role for PDE4s, showing that they play a significant part in the regulation of this facet of β_2 AR desensitization.

We have shown in the present study that inactivation of PDE4 by the PDE4 selective inhibitor rolipram enhances the isoprenaline-induced PKA phosphorylation of GRK2 (Figures 1a and 1b). We have also shown that, in the presence of rolipram, GRK2 recruitment to the membrane is enhanced (Figures 2a and 2b), as is phosphorylation of the β_2 AR by GRK2 (Figure 3a) in response to isoprenaline. This leads to the facilitation of β -arrestin recruitment (Figure 4a) and an increased rate of receptor internalization (Figure 6). It would appear that this effect of PDE4 is not subfamily-specific as siRNA-mediated knockdown of either the PDE4B or PDE4D subfamilies, which provide 30% and 60% of the total PDE4 activity in HEK-293 β_2 cells respectively [25,27], is sufficient to cause enhancement of the isoprenaline-induced PKA phosphorylation of GRK2 (Figures 1g and 1h), as well as GRK2-mediated phosphorylation of the β_2 AR (Figure 3g). This is in contrast with PDE4 control of PKA phosphorylation of the β_2 AR and consequential switching of its signalling from activation of adenylate cyclase to activation of ERK [25,28]. In this case, it is specifically the PDE4D5 isoform that has the ability to control phosphorylation of the β_2 AR via PKA [27]. In such a situation, following isoprenaline stimulation, PDE4D5 is recruited to the membrane in complex with β -arrestin and there it serves to regulate the PKA phosphorylation of the β_2 AR [25–27]. With regard to GRK2, we would suggest that its phosphorylation by PKA may initiate in the cytosol thereby increasing the ability of GRK2 to bind G $\beta\gamma$ subunits and translocate to the membrane. PKA phosphorylation of GRK2 in the cytosol is therefore likely to be sensitive to changes in global cytosolic cAMP actions, which both PDE4B and PDE4D are able to influence.

Following rolipram inhibition of PDE4, we note that isoprenaline-induced GRK2 recruitment to the membrane is transient in nature (Figure 2a). Through causing its increased phosphorylation by PKA, and thereby its affinity for interacting with G $\beta\gamma$, PDE4 inhibition will promote the ability of isoprenaline to initiate membrane recruitment of GRK. However, PDE4 inhibition will also lead to increased PKA phosphorylation of the β_2 AR and the switching of its signalling to ERK [26–29]. Such consequential increase in activated ERK can be expected to subsequently phosphorylate GRK2, thereby releasing GRK from the membrane by reducing its ability to associate with G $\beta\gamma$ [8,43]. Such actions are likely to underpin the transience of GRK2 membrane association

Figure 6 Confocal microscopy analysis of GRK-phosphorylated β_2 AR in HEK-293 cells

Shown are merged fluorescent confocal images of fixed HEK-293 β_2 cells stably expressing GFP-tagged β_2 AR (green) and stained to detect the GRK phosphorylated form (Ser³⁵⁵/Ser³⁵⁶) of the β_2 AR (red) as well as nuclei [blue; DAPI (4,6-diamidino-2-phenylindole)]. Cells were treated with isoprenaline alone (10 μ M) or isoprenaline added together with rolipram (10 μ M) or with rolipram alone in each case for the indicated times. Arrows indicate examples of GRK2-phosphorylated β_2 AR at the plasma membrane. Arrows with an asterisk indicate examples of internalized GRK2-phosphorylated β_2 AR. Images are typical of experiments performed at least three times.

Figure 7 GRK2 translocation in cardiac myocytes

(a) Cardiomyocytes were challenged with isoprenaline (10 μ M) for the indicated times either with or without, as indicated, 10 min rolipram (10 μ M) pre-treatment. Cells were harvested in lysis buffer, and GRK2 was immunoprecipitated from 300 μ g of cellular protein from each sample and blotted with an anti-(phospho-PKA substrate) antibody. Confirmation of equal GRK2 loading is shown using an anti-GRK2 antibody. (b) Cardiomyocytes were challenged with the indicated combinations of isoprenaline (10 μ M) and/or rolipram (10 μ M) and H89 (1 μ M) for the indicated times. When rolipram was added together with isoprenaline, cells were then pre-incubated with rolipram for 10 min before the addition of isoprenaline for the indicated times. In each instance, 50 μ g of membrane (P2 fraction) protein was blotted to assess the level of GRK2 present. Ctrl, control; Iso, isoprenaline; Roli, rolipram.

seen in cells challenged with isoprenaline and rolipram together. Consistent with ERK underpinning the release of GRK2 [43], under conditions of isoprenaline challenge and PDE4 inhibition,

we observed that, if ERK is inhibited with UO126, then GRK2 remains membrane-associated (Figure 2).

We also show in the present study that PKA phosphorylation of GRK2 (Figures 1a and 1e), GRK2 membrane recruitment (Figures 2a and 2c) and GRK phosphorylation of the β_2 AR (Figures 3a and 3d) can all occur in the absence of agonist in HEK-293 β_2 cells, following inactivation of PDE4s. This would suggest that, in addition to regulating isoprenaline-induced PKA phosphorylation of GRK2, with consequential effects on GRK phosphorylation of the β_2 AR and recruitment of β -arrestin, PDE4s also prevent the inappropriate membrane recruitment of GRK2 in unstimulated cells. Unsurprisingly, effects caused by rolipram alone in these cells were not as pronounced as those seen in the added presence of isoprenaline. The effect of rolipram alone on PKA phosphorylation of GRK2 and consequent membrane recruitment was not confined to HEK-293 β_2 cells, but was also observed in cardiac myocytes (Figure 7). Current dogma dictates that GRK2 recruitment to the membrane is agonist-dependent. To our knowledge, however, no one has reported the effects of PDE4 inhibition on GRK2 distribution. Also, PKC phosphorylation of GRK2 by phorbol esters in the absence of agonist has been reported to cause activation and translocation of GRK2 by relieving the tonic inhibition of GRK2 by calmodulin [40]. It is now well established that GPCRs, such as the β_2 AR, exist in equilibrium between active and inactive states and that agonist binding drives this equilibrium towards the active state. Thus, under basal conditions, such equilibrium allows for the generation of a small pool of activated $G\alpha_s$, which is responsible for basal adenylate cyclase activity, together with free $G\beta\gamma$ [6,44–46]. In the cells used in the present study, it would appear that basal adenylate cyclase activity, in the presence of PDE4 inhibition, is sufficient to allow cAMP levels to reach a magnitude whereby PKA can

Scheme 1 Control points whereby PDE4 regulates β_2 AR functioning

The schematic diagram highlights the novel role for PDE4 in regulating PKA phosphorylation of GRK2 in β_2 AR signalling. PDE4 regulates the degree of amplification of desensitization exerted when PKA becomes activated upon β_2 AR stimulation and serves to protect GRK2 from inappropriate PKA phosphorylation and translocation by fluctuations in basal cAMP levels in resting cells. β -Arrestin-recruited PDE4 regulates the ability of PKA to phosphorylate the β_2 AR and switch its coupling from G_s to G_i . For simplicity, this scheme shows key control points only, thus G_s should be taken as activating PKA by first stimulating adenylate cyclase to increase cAMP. Conversely, PDE4 should be taken as inhibiting PKA by hydrolysing cAMP. The means whereby membrane-recruited GRK2 inhibits β_2 AR coupling to G_s is through the GRK2-phosphorylated β_2 AR recruiting cytosolic β -arrestin. When β -arrestin is recruited, it brings cytosolic PDE4 to the membrane in a complex with it that now regulates the action of membrane PKA on the β_2 AR. cyt, cytosol; memb, membrane.

phosphorylate GRK2, increasing its affinity for $G\beta\gamma$ subunits and facilitating recruitment of GRK2 to the membrane.

That ERK activation is not stimulated by rolipram treatment alone in HEK-293 β_2 cells (Figure 5c) indicates that agonist stimulation is essential to allow coupling of the PKA phosphorylated β_2 AR to G_i and consequential ERK activation in these cells. The transient nature of GRK2 recruitment to the membrane seen in the presence of isoprenaline and rolipram (Figure 2a) we would suggest is contributed to by the activation of ERK causing GRK2 release from the membrane. Thus, in HEK-293 β_2 cells challenged with rolipram alone, which does not lead to ERK activation, the recruitment of GRK2 to the membrane is sustained (Figure 2c).

PDE4 enzymes are pivotally interconnected with the regulation of β_2 AR signalling (Scheme 1). β -Arrestin-recruited PDE4D5 provides the route for controlling the PKA-mediated coupling of the β_2 AR to G_i [26–28]. However, as shown in the present study for the first time, PDE4 activity, in regulating PKA phosphorylation of GRK2, may, at least in certain cells, provide a means of influencing the membrane recruitment of GRK2. There are three distinct facets to this. First, PDE4 activity is set to play a central feedback role in regulating the agonist-stimulated PKA-phosphorylation of GRK2, thereby influencing the rate of agonist-triggered desensitization. Secondly, in regulating the PKA-mediated switching of β_2 AR coupling, PDE4 activity modulates the degree of isoprenaline-stimulated ERK activation, which influences the release of GRK2 from membranes and the reversal of desensitization. Finally, PDE4 activity is positioned to protect GRK2 from phosphorylation by fluctuations in basal PKA activity. Presumably, PDE4 activity sets a threshold for PKA phosphorylation of GRK2, which normally requires agonist challenge of the β_2 AR to breach. In doing so, PDE4 prevents inappropriate membrane translocation of GRK2 from occurring in resting cells owing to PKA phosphorylation caused by fluctuations in intracellular cAMP levels. PDE4 thus ensures that, under normal conditions, GRK2 translocation is observed as being entirely agonist-dependent. PDE4 is thus set to play a fundamental role in influencing β_2 AR functioning by gating the potential for PKA

to phosphorylate GRK2 in resting cells and regulating the degree of feedback through this system in stimulated cells. The potential importance of PDE4 in preventing inappropriate activation of GRK2 in cardiac myocytes may have relevance to heart failure. This might be inferred from observations showing that, while inhibition of GRK2 can protect against heart failure [47,48], PDE4D gene inactivation in mice can lead to accelerated heart failure after myocardial infarction [49].

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